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Recent advances of research in antinutritional factors in legume seeds

Animal nutrition
Feed technology
Analytical methods

Proceedings of the First International Workshop on 'Antinutritional Factors (ANF) in Legume Seeds', November 23-25, 1988, Wageningen, The Netherlands

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Opening session

OPENING ADDRESS.
H.C. van der Plas.

There are several reasons why I feel honoured to open this first International Workshop on the role of Antinutritional Factors on animal nutrition in Wageningen.
Wageningen is the site of the National Agricultural University. In Wageningen agricultural sciences are being taught to about 6000 students in various disciplines.
Together with the veterinary faculty in Utrecht it is the only place in the Netherlands where students can obtain their M.Sc. and Ph.D. in agricultural sciences. For the Netherlands agriculture plays a very important role in the nation's economy. While in the common market most other countries have specialized in industrial production we have specialized in agriculture. We are honoured that this workshop, which is focussed on the relation between plant production and animal nutrition, takes place in Wageningen. The production of plant products has increased significantly during the last decades. This production has to be increased further to feed the growing human population of the world. In future there will also be an increasing need for protein rich feedstuffs for animal nutrition. This assumption is based on the general expectation among zootechnicans that meat producing animals will grow faster and deposit more protein in the body. These animals will need more highly digestible protein in their diet. In Europe there is a growing interest in use of protein sources other than soya and animal proteins. Legume seeds like peas, beans and lupins are crops which grow well under European climatic conditions. Therefore there is an increasing interest for use of these seeds in animal and human nutrition. However these seeds often contain Antinutritional Factors, thus limiting their use in animal nutrition. Therefore research into ANFs will be increasingly important. From this point of view this workshop will be an important medium for updating and exchanging knowledge and for the stimulation of scientists active in this area.

In the research that will be discussed in this workshop people from many disciplines have to work together. The biochemist on the one hand has to identify and to isolate the factors responsible for ANF action. Other scientists have to assess the mode action of ANFs. Also plant breeders will be needed in this field. Since animals react to these factors in the feed in a negative way, other researchers working on immunology, technology and digestive physiology will also play an important role. In that respect it is really an interdisciplinary workshop. In this field scientists of many disciplines can work together to reduce the ANF substances. An important consequence of achieving this objective, will be an increased efficiency of animal production. As a further benefit less nitrogen will appear in the waste, leading to a positive effect on the ecological equilibrium between plant production and animal utilization.
I hope that this workshop will also strengthen the cooperation between various areas of research to solve an important problem in agriculture. The progress will depend on an increased understanding of basic problems pertaining the nature of ANFs and their interactions with animals. The University wishes you a very good workshop.

WORDS OF WELCOME.
J. Huisman.

On behalf of the organizers I have the honour to welcome you on this ANF Workshop.
We are very pleased that so many specialists from the whole world have shown interest in this meeting. We had decided beforehand to limit the number of participants in this workshop. Only those scientists active in the field of ANF research have been invited. The reason is that we will try to update the knowledge on ANFs by bringing scientists actively working in this field together. We have learned that there is so much interest in this field that we would have at least 250/300 participants if there were no restrictions.
This workshop is especially aimed at the role of ANFs in animal nutrition.
However, this knowledge is also important for human nutrition because the animal can serve as a model for man.
In Europe there is a growing interest in the use of protein sources others than soya and animal protein. However, many seeds contain Antinutritional Factors, hampering these seeds for the use in nutrition for monogastric animals. This was one of the reasons that TNO and the Agricultural University started in 1985 a research programme on these factors. It was soon realized that there were important problems in this field. These were:
- the analytical methods for ANFs are not always adequate
- there is insufficient information about the way these factors are acting in the target animal. Most research is carried out with rats and chickens but only at a limited scale with pigs.
- there is insufficient information about threshold levels for ANFs
- inactivation of ANFs is mainly focussed on heat treatments. More research is needed to develop advanced (bio)technological treatments and to check possibilities in plant breeding.

These topics form the base of our ANF research programme.
In the Netherlands, we have the unique situation that different groups are working very closely together in cooperative programmes. Each group has its own speciality. Bringing them together will improve the quality and potentials of research.
Since its modest start in 1985, the programme has expanded considerably. This had been realized by the strong financial support of the Dutch animal feed industry, organized in the Commodity Board for Feedingstuffs. This Board is also the main sponsor of this ANF Workshop.
In this enlarged programme, apart from TNO and the Agricultural University, other institutes in the Netherlands as well as in other European countries are also participating.
To be more specific, the following groups are participating in our ANF-programme:

- The TNO-organization in Wageningen and Zeist.
  From the TNO-organization in Wageningen, the department ILOB, being the TNO Institute for Animal Nutrition and Physiology and the departments Biochemistry and Technology.
  From the TNO-organization Zeist the CIVO-department Analysis.

- The Agricultural University in Wageningen, departments of Animal Nutrition and Engineering.
  The research groups of TNO and the Agricultural University are active in the development of adequate ANF analysis, knowledge of the way ANFs are acting in the animal and ways to inactivate ANFs.

This research group is supported by a number of other institutes.
- State University in Utrecht, The Netherlands, department of Veterinary Pathology.
  The main research programme is focussed on study of pathological effects of various lectins in the animal.
- INRA Institute L.T.A.A. in Nantes, France.
  The main activities are the isolation and purification on large scale of trypsin inhibitors and lectins from peas.
- Oskar Kellner Institute in Rostock, German Democratic Republic.
  The main programme is focussed on the effects of ANFs on the secretion of endogenous protein and amino acids by use of the 15N technique.

Important parts of the ANF work in the Netherlands and France are incorporated in the ANF-programme of the European Eureka project "Improfeed".
The aim of this programme is improving the digestion and utilization of nutrients of feed ingredients for monogastric animals.
The Eureka project is guided by the Eureka scientific committee, consisting of four Dutch and four French representatives. We also welcome members of this committee.

Our workshop is specially focussed on the relation of ANFs in legume seeds and animal nutrition. It is evident from the programme of this workshop that the nutritional aspects are discussed in relation to various animal species like pigs, poultry, rats, guinea pigs, fish and even apes. Also some aspects of human nutrition are included in the programme. As far as I know this is the first ANF Workshop specially aimed at nutritional aspects. An important question in this respect is also, whether it is worthwhile to organize such a workshop every 3 or 4 years. Another point of discussion is, are we going to restrict ourselves to ANFs in only legume seeds or shall we extend the programme to include for instance, the catagory of glucosinolate-containing seeds. These points will also be discussed during the Workshop.
Finally, again we welcome you, and we hope that we have a succesful workshop.

ANTINUTRITIONAL FACTORS IN LEGUME SEEDS: STATE OF THE ART

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Introduction

The purpose of this presentation is to set the stage for this workshop by placing into perspective our current knowledge regarding the role of antinutritional factors (ANF) in animal and human nutrition, and, based on this information, to suggest possible directions for future research. Shown in Table 1 are examples of what might be considered the ANF which are likely to be of most significance to those who are involved in the use of legume seeds for the feeding of animals or in their use in the human diet. Time obviously does not permit me to cover each of the factors; I will confine my comments to those factors which I feel are of greatest concern to this audience. In a sense, this talk is somewhat premature in as much as many of the papers which will be presented at this workshop will no doubt contribute to a further advancement in our knowledge of ANF, and many of the unsolved problems which I will point out are in fact now being addressed. I therefore beg the indulgence of the speakers who follow me for any unintended preemption of their subject matter.

Protease inhibitors

Although the protease inhibitors are found in most legumes, those that are present in soybeans have received the most study (Liener and Kakade, 1980). Based largely on experiments involving the use of small animals such as the rat or chick, the growth-depressing effect of these inhibitors has been attributed to its effect on what is generally referred to as the negative feedback mechanism. Briefly, the inactivation of trypsin in the gut by trypsin inhibitors induces the intestinal mucosa to release cholecystokinin (CCK), a hormone which stimulates the acinar cells of the pancreas to produce more trypsin as well as other digestive enzymes such as chymotrypsin, elastase and amylase. The net result is an endogenous loss of protein rich in the S-containing amino acids leading to a depression in growth. At the same time the pancreas becomes enlarged due to hypertrophic and hyperplastic changes in morphology. Prolonged dietary exposure to raw soy flour culminates in the formation of adenomatous lesions in the pancreas of the rat (McGuiness et al., 1984; Liener et al., 1985).

Variation in species response

Most investigators have generally used trypsin of bovine origin to measure the trypsin inhibitor content of various legumes despite the fact that the nutritive value of the protein may have been evaluated in an entirely unrelated animal species. Yet in vitro studies on the inhibition of the proteases in the pancreatic juice of different animals have revealed marked differences in the degree to which these enzymes are inhibited by the protease inhibitors of various legumes (Belitz et al., 1982; Krogdahl & Holm, 1983; Rascon et al., 1985; Hanlon & Liener, 1986; Weder, 1986).

The presence or absence of the negative feedback mechanism also appears to be species dependent. Negative feedback control has been demonstrated to be operative in the rat, mouse, chicken, and hamster and is accompanied by an enlargement of the pancreas when these animals are fed raw soyflour. Other animals, however, such as the dog, calf,

pig, guinea pig, and monkey do not develop pancreatic hypertrophy despite the existence of the negative feedback mechanism, at least in the case of the pig and calf (Schneeman & Gallaher, 1986).

Experiments with human subjects have shown that the introduction of the Bowman-Birk inhibitor into the small intestine stimulates the release of digesive enzymes by the pancreas (Liener et al., 1988). The finding of an increase in plasma CCK following the feeding of a meal containing raw soy flour to human subjects (Calam et al., 1987) lends further evidence for the existence of the negative feedback mechanism in man. Other workers, however, have failed to detect an increase in plasma CCK in human subjects after the intraduodenal infusion of an extract of raw soybeans (Holm et al., 1988) or a synthetic trypsin inhibitor (Adler et al., 1988). It is evident that more research is needed in order to clarify the role of CCK in negative feedback mechanism as it relates to effect of various trypsin inhibitors on the pancreas of different species of animals.

Table 1. Examples of antinutritional factors in legume seeds.

	Distribution	Physiological effect
Proteins		
protease inhibitors	most legumes	depressed growth; pancreatic hypertrophy/hyperplasia; acinar nodules
lectins	most legumes	depressed growth; death
amylase inhibitors	most legumes	interference with starch digestion
Amino acid analogues		
β-N-oxalyl-α,β-propionic acid	Lathyrus sativus	lathyrism
β-N-methylamino-L-alanine	Cycas circinalis	neurotoxin
Glycosides		
cyanogen	lima bean	respiratory failure
vicine/convicine	Vicia faba	hemolytic anemia
cycasin	Cycas circinalis	carcinogen
oligosaccharides	most legumes	flatulence
saponins	most legumes	affects intestinal permeability
Miscellaneous		
phytate	most legumes	interference with mineral availability
tannins	most legumes	interference with protein digestibility
alkaloids	lupins	depressed growth

Species difference also appears to exist with respect to the carcinogenic effect on the pancreas of animals induced by the long-term feeding of raw soy flour. Although this effect can be readily demonstrated in the rat, other species of animals such as the mouse and hamster do not develop pancreatic nodules when fed raw soy flour for long periods of time (Liener & Hasdai, 1986). It should also be noted that raw soy flour (and presumably the trypsin inhibitor contained therein) potentiates in the rat the carcinogenic effect of subcarcinogenic doses of azaserine (McGuiness et al., 1981) and di(2-hydroxypropyl)nitrosamine (Levison et al., 1979). In terms of human exposure this raises the disquieting question as to whether the prolonged consumption of soy flour, in which the trypsin inhibitor may not have been completely inactivated, may sensitize the pancreas to the carcinogenic effect of chemical agents in our environment.

Aside from the rat (Rackis et al., 1975), information is lacking concerning the practical question of what constitutes a safe level of trypsin inhibitor in the diet for other animal

species including man. To provide such information will require studies with diets containing various levels of trypsin inhibitor activity using selected animal species. If standards are to be promulgated on the basis of such studies, a single, highly reproducible assay system for measuring trypsin inhibitor must be employed to replace the many different assay procedures now being employed by various investigators.

The significance of low levels of trypsin inhibitor activity

A question that will no doubt arise in connection with the measurement of trypsin inhibitor activities is the interpretation that should be placed on low levels of activity that are most frequently encountered with processed legumes. It is generally assumed that low residual trypsin inhibitor activity is due to the Bowman-Birk inhibitor which is regarded as a relatively heat-stable molecule. It is entirely possible that low levels of trypsin inhibitor activity may also be due to non-specific inhibition by phytate, tannins, fatty acids, or saponin. What is needed is an assay system which will specifically identify and quantitate the various trypsin inhibitors. The answer to this problem may lie in the development of specific immunoassay techniques such as an ELISA method recently described by Brandon et al. (1988) for the determination of the Kunitz soybean inhibitor, or by a procedure which involves the specific absorption of trypsin inhibitors by affinity chromatography (Roozen and deGroot, 1987).

Should trypsin inhibitors be eliminated by breeding?

Finally, we might raise the question as to whether the elimination of protease inhibitors by genetic manipulation (Hymowitz, 1986), is really a desirable goal. Are we willing to develop trypsin inhibitor free cultivars of legumes which may have enhanced nutritional properties at the expense of depriving such plants of their defense mechanism against insect and microbial predation? Nor is this the only consideration. In many legumes the Bowman-Birk inhibitor with its high content of cystine provides as much as 40% of the total amount of the S-amino acids of the total protein of the bean (Kakade et al., 1969). Thus, the genetic elimination of these inhibitors would merely serve to exacerabate the already critical deficiency of the S-containing amino acids of the bean protein. A procedure for inactivating the trypsin inhibitors of soybeans by chemical modification involving disulfide interchange (Friedman & Gumbmann, 1986) may provide an alternative solution to this problem.

Other legumes

The trypsin inhibitor content of most other legumes is considerably less than that of soybeans (Newton & Hill, 1983), but it has been shown that in many cases the feeding of the trypsin inhibitors purified from such beans will in fact depress growth and cause pancreatic hypertrophy in much the same way as the soybean inhibitors (Liener & Kakade, 1980). However, the extent to which the trypsin inhibitors of such legumes contribute to the poor quality of the raw bean is difficult to assess because of the concomitant presence of other growth inhibitors such as lectins, tannins, and perhaps other yet unidentified factors.

Lectins

Paralleling the distribution of protease inhibitors in legumes are the lectins, proteins which are characterized by their unique ability to bind to specific sugars or glycoproteins. This reaction is manifested in vitro by the agglutination of red blood cells from various species of animals. More importantly from a nutritional point of view is the fact that, by binding to the epithelial cells lining the small intestine, a series of complex events ensues which culminates in severe growth depression and ultimately in the death of the animal. The biological effects induced by the feeding of lectins derived from kidney beans and other legumes has been the object of considerable study (Liener, 1986; Pusztai, 1987) and

include the following: an impairment in transport of nutrients across the intestinal wall, intestinal hypertrophy accompanied by an increased rate of synthesis of mucosal protein, increased catabolism of liver and muscle protein, a lowering of blood insulin levels, and an inhibition of brush border hydrolases.

The presence of lectins in legumes is most often detected and quantitated by the in vitro agglutination of a suspension of red blood cells from various species of animals, the cells having been sensitized by pretreatment with trypsin or some other protease. Although in vivo toxicity of cultivars of P. vulgaris to mice and rats could be correlated with hemagglutinating activity towards trypsinated cow cells (Jaffe & Gomez, 1975; Grant et al., 1983), there is no assurance that these results can be extrapolated to other species of animals. Assuming that the toxicity of lectins towards a given species of animal is associated with its ability to bind to the intestinal mucosa of that species, what is needed is an in vitro method that will measure such binding activity. To this end Hendriks et al. (1987) have recently described an ELISA assay which permits the quantitative determination of the lectin-binding capacity of the small intestinal brush-border membrane. Although their model system involved the use of the small intestine of the cow and the soybean agglutinin in the form of a conjugate with peroxidase, this method could conceivably be adapted to the measurement of the binding of the lectins of various legume extracts to the brush-border membrane of any animal species. The predictive value of such an assay should, however, be verified by feeding the same legume to an appropriate animal species with special emphasis on the histopathological effects on the small intestine.

As far as the significance of lectins in the human diet is concerned, Bender & Reaidi (1982) have pointed out the deleterious effects associated with the consumption of raw or inadequately cooked beans, an effect which they attributed to the lectins. Still not completely understood is the role which wheat germ lectins may play in the etiology of coeliac disease (Kottgen et al., 1983; Kolberg & Solid, 1985).

In view of the proposed roles which lectins play in the defense mechanism of legumes (Jansen et al., 1976) and in the symbiotic relationship between legumes and N-fixing bacteria (Etzler, 1986), one might question the desirability of eliminating lectins by genetic manipulation. Reports of undetectable levels of lectins in some legume cultivars must be accepted with reservation since failure to detect lectin activity may be simply due to the use of an assay system which does not provide the specific receptors required for the expression of their activity. Here again the use of intestinal preparations would provide a much more relevant prediction of the potential toxicity of such legumes.

Phytate

Phytate, the salt of phytic acid, is a cyclic compound (inositol) containing six phosphate radicals. It is present to the extent of 1% to 5% of the dry weight of legume seeds. Its physiological significance lies in the fact that it readily chelates with di- and tri-valent metal ions such as calcium, magnesium, zinc, and iron to form poorly soluble compounds that are not readily absorbed from the intestines. Thus phytate has generally been regarded as an antinutritional factor which interferes with the bioavailability of minerals from plant sources (Reddy et al., 1982; Forbes & Erdman, 1983). It has been shown that high dietary calcium accentuates the effect of phytate on zinc bioavailability. The formation of Zn-Ca-phytate complexes in the upper intestinal tract of monogastric animals is believed to be a major mechanism by which phytate reduces zinc bioavailability. The bioavailability of zinc can be best be predicted by the expression: phytate x calcium/zinc molar ratio (Fordyce et al., 1987).

Although the ability of phytate to interfere with the availability of minerals accounts for its major antinutritional effect, phytate has also been shown to interact with the basic residues of proteins. It is not surprising, therefore, that phytate inhibits a number of digestive enzymes such as pepsin, pancreatin, and α-amylase. Inhibition may also result from the chelation of calcium ions which are essential for the activity of trypsin and α-amylase, or possibly to an interaction with the substrates for these enzymes. To what extent the inhibition of enzyme activity by phytate contributes to its overall antinutritional effect remains uncertain.

The phytate content of legumes can be reduced by taking advantage of the endogenous enzyme, phytase, which accompanies phytate in separate compartments of the plant tissue, or by providing an exogenous source of the enzyme from microbial sources (Liener, 1987). Thus the phytate content of various beans can be greatly reduced by simply allowing aqueous suspensions of the ground beans to undergo autolysis under appropriate conditions of time, temperature, and pH. Germination results in an increase in phytase activity which leads to a concomitant reduction in phytate levels. Fermented beans likewise have reduced levels of phytate due to the action of the phytase elaborated by various microorganisms involved in the fermentation process.

Tannins

Most legumes are known to contain appreciable levels of polyphenolic substances broadly referred to as tannins. The latter may be either hydrolyzable tannins, so called because they may be readily hydrolyzed into a mixture of carbohydrate and phenols, or condensed tannins which are complex flavonoid polymers. From a nutritional point of view the condensed tannins are the most significant,. Among the antinutritional effects attributed to these tannins is a decrease in the digestibility of the protein and carbohydrate as a result of the formation of insoluble enzyme-resistant complexes with tannins (Reddy et al., 1985). Also, as a result of the complexation of tannins with protein, enzyme reactions involved in digestion are markedly retarded by direct inhibition of the enzymes themselves. Other antinutritional effects which have been attributed to tannins include damage to the intestinal tract, toxicity of tannins absorbed from the gut, an interference with the absorption of iron, and a possible carcinogenic effect.

Rats and mice adapt to dietary tannins by induced synthesis of proline-rich salivary proteins (Mehansho et al., 1987). Since the latter show a strong affinity for tannins, these proteins may serve as a defense mechanism against the antinutritional effects of tannins. To what extent this mechanism is operative in other species of animals is not known.

Lupin alkaloids

Although alkaloids are widely distributed in the plant kingdom, the alkaloids found in lupins are of special concern to nutritionists because the lupins may provide a cheap and nutritious source of protein for animal feed and has been used as an ingredient in the human diet (Petterson, 1985). The indiscriminate use of lupins must be avoided since there are present in many species as many as twelve different alkaloids which exhibit varying degrees of toxicity (Petterson et al., 1987). However, the availability of lupin species with low alkaloid content and the virtual absence of trypsin inhibitors and lectins have given added impetus to the use of lupins.

One of the major uses of lupin seed is in the diet of pigs who can tolerate up to 0.03% of lupin alkaloids in the diet (Pearson & Carr, 1977). For human use the National Health and Medical Research Council of Australia has recommended an upper limit of 0.02% alkaloid content for lupins. Analytical methods for the determination of lupin alkaloids thus assume a very important role in assessing the safety of lupins in the diet of man and animals. A rapid colorimetric method is most commonly used for monitoring the alkaloid content of lupins (Ruiz, 1976), but this method does not distinguish among the various alkaloids. In view of the fact that the lupin alkaloids vary quite widely in terms of their toxicity (Petterson, 1985), it becomes important to be able to identify the various alkaloids that are present in a particular sample of lupin seed intended for feeding purposes. Among the various methodologies which have been proposed for the identification and quantitation of lupin alkaloids, capillary gas chromatography (Priddis, 1983) would appear to have the most sensitivity and accuracy, but obviously has its limitations for the rapid screening of lupins.

Saponins

Saponins are steroid or triterpenoid glycosides which are characterized by their bitter taste, foaming properties, and their hemolytic effect on red blood cells. They are widely distributed among plants and are present in significant levels in such legumes as alfalfa, soybeans, chick peas, and in many varieties of P. vulgaris. Much of the past research on saponins has dealt with the alfalfa saponins which reduce the growth rate of chicks, rats, and mice, but, with the exception of soybean saponins, little information is available concerning the antinutritional effects of the saponins in other legume seeds (Cheeke, 1976). Soybean saponins apparently have little effect on the growth of experimental animals; neither saponin or sapogenin could be detected in the blood of chicks, rats, and mice following the oral administration of soybean saponin (Gestetner et al., 1968). Some saponins from non-edible plants readily increase the permeability of the small intestinal mucosal cells thereby inhibiting the active transport of nutrients, but, at the same time, facilitating the uptake of materials to which the gut would normally be impermeable (Johnson et al., 1986). It is significant to note, however, that soybean saponins were much less effective in this respect.

Conclusion

The aim of this presentation has been to provide an overview of our present state of knowledge concerning some of the more important factors which serve to limit the nutritional potential of legumes as a valuable source of protein for animals as well as man. In so doing, it is obvious that there are many questions begging to be answered. It is the hope of the organizers of this workshop that, as a result of this conference, some of these answers may be forthcoming. Perhaps the most valuable outcome of this workshop will be to direct our attention to those areas which are in need of further research.

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Session lectins

BIOLOGICAL EFFECTS OF DIETARY LECTINS

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Summary

Most dietary lectins are resistant to gut proteolysis to an appreciable but variable extent. By binding to membrane receptors of epithelial cells of the small intestine and subsequent endocytosis, lectins may interfere with the digestion and absorption of nutrients, increase wasteful protein and glycoprotein synthesis and secretion, speed up cellular turnover, cause hyperplasia and modify gut immune function. A combination of some or all of these effects reduces the efficiency of nutrient utilization. As a proportion of all dietary lectins, even those which are relatively non-toxic, is systemically absorbed, lectins may affect immunity, the endocrine system and general metabolism. Thus, both soya (SBL) and kidney bean (PHA) lectins reduce blood insulin concentration for the entire period of feeding, while glucagon levels are increased by PHA. As, despite low insulin levels, rats maintain near normal blood sugar concentrations, other endocrine organs and their hormone secretion may also be affected by PHA. The net result of all the changes in hormone levels shifts the balance of general metabolism towards increased catabolism i.e. increased lipolysis, muscle breakdown and mobilization of liver glycogen. Accordingly, through the direct effects of systemically absorbed lectins or, indirectly, through the modulation of the endocrine system by gut hormones, all lectins introduced into the alimentary tract may interfere with metabolic processes of the body. With most lectins studied to date the changes induced lead to increased catabolic breakdown of all body components, proteins, lipids and carbohydrates, retard growth and affect health.
Keywords: lectins, antinutrients, gut-effects, systemic effects, growth.

Introduction

As lectins are found in most, if not all plant seeds and vegetative tissues, not surprisingly, foods derived from most cultivated plants also contain lectins. The few *ad hoc* surveys conducted in the last ten years have confirmed this. For example, Nachbar and Oppenheim (1980) found that about 30 per cent of fresh and some processed plant foods tested contained detectable haemagglutinating activity. A literature survey by the same authors showed that another 53 plants used as human foods also contained lectins. More recent surveys confirmed the ubiquitous presence of lectins in plants eaten by man and his farm animals (Grant *et al.* 1983; Liener, 1986), accordingly, the gut of both humans and animals is exposed to dietary lectins continuously (Pusztai, 1985; 1986a,b; Liener, 1986).

Abbreviations: PHA, *Phaseolus vulgaris* lectin; SBL, soyabean lectin; WGA, wheat germ agglutinin; con A, concanavalin A

Some of the dietary lectins have adverse effects on the growth and health of animals, while others may apparently be much less deleterious. It is thus of great importance to find out which of the ingested lectins interfere with the proper functioning of the digestive system, what are the main features of the reaction mechanism of the lectin-gut interactions and, most importantly, how to counteract their harmful effects on nutritional performance or, hopefully, exploit their potential beneficial properties.

Most studies up to date have been confined to the gastroenterological and nutritional properties of a few nutritionally somewhat deleterious lectins, such as PHA, con A, WGA, SBL or the lectin from the tropical legume, winged bean (*Psophocarpus tetragonolobus*). Unfortunately, we know practically nothing at all about the effects of potentially beneficial lectins, such as the tomato lectin (Kilpatrick *et al.* 1985) or their use and exploitation.

Lectins are heat-sensitive proteins. Accordingly, the biological activity of most lectins can be abolished by proper heat treatments (Pusztai, 1985). However, heat processing is expensive and, moreover, it is not always very effective. For example, in a recent survey all commercially available soya products were shown by ELISA methods to contain lectins and some contained almost as much as raw soya seeds (Prince *et al.* 1988). Thus, for the establishment of more effective and less costly strategies for the optimum utilization of lectin-containing legume seeds by monogastric animals a thorough understanding of the main features of lectin-gut interactions (Table 1) is needed.

A. LECTIN-GUT INTERACTIONS

Table 1. Main features of lectin-gut interactions.

1. Most lectins resist breakdown in the digestive system.
2. Resistant lectins bind to surface receptors of the digestive tract.
3. Receptor-bound lectins are endocytosed and induce changes in epithelial cell metabolism.
   a. Hypertrophy-hyperplasia
   b. Increased polyamine metabolism, increased absorptive capacity, etc.
4. Lectins may affect gut endocrine cells and gut hormone production.
5. Lectins may induce changes in the local (gut) immune system.
6. Lectins may interfere with the bacterial ecology of the gut, including the small intestine through increased adhesion and selective overgrowth.

1. The effects of gut proteolysis on dietary lectins

Most lectins, if not all, are resistant to proteolytic breakdown in the small intestine. Although the degree of this resistance may vary from lectin to lectin, it is usually appreciable (Pusztai, 1986a; Pusztai *et al.* 1986; Banwell *et al.* 1983; Hara *et al.* 1984). For example, up to 90 per cent of the PHA fed to rats could be recovered from the faeces fully reactive with specific anti-lectin antibodies and with its lectin activity essentially intact (Pusztai, 1980). Similar findings were made with con A (Nakata & Kimura, 1985). In fact, it is likely that resistance to proteolysis is one of the first prerequisites of toxicity. All the same, not all resistant lectins are toxic. For example, the resistant tomato lectin is essentially non-toxic (Kilpatrick *et al.* 1985).

2. Lectin binding to epithelial cells of the small intestine

Interactions of food lectins with the digestive tract begin in the oral cavity. Over half of the hot or cold aqueous extracts prepared from common foods, fruits, vegetables and seeds agglutinated erythrocytes and cells of *Streptococcus mutans* strains and reacted with human saliva (Gibbons & Dankers, 1981). Moreover, these extracts inhibited the adhesion of *S. mutans* or *sanguis* strains to saliva-coated hydroxyapatite beads. In turn, this effect can be inhibited by specific sugars, indicating that binding to teeth of the common bacteria of the oral cavity is due to bacterial adhesins (lectins). The extent of bacterial adhesion is determined by a competition between food lectins, saliva and bacteria. Indeed, it has been suggested that as salivary mucins can selectively inhibit the interactions between food lectins, bacteria, teeth and buccal epithelial cells, one of their main functions is to reduce the extent of bacterial adhesion (Gibbons & Dankers, 1981). Thus, both food and bacterial lectins are important components in the bacterial ecology of the oral cavity.

One of the main constituents of epithelial cell membranes facing the lumen of the gut are carbohydrates. However, the membranes of different structural and functional compartments in the small intestine express different types of carbohydrate structures. Membrane proteins of the less differentiated crypt cells with mainly polymannose type oligosaccharide side chains acquire more complex sugar structures as they differentiate, mature and move up the villus. Accordingly, depending on their sugar specificity, lectins react with different functional compartments of the small intestine (Etzler & Branstrator, 1974). Thus, lectins with D-mannose and/or D-glucose specificity, such as con A, pea or lentil lectins bind preferentially to the lower half of the absorptive villi. Similar binding patterns are shown by lectins specific for N-acetylglucosamine and its oligomers (WGA, tomato and potato lectins, etc.) Lectins, such as PHA, soyabean or winged bean lectins, with sugar specificities for complex type oligosaccharide side chains, bind mainly to the upper part of the absorptive villi containing highly differentiated mature absorptive and other cells. As a result of different binding sites, the effects of different lectins may vary. For example, PHA or SBL, by binding to the upper half of the villi are known to interfere with the absorption of nutrients (see, for example, Liener, 1986) and lead to extensive damage to the brush border of the proximal small intestine of rats (King *et al.* 1980a,b; 1982) and pigs (King *et al.* 1983; Begbie & King, 1985). In contrast, neither PHA nor SBL binds to or damage cells of the lower parts of the villi or the crypts. However, by increasing the length of the proliferative compartment while reducing villus height only slightly, these changes induced by PHA and SBL represent a highly significant reduction in the villus/crypt ratio (Pusztai, Ewen, Grant & Bardocz, unpublished) reflecting the activation of the cell-proliferative compartment of the small intestinal epithelium (Fig. 1a,b). Thus, both PHA and SBL are growth factors for the small intestine and induce cellular proliferation in the crypts. Although the reaction mechanism by which this proliferation is induced, is not entirely clear, it appears to be mediated by a polyamine-dependent signal transduction process which is initiated by the binding of these lectins to and their endocytosis by the brush border cells (Pusztai *et al.* 1988).

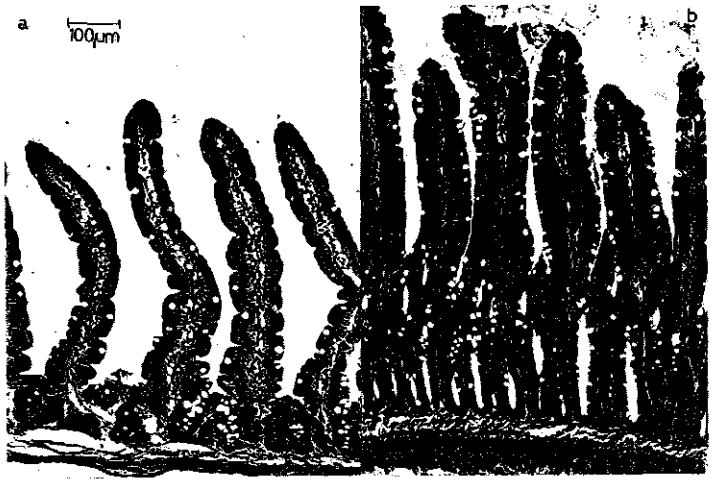

Fig.1. Light micrographs of the jejunum of rats fed on (a) control and (b) PHA-containing diets for seven days.

The results obtained with other lectins indicate that, despite some differences, there are great similarities in interactions between the gut and most lectins. For example, both con A and WGA injected into intestinal loops caused increased shedding of brush border membranes, accelerated cell loss and, by shortening the length of the villi, reduced the absorptive area of the small intestine (Lorenzsonn & Olsen, 1982). Unfortunately, the precise location of the binding sites of these lectins on the epithelium is not well known. However, the severe reduction of villus height due to con A and WGA may suggest that mannose/glucose specific lectins do not bind to the same sites as PHA and SBL.

The morphological changes observed in human coeliacs may also be lectin-related in their origin (Weiser & Douglas, 1976). As it is envisaged, the polymannose-specific lectin components of gluten-containing cereal proteins (Kottgen *et al.* 1982) and/or residual WGA (Kolberg & Sollid, 1985) first bind to surface carbohydrates of immature coeliac enterocytes (Auricchio *et al.* 1984). The ensuing changes which involve the major histocompatibility complex and cell-mediated immune hypersensitivity reactions to gluten proteins lead to a reduction in and extensive damage to the absorptive villi with a consequent depression of the absorptive potential of the gut.

3. Effects of lectins on cellular metabolism in the small intestine

After their initial binding to membrane receptors, cells take up dietary lectins by endocytosis. Thus, an appreciable part of the orally given PHA was shown inside the cells of the epithelium by indirect immunofluorescence. By immunogold staining, the endocytosed lectin was localized intracellularly in endosomic vesicles, large endosomes and lysosomes of surface cells (King *et al.* 1986). This receptor-mediated endocytosis is not confined to dietary PHA but, apparently, it occurs generally, as regardless of their sugar specificities, all lectins are preferentially endocytosed by epithelial cells (de Aizpurua & Russel-Jones, 1988). The rate of uptake of lectins by these cells was at least 4 to 5 orders of magnitude faster than that of other food antigens (Pusztai, 1988).

One of the earliest events after the transport of PHA into epithelial cells is an immediate increase, by about 80 per cent, of protein synthesis rate (Palmer *et al.* 1987). This is the opposite of what happens with cytotoxic lectins, such as ricin and abrin, which after endocytosis inactivate the 60 S ribosomal subunits and inhibit protein synthesis in eukaryotic cells (Olsnes *et al.* 1974). Although PHA-type lectins do not inactivate ribosomal subunits, the immediate stimulation of protein synthesis by PHA may still indicate that a ribosomal reaction occurs, possibly by sequestering inactive monosomes for the assembly of functional polysomes.

On prolonged feeding with PHA, the cellular metabolism of the small intestinal brush border is maintained at a highly elevated level. There is a continous stimulation of protein, DNA and RNA synthesis for the entire period of PHA exposure, the extent of which depends on the amounts of PHA in the diet (Oliveira *et al.* 1988). For example, the weight of the small intestine of rats fed on diets containing 5 per cent raw kidney bean protein doubled after ten days (Greer *et al.* 1985). The protein and DNA content of the small intestine increased by about 40 to 50 per cent, while its sugar (mucinous secretions) content doubled. No such increase occurred when the diet contained equivalent amounts of denatured PHA (Oliveira *et al.* 1988). Other lectins may have similar effects. For example, con A caused hypersecretion of mucus in both gut and nasal passages (Freed, 1982), or, when intrapharyngeally infused, it increased jejunal crypt cell production rates substantially (Weaver & Bailey, 1987). Pure soyabean lectin has also been shown to enlarge the small intestine in a dose-dependent way, and its effectiveness approached that of PHA (Grant *et al.* 1987b). Thus, despite retarding body growth, most lectins are powerful growth stimulants for the small intestine by inducing cellular hyperplasia (see Bardocz *et al.* in this issue).

4. Interaction of lectins with gut endocrine cells

Endocrine cells in the proximal small intestinal epithelium have been observed to bind dietary PHA in a manner similar to the binding of the lectin by other epithelial cells (King, Pusztai & Grant, unpublished). Moreover, PHA was visualized by fluorescent antibody staining intracellularly in gut endocrine cells. Unfortunately, there is no firm experimental evidence to indicate the extent of the involvement of gut endocrine cells in the toxic effects of lectins, although the dramatic changes in systemic endocrine organs and hormone levels on prolonged feeding with kidney bean may, at least in part, be due to indirect effects of gut hormone secretion induced by PHA binding to mucosal endocrine cells. The highly significant enlargement of the pancreas in rats given pure PHA or SBL may, in fact, be such an indirect effect mediated by gut hormones (see under systemic effects).

5. Effects of lectins on the local (gut) immune system

Several lectins are known to cause direct degranulation of mast cells *in vitro* by a mechanism not related to the production of reaginic antibodies. Similar reactions may also occur *in vivo*. The observed increased exudation of ^{125}I-labelled rat serum proteins into the small intestine of rats orally challenged with pure PHA may indeed be such an *in vivo* effect of the lectin on submucosal mast cells and the consequent increase in vascular permeability (Greer & Pusztai, 1985). This immediate and direct effect of PHA on gut mast cells could be amplified by prolonged feeding with kidney bean diets before the oral challenge with pure PHA. The anaphylactic reaction also showed a memory effect. Thus, vascular permeability on oral challenge with PHA was still higher in rats fed for five days on control diets after an

initial period of six days on a kidney bean diet, than in those animals which had been kept on the control diet for the entire eleven days of feeding (Greer & Pusztai, 1985). Accordingly, the immediate (Type-1) hypersensitivity and mild gut anaphylaxis, may contribute to the overall inefficiency of utilization of dietary proteins and the toxic effects of PHA.

Further disturbances in the proper functioning of the gut immune system have been suggested by the results of long-term experiments in which rats were fed alternately on bean proteins or casein for five days on each diet, followed again by bean-feeding. This switching of diets could be continued for 6 to 8 weeks without observing any signs of adaptation to the bean diets (Pusztai, 1980). The growth depression found with bean diets on first exposure was just as severe at the end of the diet-switching experiment as at the beginning, suggesting that the local s-IgA level in these rats was not sufficiently high to neutralize the toxic effects of the lectin. Unfortunately, neither the precise levels of or the changes in s-IgA concentrations on exposure to PHA have been established. Moreover, the underlying mechanism of the interaction of PHA with the gut immune system is not known either. Nevertheless, the existence of a somewhat general deficiency in s-IgA production in the gut is indicated by the occurrence of extensive bacterial proliferation in the small intestine.

6. Interference with the bacterial ecology of the small intestine

It has been recognised for some time that kidney bean-containing diets are more toxic for conventional than for germ-free animals (Jayne-Williams & Hewitt, 1972; Rattray *et al.* 1974). For example, while conventional rats died after a few days on diets containing 10 per cent kidney bean proteins, gnotobiotic rats of the same strain utilized about 30 per cent of these proteins and survived (Table 2). The large amounts of undegraded material accumulating in the small intestine caused by the interference with digestion and absorption and the increase in the amounts of secreted mucins on feeding rats with PHA-containing diets may facilitate bacterial overgrowth. In turn, the presence of ever-increasing numbers of bacteria further reduces the efficiency of the absorption of nutrients. Thus, PHA has been shown to cause an extensive proliferation of *E. coli* in the small intestine (Wilson *et al.* 1980; Banwell *et al.* 1983; 1985). That this is the direct result of PHA in the lumen is shown by the absence of overgrowth of *E. coli* when, Pinto III, a low-lectin bean is fed to the rats.

The bacterial proliferation may not necessarily be just the indirect result of the increased availability of substrates for the bacteria. As PHA contains high-mannose carbohydrate side-chains, its binding to the gut may provide new receptors for *E. coli* adhesion through its mannose-specific fimbrial lectins (Lis & Sharon, 1986).

The contribution of bacteria to the overall nutritional toxicity of the lectin may be more extensive than simply a competition for nutrients. Thus, endocytosis of PHA by epithelial cells is more extensive in conventional than in germ-free rats (King, Pusztai, Grant & Clarke, unpublished). Brush border cells of germ-free rats can still bind PHA with resulting damage to their microvillous membrane, just like that which occurs in conventional rats. Accordingly, the nutritional performance of even germ-free rats is poor on bean diets (Table 2). The reduction in the amounts PHA endocytosed in germ-free rats, however, lessens the extent of wasteful increases in cellular metabolism and protein, DNA and RNA syntheses which occur in conventional rats. Consequently, the utilization of bean diets by germ-free rats is superior to that of conventional rats.

In conclusion: The overall effects of the structural, biochemical and immunological lesions and bacterial proliferation induced by dietary

Table 2. Nutritional performance of (a) conventional or (b) germ-free rats on raw bean-protein (containing 10 per cent total protein).

Diet	Food intake (g/d)	Weight change (g/d)	True digestibility (%)	NPU (%)
(a)				
Casein	8.8	+ 3.3	98	90
Bean (½) + Casein (½)	4.1	- 0.9	61	20
Bean	2.8	- 5.3	All rats died after 3 days	
(b)				
Casein	6.9	+ 2.3	98	85
Bean (½) + Casein (½)	4.9	+ 1.5	85	66
Bean	4.3	+ 0.2	73	29

lectins, either directly by interfering with the digestion/absorption of food or, indirectly, by using up a large proportion of dietary proteins and energy and increasing cellular turnover and proliferation, result in reduced utilization of the diet with consequent growth retardation and poor health.

B. SYSTEMIC EFFECTS

A proportion of the dietary PHA endocytosed by brush border cells is eventually transported into the systemic circulation and internal organs of the body. Measurements of the amounts of ^{124}I-labelled PHA showed that up to 10 per cent of the toxic lectin applied intragastrically reached the blood circulation within 3 hours of the initial application (Pusztai, 1988). In contrast, the amount of systemically absorbed non-toxic tomato lectin was less than 0.1 per cent of the initial dose (Kilpatrick *et al.* 1985). Most of the absorbed PHA was bound to serum glycoproteins initially. With time, an increasing proportion became attached to blood cells, first reversibly, then gradually irreversibly as it could not be eluted from blood cells with a phosphate-buffered saline solution of fetuin, suggesting that the lectin may have been endocytosed by the cells (Pusztai, 1988).

1. Development of circulating IgG-class anti-lectin antibodies

One of the results of the systemic absorption of dietary PHA and its binding to blood cells, including immunocompetent lymphocytes, is that all animals tested so far have developed exclusive and powerful humoral anti-lectin antibodies of the IgG-class (Pusztai, 1980; Pusztai *et al.* 1981; 1983; Grant *et al.* 1985; Williams *et al.* 1984; Begbie & King, 1985) even when fed on raw beans. The absence of antibody response to other dietary proteins argues in favour of the specificity of the PHA absorption through the intestine and the immuno-suppressant effects of PHA for other antigens.

Mice and rats fed on PHA-containing diets for several weeks have been shown to develop a systemic immediate-type hypersensitivity due to the production of circulating IgE-class antibodies specific for the lectin (Pusztai *et al.* 1983). It is, not yet clear however, what contribution such an allergic hypersensitivity to PHA, and possibly to other lectins, makes to overall nutritional toxicity.

Table 3. Systemic effects of dietary lectins.

1. Development of circulating anti-lectin IgG-class antibodies.
2. Thymus atrophy with potential effects on cellular immunity.
3. Hypertrophy of the pancreas and liver.
4. Muscle atrophy.
5. Modulation of the endocrine system and increased catabolism of body protein, fat and carbohydrate.

2. Thymus atrophy

The weight of the thymus of rats fed on diets containing kidney bean protein decreased very quickly and after ten days the organ almost disappeared (Greer *et al.* 1985). The atrophy is caused specifically by PHA as the inclusion of pure PHA in egg albumin-based diets reproduced all the effects found with kidney bean (Oliveira *et al.* 1988). Although the significance of thymus involution is not known, it may have very important consequences for the functioning of the immune system. Other lectins, however, may not be as effective as PHA for inducing thymus atrophy. For example, SBL has no effects on the thymus even after 16 days of feeding rats with soya diets (Grant, Stewart, Watt & Pusztai, unpublished).

3. Liver and pancreas hypertrophy

Apart from PHA, no other lectins are known to affect both of these two important organs. Thus, although SBL induces pancreatic enlargement, it has no effect on the liver. In contrast, pure PHA has been shown to cause a slight, but significant dose-related increase in the weight of the liver (Oliveira *et al.* 1988). Since its lipid concentration was normal and the glycogen content reduced, the enlargement of the liver may have been due to accumulation of protein (Oliveira, Pusztai & Grant, unpublished). The physiological implications of these changes, however, are unclear. In addition, PHA also induces a dose-related pancreatic enlargement (Oliveira *et al.* 1988). The weight increase of the pancreas of rats which received 60 mg of pure PHA per day was almost as high as that of those rats which received a similar amount of lectin from bean diets. The reaction mechanism by which PHA mediated the enlargement is unknown. However, as insulin levels in PHA-fed rats are low (Grant *et al.* 1987c) it is unlikely to be similar to the pancreatic enlargement induced by trypsin inhibitors via a CCK-mediated pathway which leads to increased insulin production (Bonnievie-Neilsen, 1981).

Although the effects of dietary SBL on the liver are apparently negligible, it produces an appreciable enlargement of the pancreas (Grant *et al.* 1987a) due to both cellular hyperplasia and hypertrophy. The raction mechanism of these changes is, however, unknown. All the same, as with PHA and in contrast to what is seen with trypsin inhibitors, SBL depresses the concentration of circulating immunoreactive insulin.

4. Muscle atrophy

One of the effects of feeding rats on PHA-containing diets is an increased output of N through the urine indicating increased tissue catabolism (Pusztai *et al.* 1981). Direct experimental evidence for the origin of tissue protein breakdown was recently obtained by showing that with

increasing bean content in the diet there was a progressive reduction in the muscle-to-body weight ratio (Palmer *et al.* 1987). Fractional rates of protein synthesis in skeletal muscles were reduced significantly while the rates of degradation were unchanged. The net effect was a significant loss of muscle mass. Moreover, by reproducing the effect of bean diets with pure PHA, the muscle loss was clearly attributed to the lectin (Oliveira *et al.* 1988).

The strong catabolic effect of PHA on muscles may not apply to all lectins. For example, although partially purified SBL preparations, such as a soya whey fraction, induced a loss of skeletal muscle similar to that described for PHA, the pure lectin was without such effect (Grant *et al.* 1987b). Trypsin inhibitors in the whey fraction were also inactive in this respect. The lectin-depleted soya whey fraction, however, retained its activity and was as effective in reducing the weight of skeletal muscle as the original whey fraction. Unfortunately, the factor(s) responsible for muscle loss is unknown.

5. Modulation of the endocrine system and altered body metabolism

The presence of PHA in diets has profound effects on systemic metabolism. In addition to the loss of muscle, body lipid and glycogen losses were also increased (Grant *et al.* 1987c). In fact, as the decrease in the body lipid proceeded faster than any other changes, depending on the amounts of lectin in the diet, the relative concentration of body protein increased, despite the absolute loss (Pusztai, 1988). Increased lipid catabolism was also indicated by an appreciably elevated output of 3-hydroxybutyrate in the urine, while lipid absorption in the gut was reduced only slightly. The lipid loss was mainly confined to triglycerides from subcutaneous tissues of skin and tail, while the lipid content of internal organs, such as the liver, remained unchanged. Likewise, there was no change in the phospholipid content of the body. Simultaneously, the glycogen stores of the liver were also reduced. At one per cent concentration of PHA in the diet, the glycogen content of the liver was halved, while muscle glycogen was unaffected (Pusztai, 1988).

Although the underlying mechanisms of these changes in systemic metabolism are unknown, there is a general shift towards increased catabolism. This is in accord with what is known about the levels of hormones which, at least in part, have a controlling influence over metabolism. Thus, pure PHA applied intragastrically had an acute insulin-lowering effect. Moreover, circulating immunoreactive insulin levels are severely depressed in rats fed on diets containing kidney bean, PHA or soyabean (Pusztai, 1986; Pusztai *et al*, 1986; Grant *et al.* 1987c). Serum glucagon concentrations, on the other hand, showed an opposite trend. However, despite the low circulating insulin concentrations the rats were not diabetic and, their blood sugar concentration , if anything, was slightly below normal. It appears that mobilization of lipids and increased muscle catabolism due to the low insulin levels may serve to keep the concentrations of liver glycogen and blood sugar at or near physiological levels. However, as extended periods of feeding with kidney bean diets exhausted depot lipids and muscle atrophy was advanced, rats fasted overnight could no longer generate the substrates necessary for gluconeogenesis (glycerol and amino acids). Without the input of glucose from food, its concentration in blood dropped to perilously low levels. In contrast, rats fed the same amount of food but no proteins maintained physiological blood sugar concentrations (Pusztai, 1988).

Finally, the precise mechanism of the interaction between dietary lectins and the endocrine system is not understood as yet. However, reaction of PHA and possibly other lectins with gut endocrine cells may have an indirect effect on the pancreas and other endocrine organs through the enteroinsular

axis. Alternatively, the comparatively substantial amounts of systemically absorbed PHA (and other lectins) may directly interact with the endocrine system. Indeed, both processes may occur simultaneously.

In conclusion: since most lectins are resistant to gut proteolysis to an appreciable extent, they may react with components of the digesta and bind to membrane receptors of epithelial cells of the small intestine. This binding and the ensuing endocytosis may induce serious changes in the structure of the absorptive epithelium, interfere with the processes of digestion and absorption of nutrients and disrupt the metabolism of brush border cells. As a result, wasteful protein synthesis and secretion of glycoproteins may be stepped up significantly and cellular turnover speeded up with a general cellular hyperplasia occurring. Lectins may interfere with the proper functioning of the gut immune system and cause type-1 hypersensitivity reactions. Overall, these effects lead to poor utilization of nutrients, particularly proteins, and to large losses of dietary and endogenous components. Lectins may also affect systemic immunity and general metabolism with the balance of body metabolism shifting towards a general catabolic breakdown of all body constituents, lipids, carbohydrates and proteins. Apparently, most of these changes are dependent on either the direct effects on endocrine function of systemically absorbed lectins or on their indirect effects through hormones produced by gut endocrine cells. Some lectins may affect cell-mediated immunity and induce systemic hypersensitivity reactions. As different lectins may not necessarily induce all these effects, the severity of their nutritional toxicity may vary. With more toxic lectins, such as PHA, con A, winged bean lectin and, to some extent, SBL, the overall balance of the effects has serious consequences for the utilization of food, proper growth and health. Although other lectins may be less deleterious and some even be beneficial, by the nature of their fundamental reactivity they will, one way or another, interact with man and animals.

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NEW DEVELOPMENTS IN LECTIN ANALYSIS

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Summary

A method is developed with the potential of selectively measuring pathogenic lectins in animal feeds. The method is named a functional lectin immunoassay (FLIA), since lectins are detected by a combination of function and identity measurements. The potential of the FLIA method is illustrated by testing Phaseolus vulgaris 'cv processor' isolectins. (FLIA patent applied for).

Introduction

Animal feeds containing leguminous seeds are generally heat-treated in order to improve their feed value. This improvement is in part due to an increased digestibility of the feed. Further, a decreased functionality of anti-nutritional factors (ANF) is considered to be important. Amongst the ANF leguminous lectins have gained an increasing attention. Some lectins have been studied in great detail and demonstrated to be exceptionally harmful when ingested. The inactivation of lectins can be monitored using a haemagglutination assay. This assay is hampered however by both a lack in specificity and sensitivity. Furthermore, some lectins are not detected by haemagglutination. The recently developed ELISA-methods offer specificity and a higher sensitivity. At present, however, ELISA-methods cannot distinguish between functional and non-functional lectins. In this paper the concept of a Functional Lectin Immuno Assay (FLIA) is presented which provides specificity, sensitivity and the ability to measure functional lectins. As a first example a FLIA is presented for lectins from the Phaseolus Vulgaris 'cv processor' bean.

Materials and methods

Lectins were isolated from Phaseolus Vulgaris 'cv processor' beans using fetuin-Sepharose CL-4B affinity chromatography (Pusztai and Watt, 1974). Following, Phaseolus isolectins were prepared using a Mono S column equilibrated with 30 mM KH_2PO_4, pH 5.5 with 1 mM octylglucoside and eluted with a gradient of LiCl in the same buffer. Antibodies were raised against fetuin-purified lectins in New Zealand white rabbits. The antibodies were used as coating antibodies (ELISA) and as tracer antibodies (ELISA, FLIA). For the latter purpose IgG-horse radish per-oxidase conjugates (IgG-HRP) were prepared (Wilson, Nakane, 1978). Fetuin-albumin (Bovine serum albumin, BSA) conjugates were prepared following a slightly modified protocol. Porcine small

intestinal brush border membranes (BBM) were prepared as described elsewhere (Hendriks et al, 1987).

Functional Lectin Immuno Assay. Microtiterplates (96-well) were activated with glutardialdehyde (5 %, 250 µl/well, ½ h) and coated with fetuin-BSA conjugate (0.2 mg/ml fetuin, 250 µl/well, 16 h). Following, the plates were washed with ELISA buffer (25 mM MES/HCl pH 6, with 0.3 % (w/v) BSA, 0.2 % (w/v) Tween 20 and 0.9 % (w/v) NaCl) and stored in a humid box at 4 °C until use. A salt extract of Phaseolus meal and lectin test samples were diluted with ELISA buffer containing 3 % (w/v) BSA and 200 µl aliquots were added to the individual wells. After overnight incubation at 4 °C the wells were washed three times with ELISA buffer and incubated with anti lectin IgG-HRP (1/1000 dilution of conjugate; 2 hours at room temperature). Following a second washing sequence with ELISA-buffer the plates were developed for peroxidase activity after which the absorbance at 492 nm was read.

The FLIA was also carried out with a coating of porcine small intestinal BBM. With the FLIA-BBM the coating step is performed as described elsewhere (Hendriks et al, 1987) and the antibody conjugate is used in a 1/500 dilution.

Anti-Phaseolus Lectin Immuno Assay (anti PL-ELISA). The anti PL-ELISA was carried out essentially as described for the FLIA. In this case, however, a coating is used of rabbit anti Phaseolus lectin/antibodies.

Results and discussion

Functional lectins can be detected by making use of their ability to bind to a complex carbohydrate coating. Bound lectins can be sensitively identified using antibodies directed against the lectin. This is illustrated in figure 1. Here the response is shown of serial dilutions of a Phaseolus meal salt extract. Figure 1a shows the response when a coating is used of a conjugate of BSA and the bovine glycopeptide fetuin (FLIA-fetuin). In figure 1b a FLIA is used with a coating of brush border membranes representing the porcine small intestinal glycocalyx. With both FLIA methods functional lectins are detected as with an ELISA method (figure 1c). Heat treatment of the extract seems to result in complete inactivation (figure 1a, b) but reflects precipitation of the lectins since no lectin antigen is detected using ELISA (figure 1c). EDTA-treatment results in dissociation of the tetrameric lectin yielding an inactive (figure 1a, b) soluble (figure 1c) protein. Here the difference in performance between ELISA and FLIA is apparent.

In figure 2 similar experiments are shown with purified E_4 and L_4 isolectins. Where figure 2c shows that both isolectins are detected with ELISA, figure 2a and b reveal a marked difference between the FLIA-fetuin and the FLIA-BBM. With the former only E_4-isolectins are detected whereas with the latter only L_4-isolectins are detected. This can be explained by a different carbohydrate composition of the coatings and by the reported difference in carbohydrate binding specificity of the isolectins L_4 and E_4 (Miller et al, 1973).

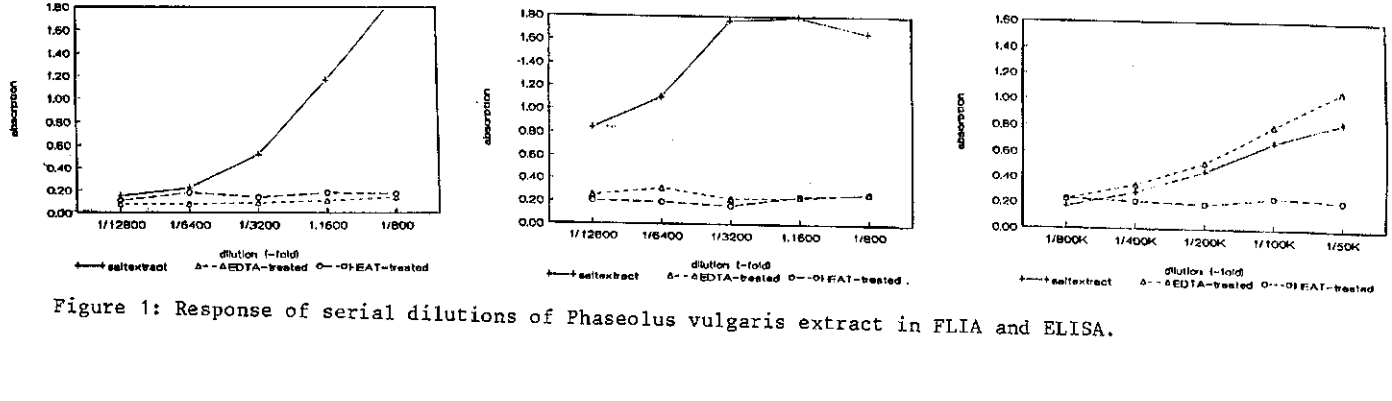

Figure 1: Response of serial dilutions of Phaseolus vulgaris extract in FLIA and ELISA.

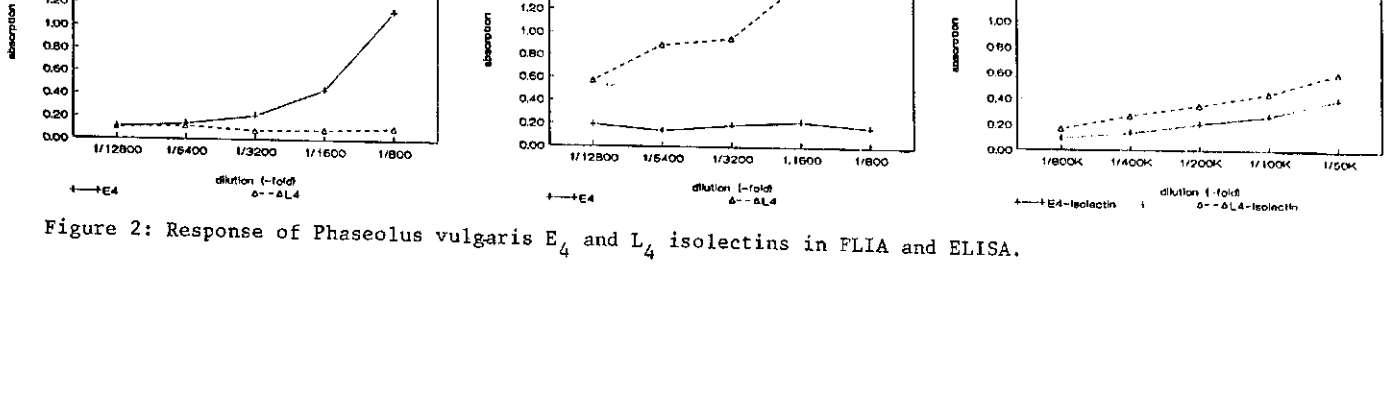

Figure 2: Response of Phaseolus vulgaris E_4 and L_4 isolectins in FLIA and ELISA.

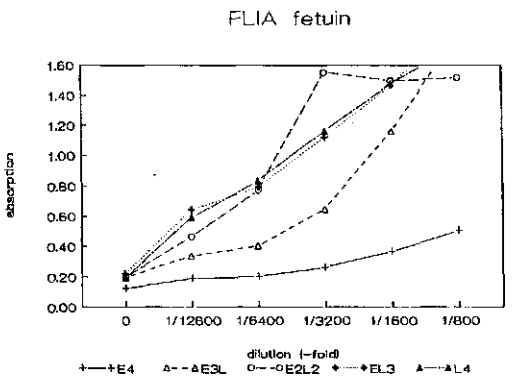

Figure 3: Response of Phaseolus vulgaris isolectins in a FLIA-fetuin assay.

In figure 3 the response is shown for all five isolectins, as measured with the FLIA-fetuin. From these data it is apparent that a lectin only requires one E-type subunit to bind to fetuin. The same is true for the FLIA-BBM where only one L-type subunit is required for detection (data not shown). This is in contrast with the results obtained with the EDTA dissociated lectins (figure 1, 2) where individual subunits did not appear to bind to the carbohydrate coating. Possibly, the native conformation of the lectin subunit which is lost upon EDTA-treatment. With the haemagglutination test the lectin tetramer has to contain at least two E-type subunits to be detected (eg L_4 and L_3E isolectins are not detected). Following the concept of the FLIA every lectin can be sensitively detected.

In conclusion, we have presented the concept of a new type of assay for functional lectins and tentatively named it FLIA. The performance of the assay is illustrated with a FLIA-fetuin and a FLIA-BBM for Phaseolus vulgaris lectins. Following the concept of the FLIA specific assays can be developed for the different leguminous lectins occurring in animal feedstuffs.

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LOCAL (GUT) AND SYSTEMIC RESPONSES OF RATS TO DIETARY SOYABEAN (GLYCINE MAX) PROTEINS

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Summary

Dietary soyabean lectin depressed rat growth, induced cellular hypertrophy and hyperplasia in the pancreas and stimulated small intestine crypt cell proliferation. Trypsin inhibitors (Kunitz + Bowman-Birk) reduced growth rate and induced pancreas hypertrophy and hyperplasia. Other anti-nutritional factor/s, devoid of lectin or trypsin inhibitory activity, caused a loss of muscle. The poor performance of soyabean-fed animals is thus due to the combined effects of these anti-nutritional factors upon body metabolism.

Prolonged soyabean feeding, particularly in conjunction with a high unsaturated lipid intake, appeared to increase the incidence of pancreas neoplasia in rats.
Key words: soyabean proteins, intestine, pancreas, muscle, pancreas neoplasia.

Introduction

As a result of interference with both local (gut) and systemic metabolism, the growth of young rats on diets containing raw soyabean (*Glycine max*) was much poorer than that achieved with animal protein-based diets (Grant *et al.* 1986). It has now been shown that the impaired performance is due to the combined effects of lectin, trypsin inhbitors and other anti-nutritional factors upon general body metabolism.

Results and Discussion

1. Growth

Both the purified lectin and trypsin inhibitors significantly reduced the growth of rats (Table 1). The lectin appeared primarily to impair retention of absorbed dietary nitrogen, whereas trypsin inhibitors lowered both digestion of protein and retention of absorbed nitrogen (Grant *et al.*, unpublished). The effects of the lectin and trypsin inhibitors were apparently additive since the growth depression with soyabean whey [pH 4.8 soluble] proteins which contained the bulk of the trypsin inhibitors and lectin was greater than with either purified component.

2. Small intestine

Purified lectin or whey proteins induced considerable enlargement of the jejunum within 7 days mainly as a result of cellular hyperplasia [increase in DNA] (Table 1). The ileum was altered only to a small extent by lectin or whey proteins (results not given), indicating that the overall small intestine enlargement (Grant *et al.* 1987a) was primarily due to cellular proliferation in the jejunum.

Table 1. Changes in dry weight and composition of a 20 cm section of jejunum upon feeding of rats with various soyabean proteins for 7 days. [Initial weight 85 g; food intake 7 g/rat/day; n = 4 for all treatments; results are given as means ± SE].

Diet	10% lactalbumin control	10% whey proteins	0.75%[α] lectin	2.8%[α] trypsin inhibitors
Body weight gain (g)	9.8±0.5	3.5±0.6	6.0±0.9	6.2±0.4
Jejunum weight (mg)	157±10	198±11*	238±17*	161±8
DNA (mg)	3.7±0.4	4.4±0.3*	5.0±0.2*	3.4±0.4
Protein (mg)	125±7	161±6*	178±7*	123±4
Protein/DNA (mg/mg)	34±2	36±1	35±1	36±1
Total polyamine (nmol/20 cm)	1000±50	1650±100*	2100±80*	990±50

* Significantly different from control ($P<0.01$). [α] lectin and Kunitz & Bowman-Birk inhibitors were prepared as before (Grant *et al.* 1987a) and incorporated in control diet at levels approximating that in the whey protein fraction. Tissue analysis as described previously (Grant *et al.*, 1987a). Polyamines determined by the method of Seiler and Knödgen (1980).

Figure 1. Section through jejunum from rats fed either control [A] for lectin [B] diets (Scale bar = 125 µm).

Feeding of lectin led to considerable alterations to jejunal morphology (Figure 1). The depth of the crypts were greatly increased. As a result the villus/crypt ratio was approximately 3:2 compared with 12:2 in controls. Thus dietary soyabean lectin appeared to induce crypt cell proliferation in a manner similar to that found with the toxic kidney bean lectin. Disruption of the microvilli was also apparent following lectin consumption. Again, this was similar to that observed upon feeding of the kidney bean lectin.

Cellular hyperplasia induced in the small intestine by trophic stimuli, such as hormones or growth factors, is mediated via a polyamine (putrescine, spermidine and spermine) dependent pathway (Luk & Yang, 1987). Upon lectin feeding there was an appreciable increase in the total polyamine content of

the gut (Table 1) and in the ratio of spermidine to spermine. Also, the small intestine enlargement was previously found to be, in part, prevented by treatment of rats with α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, the rate limiting enzyme of *de novo* polyamine synthesis (Grant *et al*, 1988). Thus, the lectin-induced changes appeared to be mediated through a polyamine-dependent pathway in a manner similar to that for other trophic stimuli. This would suggest that crypt cell proliferation may have been induced by gastrointestinal hormones released as a result of the interaction of dietary lectin with gut endocrine cells. Alternatively, lectin taken up into the circulation may, by binding to appropriate receptors in the crypts, have mimicked endogenous growth factors and stimulated crypt cell production.

Table 2. Changes in pancreas weight and composition induced by feeding of diets containing soyabean lectin or trypsin inhibitors for 7 or 16 days . [Initial weight 85 g; food intake 7 g/rat/day; diets as per Table 1; n = 4 for all treatments; results given as means ± SE].

Diet	7 days			16 days		
	control	lectin	trypsin inhibitors	control	lectin	trypsin inhibitors
Pancreas dry weight (mg)	121±12^{a}	168±9^{b}	177±8^{b}	145±12^{c}	196±10^{d}	184±9^{b}
DNA (mg)	2.3±0.1^{a}	2.4±0.2^{a}	2.4±0.2^{a}	2.7±0.1^{b}	3.3±0.1^{c}	3.1±0.1^{c}
Protein (mg)	73±12^{a}	105±11^{b}	109±10^{b}	87±1^{c}	133±7^{d}	123±4^{d}
Protein/DNA (mg/mg)	32±5^{a}	44±4^{b}	43±3^{b}	32±1^{a}	40±2^{b}	40±2^{b}

Values in a row with different superscripts are significantly different ($P<0.01$)

3. Pancreas

Dietary lectin and trypsin inhibitors (Kunitz + Bowman-Birk) each induced pancreatic enlargement (Table 2). In both cases the increase in the initial 7 days was due to cellular hypertrophy [increase in protein/DNA ratio]. At 16 days both cellular hyperplasia and hypertrophy were evident. Therefore, the enlargement caused by raw soyabean or soyabean whey protein feeding was probably due to the combined effects of lectin and trypsin inhibitors upon the pancreas (Grant *et al*, 1987b). Circulating insulin levels were found to be low in soyabean-fed rats (Pusztai *et al.*, 1986). Thus since trypsin inhibitors did not alter blood insulin levels (Goke *et al.*, 1985), it is possible that the soyabean lectin, alone and/or in combination with other factors, impair insulin production and/or secretion and thereby reduces its concentration in the blood.

Pancreas enlargement persisted upon long-term (up to 2 years) feeding with a raw soyabean diet. There was also a significant increase in the occurrence of pre-neoplastic changes and pancreas tumours after 1-2 years (Grant *et al.* unpublished). Comparison with other studies suggests that the incidence of neoplasia is dependent on the level of both soyabean protein and unsaturated lipids in the diet (Table 3).

Table 3. Effect of soyabean proteins and unsaturated lipids upon incidence of pancreas cancer in rats.

Study	1	2	3	3	4
Experimental period	2 years	2 years	2 years	2 years	1.5 years
% dietary protein	10	40	10	22	24
% trypsin inhibitor	0.4	>2.0	up to 0.6	1.3	0.1
Protein source*	RSM	RSM	DSM/SPI	DSM	SPI
% dietary lipid	22	20	8	8	10
Lipid source**	SO+CO	SO	CO+L	CO+L	CO
% incidence***	8-15	10-15	1-1.5	6	0

1 Grant *et al.*, (unpublished). 2 McGuiness *et al.* 1984. 3 Gumbmann *et al.* 1985. 4 Richter & Schneeman, 1987. *RSM, raw soyabean; DSM, defatted soyabean; SPI, soya protein isolate. **SO, soyabean oil; CO, corn oil; L, lard. *** spontaneous incidence was up to 1%.

No pancreatic lesions were observed after feeding a 1.8% trypsin inhibitor diet, based on egg white, for 1.5 years (Richter & Schneeman, 1987). In contrast acinar adenoma and nodular hyperplasia were observed upon feeding of soyabean diets, containing 0.6% trypsin inhibitor, over the same period of time (Spangler *et al.* 1985). This suggests that, in addition to trypsin inhibitors, other proteins, possibly the lectin, mediate the pancreas changes found upon long-term feeding with soyabean.

Elevated dietary levels of unsaturated lipids can potentiate the effects of carcinogens upon the pancreas (Roebuck, 1986). Therefore the high incidence of pancreas neoplasia found in our studies and by McGuiness *et al.* (1984) may have been due to synergism between trypsin inhibitors, lectins and unsaturated dietary fats.

4. Muscle

Feeding of whey proteins to rats resulted in a considerable reduction in gastrocnemius and plantaris muscle weights (Grant *et al.* 1987a). These muscles are thought to be reflective of skeletal muscle as a whole and, therefore, the changes may indicate a general loss of skeletal muscle. These effects did not appear to be due to either trypsin inhibitors or lectin.

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STIMULATION OF POLYAMINE SYNTHESIS AND GROWTH OF THE SMALL INTESTINE BY DIETARY KIDNEY BEAN LECTIN

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Summary

Phaseolus vulgaris lectin (PHA)-induced hyperplastic growth of the small intestine was dependent on a simultaneously occurring increase in tissue polyamine concentration. However, most of the increase in the amounts of polyamines was not due to local synthesis *via* ornithine decarboxylase (ODC) but to a PHA-induced increase in the uptake of the polyamines needed for cellular proliferation. The main source of polyamines for the gut was the blood, with smaller amounts derived from food. However, polyamine concentration in circulating blood is usually very low, therefore, most of the polyamines needed for the PHA-induced cellular proliferation in the small intestine must ultimately derive from internal organs and/or tissues of the body. Neither the source nor the mechanism of polyamine release into blood circulation, due to the initial stimulus by dietary PHA reaching the small intestine, is known. All the same, stimulation of wastefully increased protein, DNA and RNA syntheses, high metabolic activity and cell turnover rates *in brush border cells through a polyamine-dependent mechanism* contributes to and exacerbates the nutritional toxicity of dietary PHA.
Keywords: *Phaseolus vulgaris* lectin; polyamines; toxicity; gut; growth

Introduction

It is now generally recognized that the lectin, PHA, is responsible for the known toxicity for monogastric animals of diets based on raw kidney bean (*Phaseolus vulgaris*) proteins. Clearly, for the establishment of effective strategies to counteract such a toxic effect, exploratory studies on the mechanism of the PHA - small intestine interaction are of great importance. The extraordinarily high resistance to gut proteolysis of dietary PHA has been well established. The fully reactive lectin in the gut lumen binds to brush border cells of the proximal small intestine and this leads to extensive damage to the microvillous membrane of epithelial cells (for details see Pusztai in this issue). In addition, a proportion of the receptor-bound lectin is absorbed by these surface cells. However, it is not clear whether this binding of PHA to class-1 (information) receptors or the endocytosis of the class-2 receptor-bound PHA is mainly responsible for the immediate and dramatic changes in epithelial cell metabolism when the lectin reaches the small intestine. The acute lectin effects resulting in considerable increase in protein synthesis, mucin accumulation/secretion and a generally elevated metabolic state of the brush border cells are followed by a dose-dependent hyperplastic enlargement of the small intestine on prolonged exposure to dietary PHA. Accordingly, despite its overall growth-depressant effects for the animal, PHA behaves as a powerful growth-factor for the small intestine (Pusztai *et al.* 1988). Similar mucosal growth occurs in response to a number of other, more physiological stimuli, such as lactation, weaning, partial resection or obstruction of the lumen (for refs. see Pusztai *et al.* 1988). In fact, one of the strongest stimuli for growth of the gut is feeding, particularly after a period of

fasting, and the size of the small intestine is mainly dependent on the amounts of food in the lumen. However, growth stimulation by PHA occurs despite a reduced food intake and poor luminal nutrition. The maintenance of the wasteful processes of increased protein, DNA, RNA syntheses and cell turnover have a high nutrient and energy cost for the animal. Thus, the already poor nutrient utilization is further reduced with a resulting exacerbation of the nutritional toxicity of the dietary PHA.

Growth factors induce gut hyperplasia and increase polyamine metabolism

When growth of the small intestine is stimulated by various growth factors there is always a considerable and coincident increase in the polyamine (putrescine, spermidine and spermine) contents of the tissue. Additionally, there is also an increase in the concentration and/or activity of most of the enzymes which control the synthesis and metabolism of polyamines. In fact, the presence of even simple aliphatic amines and, particularly, of polyamines in the lumen stimulates not only mucosal growth but also an increase in the activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of *de novo* polyamine synthesis (Hosomi *et al.* 1987; Luk & Yang, 1987; 1988). There is also a rise in the activity of S-adenosyl-methionine decarboxylase, a second enzyme of polyamine biosynthesis. Moreover, when *de novo* putrescine biosynthesis is blocked by the inhibition of ornithine decarboxylase with α-difluoromethylornithine (DFMO), mucosal growth is also stopped. Thus apparently, small intestinal growth may have an absolute requirement for the concurrent synthesis and metabolism of polyamines and the various growth factors stimulate growth through the activation of enzymes which control polyamine metabolism.

PHA-induced growth of the small intestine and its dependence on polyamines

The considerable enlargement of the small intestine caused by dietary PHA has also been found to be accompanied by substantial increases in the amounts of polyamines in the tissue as compared with those obtained from pair-fed (lactalbumin) control rats (Table 1). This effect was particularly striking with jejunal tissues. By the inclusion of DFMO in the diet, most of the lectin-induced increases in DNA and protein contents and the simultaneously elevated polyamine concentrations were reversed. Moreover, most the the PHA-induced growth was also blocked (Table 2).

The dependence on increased tissue polyamine concentration of cellular proliferation in the small intestine was further corroborated by the finding that, by the incorporating polyamines in kidney bean diets, PHA-induced growth could partially be restored, even after DFMO treatment (Table 2). Clearly, although the synthesis of putrescine was considerably reduced after inhibition of ODC activity by DFMO the gut could, apparently, still absorb polyamines from the food and, therefore, proliferation could again proceed to a limited extent (Table 2). However, even relatively large amounts of luminally applied polyamines were not fully effective in restoring PHA-induced growth to the DFMO-treated small intestine (Table 2). The results, therefore, suggest a source other than the food for the main supply of polyamines in the DFMO-treated small intestine. Indeed, some growth factors, such as gastrin, are known to stimulate proliferation by increasing the uptake of polyamines from circulation rather than by inducing polyamine synthesis in the gut *via* ODC. (Seidel *et al.* 1985; Majumdar & Johnson, 1987). It appears that the main effect of PHA was also to increase the uptake

Table 1. The growth and polyamine content of parts (20 cm) of the small intestine from rats (n = 6 for each treatment) pair-fed lactalbumin control or kidney bean-containing diets for three days (means with SE).

Diet	Jejunum				Ileum			
	Control		Kidney bean		Control		Kidney bean	
	means	SE	means	SE	means	SE	means	SE
Wet weight (mg)	1022	143	1361	82	734	116	950	77
Protein (mg)	112	7	147	6	82	9	101	9
Polyamines:								
nmoles	409	72	793	130	393	72	551	117
spermidine/spermine	1.8		2.4		1.5		1.9	

Table 2. Composition of jejunum (20 cm) of rats (n = 5 for each group) fed on kidney bean diets for 7 days with or without DFMO as compared with that of pair-fed lactalbumin controls. Some of the diets containing DFMO were also supplemented with putrescine (P) or spermidine (Sp) + spermine (S).

Diet	Protein (mg)		RNA (mg)		DNA (mg)		Polyamines (nmoles)	
	means	SE	means	SE	means	SE	means	SE
Control	93.8	3.0	7.5	0.3	5.4	0.4	934	98
Kidney bean								
- DFMO	153.7	10.9	21.9	3.0	8.0	0.6	1997	144
+ DFMO	109.4	13.2	12.9	1.8	5.4	0.3	1050	128
+ DFMO + P	133.6	15.4	17.2	2.1	6.6	0.8	1253	232
+ DFMO + S + Sp	134.0	8.1	16.0	1.5	6.5	0.6	1330	110

of polyamines needed for gut growth chiefly from the circulating blood through the basolateral membrane, and only to a small extent from the food (Table 3). However, polyamine concentration in blood is low and, therefore, the polyamines used for supplying the small intestine *via* the blood must have originated from other organs and tissues of the body.

The mechanism of this release and the tissues involved are not yet known. Although the physiological effects of the loss of polyamines on the proper functioning and cellular metabolism of internal tissues are unknown, these effects may be appreciable. As polyamines are obligatory components of tissue regeneration and renewal, the overall balance of metabolism of these tissues may be shifted towards increased breakdown. Thus, the PHA-induced wasteful growth of the small intestine may not only use up the much needed protein and energy content of the food, but by increasing catabolic breakdown in the body for the release of polyamines, it may also waste endogenous

Table 3. PHA-induced labelled polyamine uptake from blood circulation.

Stimulant:	Length (cm)		Dry weight (g)		Absorbed polyamines (nmoles/h) Putrescine		Spermine	
	means	SE	means	SE	means	SE	means	SE
Control	87	3	0.53	0.02	1.01	0.03	1.01	0.03
PHA	93	2	0.84	0.02	1.53	0.04	2.73	0.03

tissue components. As the general nutrition status of the animal on such diets is already poor, replacement of wasted tissues is becoming progressively more and more difficult and growth impossible.

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THE EFFECT OF LECTINS AND MONOSACCHARIDES ON THE IN VITRO ATTACHMENT OF E. COLI F17$^+$ TO INTESTINAL CALF VILLI AND IMMOBILIZED GLYCOPROTEINS

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Summary

In vitro, E. coli strain 25KH09st, expressing F17 fimbriae, attach avidily to calf intestinal villi. Attachment can be inhibited by N-acetyl-D-glucosamine, or by treating the villi with N-acetyl-D-glucosamine specific lectins. E. coli F17$^+$ isolates display carbohydrate binding heterogeneity, as was shown by binding studies using Eupergit-C-glycoprotein beads. These observations are of paramount importance when receptor analogs are envisaged to be used as attachment inhibitors in vivo.
Keywords: F17 fimbriae, bacterial lectins, plant lectins, Eupergit-C-glycoprotein beads.

Introduction

Attachment of pathogenic bacteria to mucosal surfaces of the host is now generally recognized as an initial, but essential step in pathogenesis. Binding of gram-negative bacteria to mucosal surfaces is in most instances mediated by proteinaceous appendages, called fimbriae, which protrude from the surface of the bacteria and which recognize carbohydrate residues of mucosal glycoconjugates [Gaastra & De Graaf, 1982; De Graaf, 1986; Clegg & Gerlach, 1987]. Although E. coli are advantageous commensals of the large intestine of mammals, some strains are able to colonize other niches and are the causative agents of both intestinal and extra-intestinal diseases in man and his domestic animals. Enterotoxigenic E. coli strains induce diarrhoea by attaching to the intestinal mucosa and by producing enterotoxins that provoke the influx of sodium ions and water into the intestinal lumen.

Neonatal diarrhoea in calves has generally been ascribed to colonization of the intestine by E. coli strains producing K99 and/or F41 fimbriae [Gaastra & De Graaf, 1982]. More recently however another fimbrial antigen, designated Att25 in Belgium [Pohl et al., 1982, 1984] and F(Y) in France [Girardeau et al., 1980; Contrepois & Girardeau, 1985; Morris et al., 1985] has been shown to be implicated in neonatal bovine coligenic diarrhoea as well. Since the physico-chemical properties of the major subunit of Att25 and F(Y) have been shown to be identical, they are now called F17 fimbriae. Epidemiological studies revealed that E. coli F17$^+$ strains are also associated with other outbreaks of coligenic diarrhoea in other countries of the European Community [P. Pohl, to be published] as well as in Japan [Shimizu et al., 1987]. Furthermore, Pohl et al. [1983] and Shimizu et al. [1987] reported that E. coli F17$^+$ strains may be responsible for enterocolitis.

Materials and Methods

Bacterial strains and culture conditions

All *E. coli* F17$^+$ isolates used in this study have been recovered from diarrheic calves and were typified at the National Institute of Veterinarian Research of Belgium. Bacteria were grown at 37°C, either on LB-agar pH 7.3 plates for 40 hours, or in liquid medium until stationary phase.

Lectins

Canavalia ensiformis (Con A), *Pisum sativum* (PSA) and *Lens culinaris* (LCA) lectins were purified by affinity chromatography on Sephadex G-75 as described previously [Van Driessche et al., 1982]. *Solanum tuberosum* lectin (STA) was purified by affinity chromatography on chitin as in [Van Driessche et al., 1983]. The lectins from the embryo of *Triticum vulgaris* (WGA), and from the seeds of *Arachis hypogaea* (PNA), *Glycine max* (SBA), *Dolichos biflorus* (DBA) and *Ulex europaeus* (UEA) were generously provided by Kem-en-Tec, Copenhagen. F17-fimbrial lectins were purified as described by Schoup et al. [1987].

In vitro attachment assay

In vitro attachment of *E. coli* F17$^+$ to small intestinal calf villi was performed as described by Girardeau [1980]. Oxidation of villar glycoconjugates was performed in the dark in 0.2 M sodium acetate buffer pH 4.5 containing 10 mM sodium metaperiodate.

Coupling of glycoproteins to Eupergit-C beads

Hen egg white ovomucoid, fetuin or bovine submaxillary gland mucin (all from Sigma Chem. Comp.) were coupled to Eupergit-C beads in 1 M potassium phosphate, and attachment and attachment inhibition were performed as described previously [Van Driessche et al., 1988].

Agglutination of yeast cells

Agglutination of *Saccharomyces cerevisiae* cells was performed on microscopy glasses either at 4°C or at 25°C. Twenty μl of yeast cell suspension (1 g, wet weight, per 10 ml phosphate buffered saline) was mixed with an equal volume of bacterial suspension. Agglutination was scored either with the naked eye or by light microscopical examination.

Results

Attachment of *E. coli* strain 25KH09st to small intestinal calf villi and to Eupergit-C-glycoprotein beads

E. coli strain 25KH09st readily attaches *in vitro* to small intestinal calf villi. Attachment is most prominent at the villar tip. When, prior to addition of the bacteria, the villi were incubated with purified F17 fimbriae (1 mg/ml), no attachment of bacteria was observed. Similarly, incubation of villi with either of the lectins WGA, STA or PNA (at a final concentration of 250 μg/ml) inhibited bacterial adhesion. On the other hand the lectins LCA, PSA, Con A, UEA, DBA or SBA had no effect. When the saccharides of the villar glycoconjugates had been oxidized with

sodium metaperiodate, a reagent that cleaves the carbon-carbon bond between vicinal hydroxyl groups of sugars, no attachment was observed.

When the bacteria were incubated for 15 min. with N-acetyl-D-glucosamine, prior to being exposed to calf villi, no adhesion could be observed. All other sugars tested, i.e. D(+)glucose, D(+)glucosamine, methyl-α,D-glucopyranoside, mannose, methyl-α,D-mannopyranoside, maltose, D(-)fructose, α,L(-)fucose, lactose, D(+)galactose, D(+)galactosamine, N-acetyl-D-galactosamine or N-acetylneuraminic acid, were without effect.

F17 fimbriae producing E. coli strain 25KH09st attached to Eupergit-C-ovomucoid and Eupergit-C-(bovine submaxillary gland mucin). In agreement with the binding characteristics of the strain to calf villi, attachment to the glycoprotein derivatized Eupergit beads was only inhibited by N-acetyl-D-glucosamine. Oxidation of the saccharide chains of the glycoproteins abolished attachment completely.

Carbohydrate binding properties of other F17 fimbriae producing E. coli isolates

The carbohydrate binding characteristics of different E. coli $F17^+$ isolates were studied using the Eupergit-C-glycoprotein system. Electron microscopical examination revealed that none of the isolates produced type-1 (mannose-sensitive) fimbriae. The results of the attachment of different $F17^+$ isolates on Eupergit-C-glycoprotein beads are summarized in Table 1. In control experiments, where sodium metaperiodate oxidized

Table 1. Adhesion properties of various F17 isolates on Eupergit-C-glycoprotein conjugates: ovomucoid (OVO), fetuin (FET) and mucin (MUC), and agglutination of Saccharomyces cerevisiae cells.

	Adhesion properties						Agglutination
	Non-derivatised beads	OVO (PBS)	FET (PBS)	MUC (PBS)	MUC (PBS/mann)	MUC (PBS/GlcNac)	(PBS)
Contrepois	+	+	+	+	+	+	+
ppL2	-	+	+	+	+	+	+
1860	some/particle	+	-	+	+	-	-
F(Y)	-	-	-	+	+	-	-
F17u	-	+	-	+	-	-	nd
32KH85	-	-	-	+	+	-	-
33KH85	-	-	-	+	-	-	-
43KH85	-	-	-	+	+	-	-

	OVO (PBS/mann/GlcNac)	FET	MUC
Contrepois	-	-	-
ppL2	-	-	-

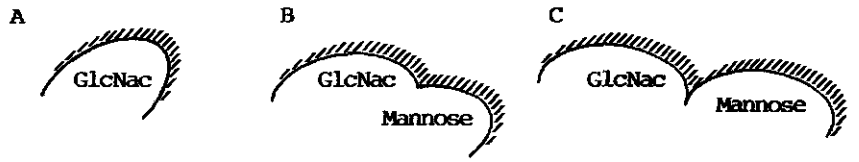
PBS: phosphate buffered saline, i.e. 10 mM potassium phosphate + 150 mM NaCl, pH 7.2; GlcNac: N-acetyl-D-glucosamine, final concentration 100 mM; mann: mannose, final concentration 100 mM; nd: not determined.

beads were used, no attachment at all was observed. Since all isolates bind to Eupergit-C-mucin beads, these beads were used to study the attachment-inhibition by N-acetyl-D-glucosamine and mannose (Table 1). Some isolates, although devoid of type-1 fimbriae, agglutinated yeast cells (Table 1). In all cases, agglutination could be completely inhibited by mannose.

Discussion

The results presented in this paper and elsewhere clearly show that F17 fimbriae are implicated in the adherence of E. coli F17$^+$ to calf small intestinal villi. Indeed, purified F17 fimbriae effectively block attachment of F17 fimbriated E. coli. Besides, when grown in conditions where no F17 fimbriae are produced [Lintermans et al., 1988], the bacteria fail to attach. Since sodium metaperiodate oxidation of the villi completely abolishes adherence, it can be concluded that F17 fimbriae recognize glycoconjugates of the intestinal mucosa. The adherence-inhibition of strain 25KH09st caused by GlcNac, as well as by GlcNac-specific lectins such as WGA and STA, demonstrates that the fimbriae of this strain at least are GlcNac specific. Since galactose does not inhibit attachment, adhesion inhibition observed by PNA must be attributed to steric hindrance and points to the presence of a galactose residue in the immediate vicinity of the GlcNac residues in the receptor molecule. The implication of GlcNac in the binding of strain 25KH09st to glycoconjugates is further evidenced from the fact that it inhibits attachment of this strain to Eupergit-C-glycoprotein beads.

From our studies on the binding properties of different E. coli F17$^+$ isolates to Eupergit-C-glycoprotein beads, it can be concluded that F17 fimbriae constitute a heterogeneous group of carbohydrate binding proteins. The same conclusion can be drawn from the attachment inhibition studies, as well as from the yeast agglutinating properties of some strains. Based on the inhibition by simple sugars of attachment of the E. coli F17$^+$ isolates onto Eupergit-C-glycoprotein beads, three major groups can be recognized. A first one consists of E. coli F17$^+$ that can be prevented from binding by GlcNac. The attachment of the second group is inhibited by either GlcNac or mannose, while attachment of E. coli F17$^+$ of the third group is only inhibitable by a mixture of both sugars. Recently it was shown by Lintermans et al. (submitted for publication) that F17 fimbriae production and attachment to either calf villi or Eupergit-C-glycoprotein beads are properties that can genetically be separated, indicating that F17 fimbriae are built up of at least two different types of subunits, i.e. major structural subunits and minor adhesive subunits. Our results described in this paper clearly show that the adhesive subunits of different isolates may be quite different. Whether a similar heterogeneity exists at the level of the major structural subunit is actually under investigation. Similarly, at present we can only speculate about the differences or relatedness among the various types of adhesive subunits. From the information available until now, the following models depicting the carbohydrate binding sites of the different recognized adhesins can be suggested (see figure below). (A) Isolates that are solely inhibited by GlcNac should contain small sugar binding site(s) that fit this sugar. (B) An extended binding site built up of a GlcNac and a mannose subsite might explain the binding properties of adhesins that can be blocked by either GlcNac or mannose. In this case, saturation of either subsite might prevent binding of the isolates to glycoconjugates. Most probably both subsites are partially overlapping. (C) Isolates which require both GlcNac and mannose for



attachment inhibition should either contain an extended binding site consisting of two subsites, one specific for GlcNac and the other one for mannose. Occupation of both subsites is necessary in order to prevent attachment. Alternatively, these isolates might contain two different types of adhesins, i.e. GlcNac specific fimbrial adhesins and a mannose-specific non-fimbrial membrane bound adhesin. Upon saturation of either adhesin with its complementary sugar, binding would still be possible by the second one. Yeast cell agglutination by these strains would be due to the presence of the mannose-specific adhesin.

The observed carbohydrate binding heterogeneity of E. coli $F17^+$ isolates is of special practical importance when the use of receptor analogs is envisaged to prevent bacterial attachment to the intestinal mucosa. Indeed, receptor analog therapy might only be an alternative for vaccination or antibiotic therapy as far as those receptor analogs can be identified and purified in large quantities that will effectively saturate the different carbohydrate binding sites presented at the bacterial surface. Our results show that oligosaccharides of bovine submaxillary gland mucin are potential attachment blockers since these are the only ones identified until now that are recognized by all $F17^+$ isolates tested.

Instead of using receptor analogs to block the carbohydrate binding sites of the bacterial lectins, plant lectins may also be envisaged to be used as mucosal receptor blockers in attempts to prevent bacterial attachment. Our results clearly show that, at least in vitro, STA, WGA and PNA effectively prevent E. coli $F17^+$ from binding to small intestinal calf villi. The outstanding studies of I. Liener [1986], A. Pusztai [1986] and others have indicated however that some lectins at least, when administered in an active form, may be harmful for mammals (see also contributions of Liener and Pusztai to these Proceedings). Since tomato lectin, which is structurally very similar to STA, was shown by Kilpatrick et al. [1985] to bind to villi of both duodenum and ileum of rats without inducing pathological changes, it may be expected that STA or tomato lectin might be used in vivo as an attachment blocker.

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PATHOLOGICAL CHANGES OF THE SMALL INTESTINAL MUCOSA OF PIGLETS AFTER FEEDING PHASEOLUS VULGARIS BEANS

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Summary

In this bio-essay the pathological effects of diets containing Phaseolus vulgaris beans, on the mucosal structure of the jejunum in pigs have been studied. By stereomicroscopy the changes in the three-dimensional structure of the villi were examined, whereas the villus/crypt ratio was analysed by morphometrical measurements. The histological structure was also examined.
The jejunal mucosa of the animals fed the diet with Phaseolus vulgaris beans proved to be characterized by a state of hyperregenerative mucosal atrophy.
Activity of three brush border enzymes was determined biochemically. The activity of alkaline phosphatase in the test animals did not differ significantly from the control ones, whereas the activity of aminopeptidase and sucrase were statistically significant lower in the test animals.
Keywords: Phaseolus vulgaris agglutinin, small intestinal mucosa, pig.

Introduction

Seeds of leguminosae can be highly toxic to man and animal (Liener, 1986). These seeds contain a number of antinutritional factors (ANF), like protease inhibitors, tannins and lectins, which may count for a less efficient utilization of the plant's proteins (Liener, 1974; Lorenz-Meyer et al, 1985). The lectins may account for various morphological and functional changes in the epithelium of the small intestine in man and animal when fed orally (Pusztai, 1987). They can reach the intestine in an active form (Hara et al, 1984, Greer, 1983, Nakata and Kimura, 1985) where they bind to the carbohydrate chains of glycoconjugates, as present in the mucus and the glycocalyx (Egberts et al, 1984). They may provoke a precipitation of the mucins, damaging the outermost mucosal barrier (Mouwen et al, 1983) and of the glycocalyx, influencing the activity of the brush border enzymes (Erickson, 1985, Nakata & Kimura, 1985). In rats, after oral intake of Phaseolus vulgaris agglutinin (PHA) Greer (1983) found an increased mucus production in the intestine. Rouanet et al. (1985),in rats, noted a shortening of the microvilli and desquamation of the enterocytes. Lorenz-meyer et al. (1985) observed a reduction of the length of the villi and an increase in depth of the crypts in rats.
The fact that over the past years more seeds of leguminosae, containing ANF, are added to the diets of production animals (e.g. pigs) which,

without proper heat treatment, results in a less efficient feed conversion, diminished growth, intermittant scouring, wasting and sometimes death, led to this study in pigs.

Materials and Method

At random obtained piglets, Great York x Dutch landrace were weaned at the age of two weeks and accustomed to the testcages during a two weeks period. The test period lasted three weeks, during which the animals were grouped in 2 groups of 12 animals each. During the first five test days the test animals were habituated to the testdiet. The control diet did not contain any Phaseolus vulgaris beans, while the test diet was supplemented with 20% untreated Phaseolus vulgaris beans (commercially obtained), with a medium rich lectin content. The diets were equalized for contents of crude protein, amino acids, and nett energy. The animals were fed at a level of 4% of the bodyweight.
At the end of the test period 7 piglets of each group were anesthetized with Fluothane®, nitrous oxide and oxygen, administered by facemask. The abdomen was opened and samples from three different places of the jejunum were taken, 0.5 m distal of the ligament of Treitz, the middle of the jejunum and 0.5 m proximal to the ileocaecal ligament. Subsequently the animals were euthanized with T61® intracardially. From each place one sample was fixed in a 4% buffered formaline solution for stereomicroscopical and histological examination and one was deepfrozen for biochemical determination of the brush border enzymes.
For the histological investigation serial paraffin sections 5µ in thickness were cut and stained hematoxylin-eosin and periodic acid Schiff reagens.
The animals were weighed at the beginning of the test period, after one week and at the end of the test period.

Stereomicroscopy

The mucosa was studied by means of a dissecting microscope and graded according to Mouwen (1972).

Histology

To characterize the jejunal mucosa morphologically the length of the villi and the depth of the crypts were measured using a TEA Image manager (TIM) system. The villus/crypt ratio was calculated. The histological investigation was directed to the determination of the density of goblet cells, in thirty crypts as well as in thirty villi, the density of the intra-epithelial lymphoid cells per 300 enterocytes on the villi and the mitotic activity in the crypts (number of mitoses in 300 crypt epithelial cells).

Biochemistry

The activity of alkaline phosphatase was determined according to the method of Bessey et al.(1946). For aminopeptidase and sucrase activities the method of Bergmeyer (1974) was used.

In statistical calculations of the results, statistical significance was accepted at the $p \leq 0.05$ level (Wilcoxon rank test and ANOVA).

Results

During the test period there was a serious growth inhibition, even weight loss in the test group animals.

The mucosal surface of the test animals appeared to be covered with more mucus than that of the control ones.

The stereomicroscopical investigation indicates a clear change in the three-dimensional image of the villus structure of the test animals, when compared with the control ones. In the test group, a shift has taken place towards the higher gradations. Predominantly shortened and abnormally shaped villi, like tongue-, leaf-, ridgeshaped and convoluted villi were present. Even flat areas, where the villi have completely disappeared occurred. Statistical calculations showed a significant difference between the control group and the test group (figure 1).

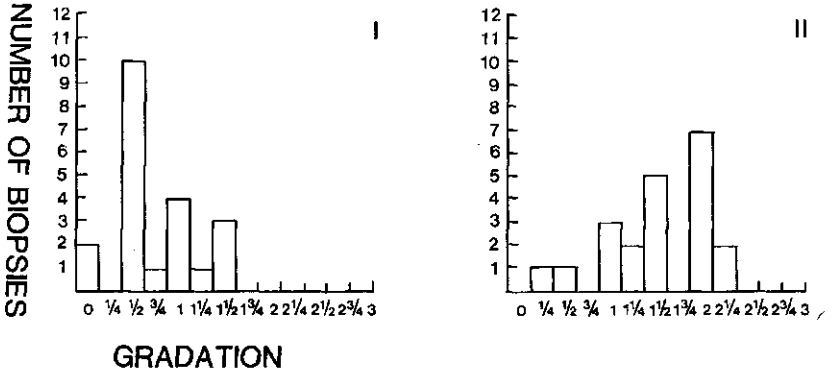

Figure 1 Mucosal pattern as graded by dissecting microscopy in the jejunum of the control animals (I) and the test animals (II). Depending on the degree of alteration of the intestinal villi a grading from 0 to 3 was used.

0 a normal villous pattern with almost all finger-shaped villi.
½ mixed finger- and tongue-shaped villi
1 a pattern with predominantly long to short tongue-shaped villi with few finger-shaped and leave-shaped ones.
1½ predominantly short tongue- and leaf-shaped villi with few long tongue- and ridge-shaped villi.
2 mixture of short tongue-, leaf-, ridge-shaped and convoluted villi.
2½ similar to grade 2 with flat areas, indicating a highly abnormal pattern

In the morphometrical measurements the length of the villi was drastically reduced in the test animals, whereas the depth of the crypts had increased. A significant reduction of the ratio of villous height to crypt depth was calculated for the test group in comparison to the control group. The differences between the test and the control groups were statistically significant.

The number of intra-epithelial lymphoid cells (I.E.L.) in the test animals was lower than in the control animals. The index of mitosis (I.M.) was highest in the test animals. The number of goblet cells on the villi (G.C.V.) of the test animals was lower than in the control ones. The number of goblet cells in the crypts (G.C.C.) was highest in the test animals (fig. 2).

All differences described were statistically significant.

	I.E.L		I.M.		G.C.V		G.C.C.	
animals	mean	sem	mean	sem	mean	sem	mean	sem
control	21.7	1.0	5.4	0.6	12.0	0.8	16.7	0.7
test	14.9	0.9	14.4	0.5	7.6	1.0	20.8	1.2

Table 1. Numbers of intra-epithelial lymphoid cells (I.E.L.), villous (G.C.V) and crypt (G.C.C.) goblet cells, and index of mitosis (I.M.) in pigs from the test and the control groups.

The activity of alkaline phosphatase in the test animals did not differ significantly from that in the control animals. However, the activity of aminopeptidase and sucrase was statistically significant lower in the test animals.

Discussion

The stereomicroscopical, histological, morphometrical and biochemical findings indicate that the mucosa of the piglets fed diets containing Phaseolus vulgaris beans differs from that in the piglets fed the control diet. The number of intra-epithelial lymphoid cells in the test animals is statistically significant lower than in the control animals. Its significance is not known yet.
The fact that a smaller amount of goblet cells was found on the villi of the test animals may be due to a depletion of the goblets cells. This corresponds with the appearance of more mucus on the intestinal mucosal surface of the test animal and agrees with the findings of Greer (1983).
The shortening of the villi, the increased depth of the crypts, the decreased villus/crypt ratio together with the elevated index of mitosis are illustrative for hyperregenerative villus atrophy (van der Vegt, 1963) in the test animals.
The changes of the mucosa of the jejunum may be caused by the lectins present in the unheated Phaseolus vulgaris beans. The binding of the lectins to the glycoconjugates of the epithelium may cause degeneration of the epithelium, leading to the hyperregenerative villousatrophy.

A consequence of the morphological changes is a reduction of the digestive and absorptive surface of the mucosa, counting for the diminished growth and even loss of weight of the test animals. However, also systemic effects may play a role (Greer, 1983).

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CELL BIOLOGICAL EFFECTS OF PURIFIED PHASEOLUS VULGARIS ISOLECTINS ON DIFFERENTIATED HUMAN COLON CARCINOMA CACO-2 CELLS.

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Summary

Cell biological effects of purified isolectins from Phaseolus vulgaris 'cv Processor' beans on differentiated human colon carcinoma Caco-2 cells have been investigated. PHA-L4 and PHA-E4 isolectins were found to stimulate the incorporation of ^{14}C-thymidine and ^{3}H-glucosamine into the trichloroacetic acid insoluble fraction of the Caco-2 cells, whereas no change in the uptake of ^{14}C-uridine was observed.
In comparison with the PHA-L4 isolectin the concentration of PHA-E4 isolectin required to stimulate the incorporation was lower.
After heat treatment of the PHA-E4 isolectin, the uptake of radioactive precursors did not differ significantly from control Caco-2 cells.

keywords: Phaseolus vulgaris agglutinin (PHA); PHA-isolectins; in vitro.

Introduction

Seeds of leguminosae contain antinutritional factors as lectins, trypsin inhibitors and tannins. When fed to man or animal, these seeds may result in a less efficient utilization of the plants protein, unless subjected to some form of heat treatment (Liener, 1974). Plant lectins, which can reach the intestinal tract in an active form (Greer, 1983; Nakata and Kimura, 1985) may induce various pathological changes in the epithelium of the small intestine, thus reducing its digestive and absorptive area (Lorenzsonn & Olson, 1982; Rouanet et al., 1985; Pusztai, 1987). In spite of numerous studies, the pathogenesis of lectin induced changes in the small intestinal epithelium is not yet understood.
In this study we have investigated the cell biological effects of different isolectins from Phaseolus vulgaris 'cv Processor' bean, using a human colon carcinoma cell line (Caco-2). This Caco-2 cell line was demonstrated to develop similar characteristics as small intestinal enterocytes (Pinto et al., 1983; Chantret et al., 1988). Several radioactive precursors, ^{3}H-glucosamine, ^{3}H-fucose ^{14}C-thymidine and ^{14}C-uridine were used as indicators for lectin induced changes in the metabolic processes of the Caco-2 cells.

Materials and method

Crude lectins were prepared from Phaseolus vulgaris 'cv processor' beans, using fetuin Sepharose affinity chromatography essentially according to Pusztai & Watt (1974). The crude lectin fraction was then separated on a Mono S high resolution ion exchange column, equilibrated with 30 mM K_2HPO_4, pH 5.5 with 1 mM octylglucoside and eluted with a gradient of 0 to 200 mM LiCl in the same buffer. This yielded five isolectin fractions, which were identified using PA-gel-electrophoresis and haemagglutination.

In all experiments tissue culture 24 flat bottom well plates were used. The cells were seeded at 8.10^4 cells/well with 1 ml of culture medium composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 50 µg of gentamycine/ml, 10 mM sodium bicarbonate, 25 mM Hepes and 20 % (v/v) fetal calf serum (FCS) added and cultured at 37°C in a humified atmosphere of 5% CO_2 in air. A monolayer of cells was formed. On day 21 the culture medium was replaced by different concentrations of the isolectins in 0.5 ml of DMEM. After 44 hours incubation ^{14}C-thymidine (0.05 µCi), ^{14}C-uridine (0.05 µCi), ^{3}Hfucose or ^{3}H-glucosamine (1 µCi) were added and incubation was continued for 4 hours. The incorporation was stopped by adding 1 ml of 10% TCA (4°C). Next the monolayer was washed 2 times with 1 ml of PBS, pH 7.3, fixed with 1 ml of methanol (100%) and dissolved in 0.5 ml of 0.1 N NaOH. The incorporated radioactivity was determined by liquid scintillation counting. The relative incorporation of ^{3}H-glucosamine and ^{3}H-fucose for glycosylation, ^{14}C-thymidine for DNA-synthesis and ^{14}C-uridine for RNAsynthesis in lectin-incubated Caco-2 cells and control Caco-2 cells were compared (table 1). The relative incorporation is defined as a factor by which the response is increased or decreased as compared to cell cul tures which were not incubated with lectins.

The haemagglutinating activity of the PHA-isolectins was determined using rabbit erythocytes.

Results

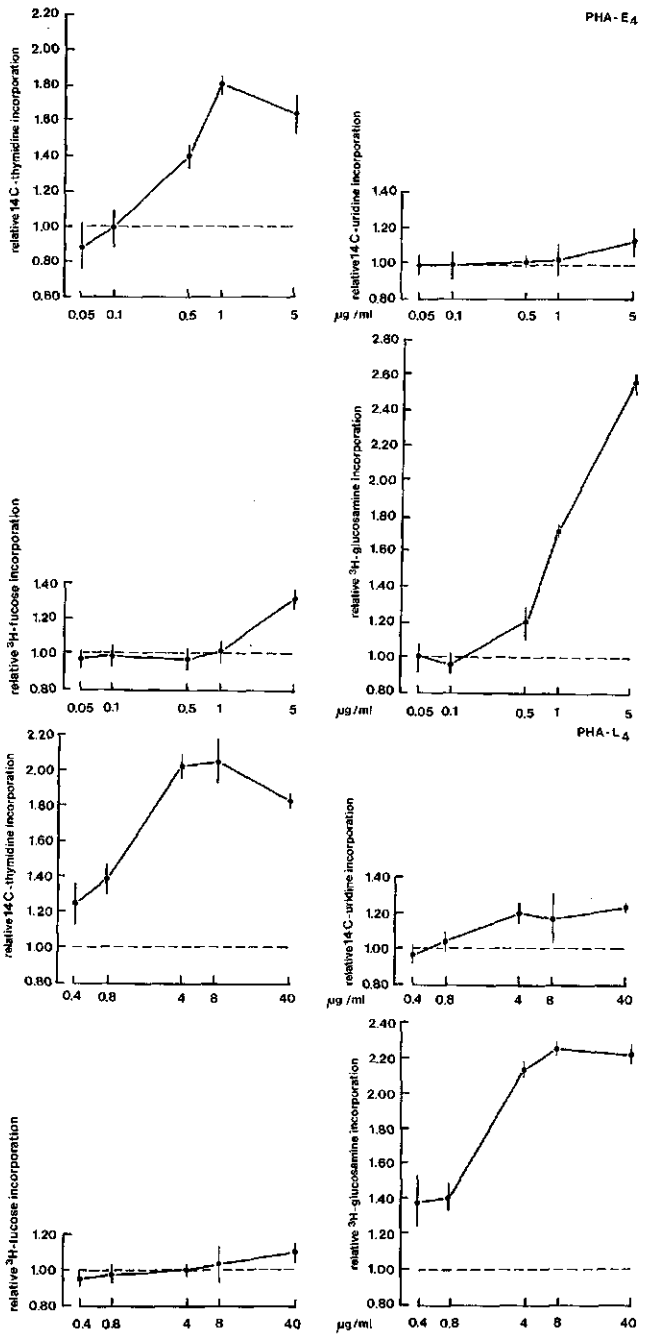
All isolectins increased the relative incorporation of ^{3}H-glucosamine and ^{14}C-thymidine, whilst the incorporation of ^{14}C-uridine and ^{3}H-fucose did not differ from the incorporation of these precursors in the control Caco-2 cells. There was a dose-related respons for the effect of PHA-L4 and PHA-E4 on the relative incorporation of the precursors (Fig. 1 and table 1). Although data are not shown here, a similar picture can be drawn for the PHA-L3E, PHA-L2E2 and the PHA-LE3. Whereas the concentration of PHA-E4 necessary to achieve these results was far less than the concentration of PHA-L4.

The cells, incubated with heat inactivated PHA-E4-isolectin or components from the buffer solution in which the isolectins were delivered showed no difference from the control cells with respect to the incorporation of the radioactive precursors.

The number of cells/well remained the same during the experiment regardless the concentrations of isolectins used.

The PHA-L isolectins and PHA-E4 showed a weak and a strong haemagglutinating activity respectively (Table 1).

Figure 1. Relative incorporation of ^{3}H-glucosamine, ^{3}H-fucose, ^{14}C-thymidine and ^{14}C-uridine by differentiated Caco-2 cells following incubation with PHA isolectins.



PHA-	L4	L_3E	L_2E_2	LE_3	E_4
^{3}H-glucosamine	++	++	++	++	+++
^{3}H-fucose	0	0	+	+	+
^{14}C-thymidine	++	++	++	++	++
^{14}C-uridine	0	0	0	0	0
HA/mg protein	770	710	7,140	9,090	18,180

Table 1. The effect of PHA isolectins upon the relative incorporation of ^{3}H-glucosamine, ^{3}H-fucose, ^{14}C-thymidine and ^{14}C-uridine by differentiated Caco-2 cells, also shown is the haemagglutination activity of the isolectins in haemagglutinating units per mg protein (HA/mg protein. One haemagglutinating unit is defined as the smallest amount of sample necessary for agglutination under test conditions, Valdebouze et al., 1980).
0 = relative incorporation is 1.0
+ = relative incorporation is 1.2 - 1.5
++ = relative incorporation is 1.5 - 2.0
+++ = relative incorporation is 2.0

Discussion

All isolectins induce an increase of the relative incorporation of ^{3}H-glucosamine and ^{14}C-thymidine, showing a dose-related response. In a parallel experiment using commercial PHA-E and PHA-L an enhanced incorporation of ^{35}S-methionine in Caco-2 cells was found (Koninkx, personal communication). So the increase in the relative incorporation of ^{3}H-glucosamine indicates an increased glycosylation and/or increased glycoprotein synthesis of the Caco-2 cells. The fact that there is no change in amount of RNA in the cells, suggests that the increased glycoprotein synthesis may be due to a translational control of the glycoprotein synthesis. The augmented incorporation of ^{14}C-thymidine, without an increase in the number of cells, may point to the synthesis of DNA for other purposes than an increase of numbers of cells, e.g. for the repair of DNA damaged by the isolectins. Further studies to elucidate this subject are necessary.
In spite of the fact that the PHA-L4 and PHA-L3E isolectins show a weak haemagglutination activity, they have a stimulatory effect on the relative incorporation of radioactive precursors in the Caco-2 cells. Since the haemagglutinating activity of an isolectin is mostly restricted to the E-subunit, the negative results in the haemaglutination test may be due to the fact that these isolectins contain no or just one E subunit.

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ACTIN CYTOSKELETAL LESIONS IN DIFFERENTIATED HUMAN COLON CARCINOMA CACO-2 CELLS AFTER EXPOSURE TO SOYBEAN AGGLUTININ

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Summary

We have investigated the effects of soybean agglutinin on the cytoskeletal element actin in differentiated Caco-2 cells. The actin cytoskeleton of the cells was visualized by fluorescence microscopy using NBD-phallacidin as a specific marker for F-actin. Using the deoxyribonuclease-I inhibition assay depolymerization of F-actin could be established after exposure for 15 min. to soybean agglutinin (SBA). In addition, an increase of intracellular G-actin was measured. The induction of depolymerization could be prevented by incubating the Caco-2 cells with SBA and N-acetylgalactosamine simultaneously. The increase in the amount of G-actin appeared to be correlated with a shortening of microvilli of the Caco-2 cells.
Keywords: soybean agglutinin, actin, depolymerization, Caco-2 cells.

Introduction

The human colon carcinoma cell line Caco-2, represents an unique in vitro model for investigations on structural and functional properties of differentiated enterocytes (Pinto et al., 1983). Since differentiated Caco-2 cells exhibit characteristics of small intestinal enterocytes and soybeans are common components in the diet of man and animal (Liener, 1974; Liener, 1979), we decided to study the effects of SBA on the actin cytoskeletal filaments in differentiated Caco-2 cells.

Materials and methods

For cell culture Dulbecco's modified Eagle medium was used, supplemented with 1% (v/v) non-essential amino acids, 50µg of gentamycin /ml, 25 mM Hepes, 10 mM sodium bicarbonate and 20% (v/v) fetal calf serum (FCS). The cells were seeded at 8.10^4 cells/well containing 1 ml of culture medium. Incubation with SBA was performed in culture medium containing 2% (v/v) of the serum substitute Ultroser G and 1% (v/v) FCS. NBD-phallacidin was used as a specific marker for F-actin (Barak et al., 1980). The percentage of G-actin in differentiated Caco-2 cells was determined according to the assay of Blikstad et al. (1978) modified by Raemakers et al. (1981). For ultrastructural studies, fixation and embedding of the cell monolayers was performed in situ (Pinto et al., 1983).

Results

The effect of SBA on the percentage G-actin in the cytoplasm of differentiated Caco-2 cells

When differentiated Caco-2 cells were incubated with SBA and subsequently stained with NBD-phallacidin, no change was observed in the fluorescence pattern of F-actin between SBA-incubated and control cells. However, using the biochemical approach a significant increase in the percentage of G-actin in Caco-2 cells could be established at 15 min. after addition of SBA. The effect was inhibited by silmultaneous administration of SBA and N-acetylgalactosamine. In addition, incubation of differentiated Caco-2 cells for two hours in the presence of graded amounts of SBA revealed an increase in the percentage of G-actin. At concentrations of 10 and 20µg/ml a significant increase was achieved ($p < 0.05$) (Figure 1).

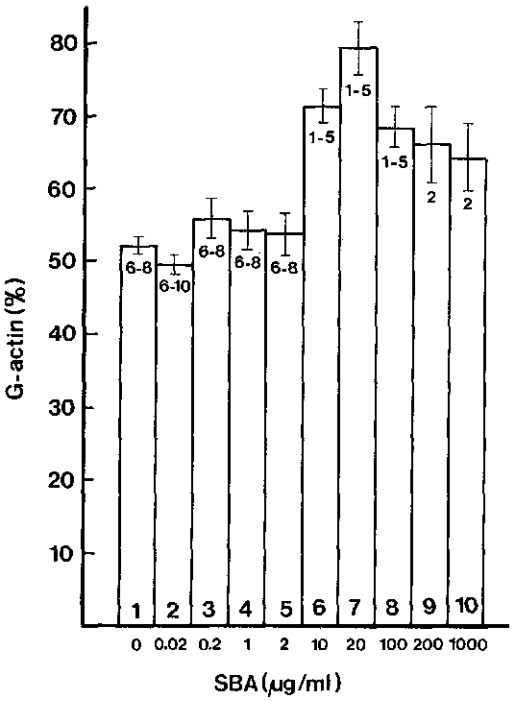

Fig.1 Changes in the percentage of G-actin in differentiated Caco-2 cells after incubation with SBA. Two different passages have been used to establish this effect. In each passage the mean percentage of G-actin of the SBA-concentrations was detemined using quadruplicate cultures. The results are expressed as the mean percentage of G-actin ± SEM. The numbers at the top of the columns indicate with which columns the concentration of SBA in question differs significantly ($p < 0.05$).

Ultrastructural changes in the microvilli of differentiated Caco-2 cells after incubation with SBA

Scanning electronmicroscopic studies revealed that after incubation of differentiated Caco-2 cells for two hours with 20 µg of SBA/ml, aggregation of the microvilli appeared to be induced. Aggregation of the microvilli was absent in control Caco-2 cells. In comparison with control cells transmission electronmicroscopic studies of SBA-incubated cells clearly exhibited the presence of shortened microvilli (Figure 2).

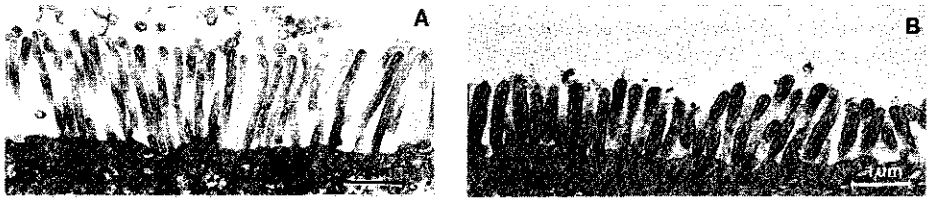

Fig. 2 Microvilli of control Caco-2 cells (A). Shortened microvilli of Caco-2 cells after incubation with SBA (B).

Discussion

Although at the cellular level no difference was found in the fluorescent pattern of F-actin between SBA-incubated and control cells, the biochemical approach clearly demonstrated a significant increase in the percentage of G-actin in differentiated Caco-2 cells in a dose-related fashion. The conversion of F-actin into G-actin, leading to an increase in the percentage of G-actin could already be established at 15 min. after addition of SBA. The conversion was completely inhibited by simultaneous incubation with N-acetyl galactosamine, indicating that the effect was related to binding of SBA to carbohydrate residues of the Caco-2 cells. The short period of incubation needed to accomplish the increase of G-actin suggests that the agglutinin effects result from cell surface receptor-agglutinin interaction rather than an increased biosynthesis of G-actin.

In rats the appearance of morphological alterations in the cytoskeletal structure was found to be correlated with the appearance of shortened microvilli (King et al., 1982; Lorenzsonn & Olson, 1982; Sjölander et al., 1986). Also, after incubation with SBA the microvilli covering the Caco-2 cells were shortened (figure 2). The actin cytoskeletal changes which appear to be the result of the interaction between SBA and its specific sugar on the cell surface membrane probably play a role in the pathogenesis of the microvillous abnormalities.

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FUNCTIONAL EFFECTS OF PHASEOLUS VULGARIS AGGLUTININ ON DIFFERENTIATED HUMAN COLON CARCINOMA CACO-2 CELLS

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Summary

Differentiated human colon carcinoma Caco-2 cells have been used to study the effects of Phaseolus vulgaris agglutinin (PHA). Following incubation for 48 hours with PHA-L and PHA-E respectivily the incorporation of precursors for DNA-, RNA-, and glycoprotein synthesis into the trichloro-acetic insoluble fraction of the Caco-2 cells was determined. Both PHA-L and PHA-E stimulated the uptake of ^{14}C-thymidine and ^{3}Hglucosamine, whereas neither PHA-L nor PHA-E were able to influence the uptake of ^{14}C-uridine. With respect to ^{3}H-fucose the stimulatory effect remains confined to PHA-E. Since PHA-L and PHA-E were tested at the same concentration, it is obvious that PHA-E is more effective than PHA-L. The changes in the uptake of radioactive precursors were lost after heat treatment of PHA-E.

Introduction

Phaseolus vulgaris agglutinin (PHA) binds through its sugar-reactive sites to the epithelial cells of the small intestine and disrupts the luminal surface (King et al., 1982; King et al., 1986). In addition, lectins are known to interfere with cellular processes in different types of cells.
Essentially all metabolic processes examined in PHA-treated lymphocytes are stimulated, though to varying degrees, and at different times after exposure to the mitogen (Hume & Weidemann, 1980) There are few studies on PHA in relation to cell metabolism in enterocytes. In the present study, using differentiated human colon carcinoma Caco-2 cells, which display both structural (microvilli, tight junctions) and functional (brush border enzymes) characteristics of small intestinal enterocytes (Pinto et al., 1983), the effects of PHA-L and PHA-E on the uptake of thymidine, uridine, glycosamine and fucose was investigated.

Materials and Methods

Tissue culture 24 flat bottom well plates were used in all experiments. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 50µg of gentamycine/ml, 10 mM sodium bicarbonate, 25 mM Hepes and 20% (v/v) FCS and cultured at 37°C in a humified atmosphere of 5% CO_2 in air. The cells were seeded at 8.10^4 cells/well containing 1 ml of culture medium. On day 21 the cell culture medium was replaced by 0.5 ml of DMEM containing different concentrations of PHA-L and PHA-E respectivily. After incubation for 44 hours ^{14}C-thymidine (0.05 µCi), ^{14}C-uridine (0.05

μCi), ^{3}H-fucose (1 μCi) or ^{3}H-glucosamine (1 μCi) was added and incubation was continued for 4 hours.
The incorporation was stopped by adding 1 ml of 10% TCA (4°C). Subsequently the monolayer was washed 2 times with 1 ml of PBS, pH 7.3, fixed with 1 ml of methanol and dissolved in 0.5 ml of 0.1 N NaOH. The incorporated radioactivity was determined by liquid scintillation counting.

Results

Incorporation of the precursors for DNA-, RNA-, and glycoprotein synthesis by differentiated Caco-2 cells following incubation with PHA-E or PHA-L

When PHA-L was added to the culture medium and incubation was performed for 48 hours, the relative incorporation of ^{14}C-thymidine and ^{3}H-glucosamine appeared to increase with increasing lectin concentrations, whereas no change in the relative incorporation of ^{14}C-uridine and ^{3}H-fucose could be observed (Figure 1). In comparison with PHA-L the stimulatory effect expressed as the relative incorporation of ^{14}C-thymidine and ^{3}H-glucosamine appeared to be significantly higher for PHA-E. Moreover, PHA-E concentrations as low as 40 μg/ml already displayed a maximal stimulatory effect with respect to the relative incorporation of ^{14}C-thymidine, whereas the relative incorporation of ^{3}H-glucosamine amounted to 85%. As opposed to PHA-L a marked increase of the relative incorporation of ^{3}H-fucose was observed after incubation. The relative incorporation of ^{14}C-uridine did not change irrespective of the PHA-concentration. The changes in the relative incorporation of ^{14}C-thymidine, ^{35}S-methionine (not demonstrated in Figure 1), ^{3}H-fucose and ^{3}H-glucosamine vanished, when prior to incubation PHA-E was heated for 15 min at 100°C (Table 1).

Table 1. The effect of heat treated PHA-E (200 μg/ml) upon the relative incorporation of radioactive precursors by differentiated Caco-2 cells.

Precursor	Relative incorporation	Relative incorporation
	no heat-treatment	heat-treatment
^{14}C-thymidine	1.71 ± 0.04	1.03 ± 0.06
^{14}C-uridine	0.95 ± 0.07	1.05 ± 0.07
^{35}S-methionine	1.45 ± 0.02	1.00 ± 0.03
^{3}H-fucose	1.62 ± 0.04	0.99 ± 0.05
^{3}H-glucosamine	1.96 ± 0.09	1.02 ± 0.05

Fig.1. Relative incorporation of ^{14}C-thymidine, ^{14}C-uridine, ^{3}H-glucosamine and ^{3}H-fucose by differentiated Caco-2 cells following incubation with PHA-L and PHA-E, respectivily. Two different passages of the cells have been used to establish the effect of PHA-L and PHA-E. The results are expressed as the mean relative incorporation ± SEM. For each passage of the cells the relative incorporation of all lectin concentrations was determined using quadruplicate cultures.

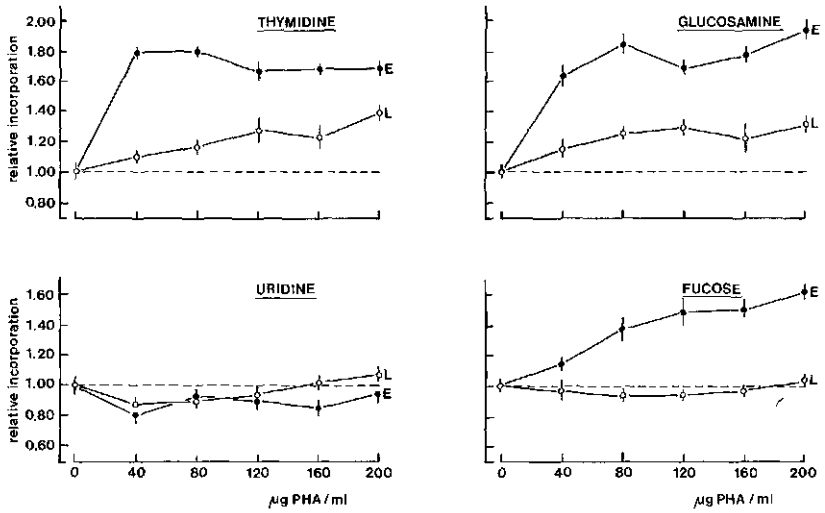

Fig.1

Discussion

PHA-L and PHA-E have been used to investigate whether or not any difference could be determined in their effect upon the cell metabolism of differentiated Caco-2 cells. Since incubation was performed with the very same PHA-concentrations, the results demonstrate, that PHA-E stimulates the incorporation of thymidine, fucose and glucosamine to a greater extent than PHA-L. On the other hand, neither PHA-L nor PHA-E is able to stimulate the uptake of uridine. The data presented here further indicate, that the established changes in the cellular metabolism do not correlate with the agglutination activity of the isolectins (no erythrocyte agglutination for PHA-L, erythrocyte agglutination for PHA-E). After incubation of differentiated Caco-2 cells with heat-treated PHA-E, the stimulatory effect of PHA-E, resulting in an increased incorporation of radioactive precursors, disappears.

Although data are not shown here, the DNA content per well does not change with increasing lectin concentration. For that reason DNA synthesis most probably does not account for the increased uptake of thymidine. Whether the uptake is due to an increased DNA repair is presently unknown.

In the presence of PHA-E the relative incorporation of methionine (Table 1), fucose and glucosamine increases, reflecting sustained synthesis of (glyco)proteins. At the same time the relative incorporation of uridine does not change as compared to control Caco-2 cells. For that reason the data presented here suggest that the increased rate of synthesis of (glyco)proteins is due to a translational rather than transcriptional control of (glyco)protein synthesis.

PHA-L as well as PHA-E induce a number of cell metabolic changes within the Caco-2 cells. Further experiments are necessary to elucidate the regulatory mechanisms of these events.

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MATURATION OF PEA LECTIN; A COMPARISON WITH OTHER LEGUMINOSAE LECTINS

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Summary

Using preparative isoelectric focusing, affinity purified lectin from immature pea seeds can be separated into different fractions, one of which is built up of 28 kd chains (γ-chains). γ_2 molecules display both carbohydrate binding and haemagglutinating properties. Processing of γ_2 into $\alpha_2\beta_2$ molecules is accompanied by only minor changes in the conformation of the lectin. The biosynthesis, transport and post-translational processing steps of different Leguminosae lectins are compared.
Keywords: Pea (Pisum sativum L.) lectin, Leguminosae lectins, protein processing.

Introduction

Cell agglutinating and/or glycoconjugate precipitating proteins from non-immune origin, called "lectins", were first described by Stillmark at the end of the previous century [Franz, 1988]. Hundred years later it is generally recognized that lectins are not solely confined to plant tissues, but rather are widely distributed in nature (see Liener et al., [1986] for reviews on plant, animal and bacterial lectins). Today, over 100 lectins have been purified and characterized, some of them only superficially, but others in great detail. Especially the seeds of legume plants have proven to be excellent lectin sources, since up to 15% of the total protein content of these seeds may consist of lectin. This might be a principal reason why legume lectins have by far been most thorougly investigated (see Van Driessche [1988] for a recent review).

In this paper we describe the different processing steps which convert the precursor of the lectin from pea seeds (Pisum sativum L.) into its mature form and compare the protein maturation of pea lectin with that of some other types of Leguminosae lectins.

Materials and Methods

Extraction and purification of pea seed lectin, its subunits and its precursor

Pea lectin (var. "Wonder van Kelvedon") from both mature and immature seeds was purified by affinity chromatography on Sephadex G-75 as described previously [Van Driessche et al., 1982], except that the extraction time was shortened to two hours.

Purification of the pea lectin subunits and the natural fragments was performed as reported previously [Van Driessche et al., 1976].

Preparative isoelectric focusing was used to purify the pea lectin precursor starting from affinity purified pea lectin isolated from immature seeds. Isoelectric focusing on a preparative scale was performed on an LKB 2117 Multiphor apparatus in a 3.5-10/5-8 (Ampholines 1/1 by volume) pH gradient as recommended by the manufacturer (LKB-

application note 198) using Biogel P-200 as supporting medium. The homogeneity of the different protein fractions was controlled by analytic isoelectric focusing.

Analytical methods

SDS-PAGE was performed on linear 12.5-25% polyacrylamide gels using the buffer system of Laemmli [1970].

Haemagglutination assays were performed in micro-titer plates with non-trypsinized rabbit erythrocytes.

Circular dichroism measurements were performed on a Cary 61 spectro-polarimeter. The mean residual ellipticity [θ], in degrees.cm^2.dmole^{-1}, was calculated as: [θ] = (MRW/100) x θ/c.d where c is the protein concentration in g/ml and d is the optical path length in dm; the mean residual weight (MRW) is 110.

Results

When affinity purified pea seed lectin preparations isolated from mature and immature seeds are compared by SDS-PAGE, the banding pattern is identical except for a 28 kd polypeptide which is prominently present in the lectin preparations from immature seeds, while only trace amounts are present in the preparations from mature seeds. The 18 kd and 5.8 kd polypeptides correspond to respectively the β- and α-chains of pea lectin, whereas the 8.5 kd and 6.5 kd bands were previously shown to be naturally occuring fragments of the β-chain [Van Driessche et al., 1988]. The α- and β-subunits, as well as the natural β-chain-derived fragments can be purified to homogeneity by gel filtration on Biogel P-100 in 6 M guanidine-HCl [Van Driessche et al., 1976].

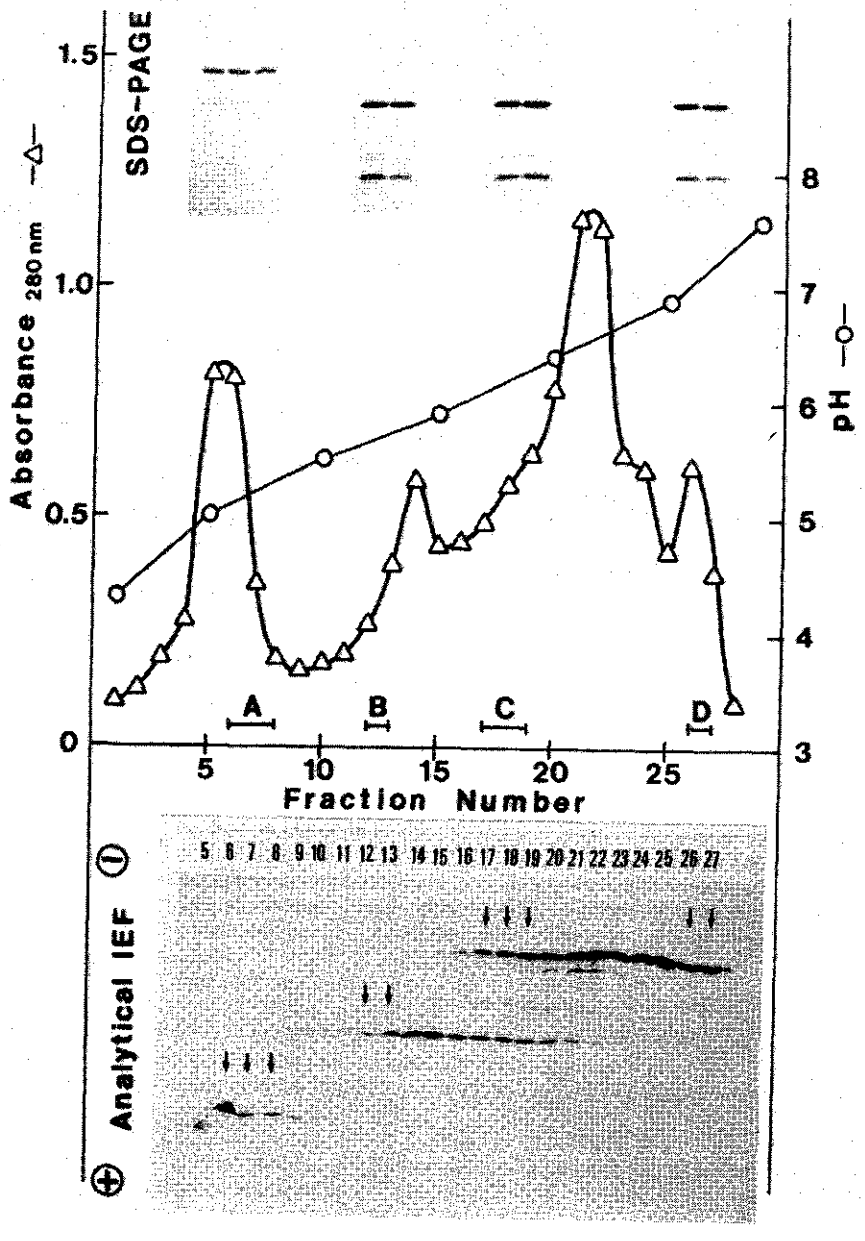
All attempts to isolate the 28 kd chain from immature seed lectin by this procedure were unsuccessful. Upon preparative isoelectric focusing of affinity purified pea lectin isolated from immature seeds, a fraction with a pI of 5.2 could be obtained which consists of 28 kd subunits (Figure 1, fraction A). This fraction binds onto a Sephadex G-75 column and agglutinates rabbit erythrocytes in a glucose/mannose inhibitable manner. The 28 kd chain was shown by automatic sequencing to have a N-terminal sequence identical to that of the β-chain [Van Driessche et al., 1988]. Besides we showed that the 28 kd polypeptide contains both β- and α-chain peptides [Van Driessche et al., 1988].

Circular dichroism measurements of pea lectin prepared from mature seeds ($\alpha_2\beta_2$ subunit structure) and of the purified isolectin pI 5.2 (γ_2) reveal that both have essentially the same secondary structure, while differences in the tertiary structure are evidenced in the near UV-region of the spectrum (Figure 2).

Discussion

When isolated from immature seeds, affinity purified pea lectin is essentially composed of three types of subunits, i.e. α-, β- and γ-subunits. Using preparative isoelectric focusing a carbohydrate-binding, haemagglutinating fraction can be isolated consisting exclusively of 28 kd subunits (γ-chain). This chain was previously shown to contain peptides derived from both the α- and β-chains, indicating that the 28 kd polypeptide is a precursor molecule from which the α- and β-subunits are derived. Since the γ-chain is prominently present in immature seeds, but only in extremely minor quantities in mature seeds, it can be concluded that proteolytic processing of the precursor is maturation-dependent.

Figure 1. Separation of affinity purified pea lectin from immature seeds by preparative isoelectric focusing. Fractions were pooled as indicated by the arrows.



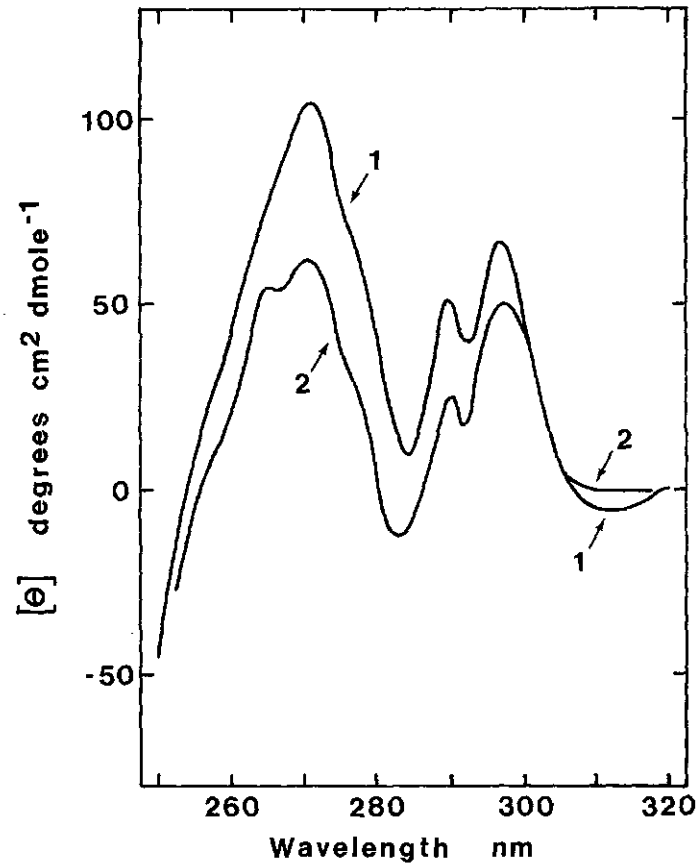


Figure 2. Comparison of circular dichroism spectra of pea lectin $\alpha_2\beta_2$ (spectrum 1) and pea lectin γ_2 (spectrum 2).

From the comparison of the circular dichroism spectra of both (γ_2) and ($\alpha_2\beta_2$) pea lectin, it can be concluded that the proteolytic processing which converts the precursor chain into the mature subunits is not accompanied by major structural changes. These findings are in agreement with the fact that γ_2 molecules behave as active lectins.

From our data, in combination with those of Higgins and coworkers [1983a, 1983b], the following picture can be drawn for the biosynthesis, processing and transport of pea lectin, which is a typical member of the two-chain legume lectins: (i) The lectin mRNA is translated into a pre-prolectin in association with the rough endoplasmatic reticulum (RER). (ii) In the RER, the presequence is cotranslationally removed to yield the prolectin (γ_2). (iii) The prolectin moves towards the protein bodies [Van Driessche et al., 1981] where it is proteolytically processed to yield the α- and β-subunits. As a result, a peptide of 25 residues is removed. Some β-chains are further degraded to produce the naturally occuring fragments (6.5 kd and 8.5 kd). A comparison of the C-terminal part of the α-chain sequence as derived from protein- [Richardson et al.,

1978] and cDNA-sequence analysis [Higgins et al., 1983a] reveals that a small peptide (Ala-Ala-Asp-Ala) is also post-translationally removed from the α-chains. Since the two pea isolectins (pI 5.8 and pI 7.0) only differ in their lysine content, and since the α-chains display charge heterogeneity when submitted to electrophoresis in acid urea [Van Driessche et al., 1976], it is tempting to suggest that this charge heterogeneity results from the splitting off of a very small peptide from a fraction of the α-chain molecules, by which the lysyl-residue in the vicinity of the C-terminus (Lys-240) is removed. This view on the ontogeny of the pea isolectins is supported by the fact that only one band is obtained upon electrophoresis of pea lectin which is built up of 28 kd polypeptides (results not shown).

Another two-chain Leguminosae lectin, that of Vicia faba seeds (favin), has been shown by Hemperly et al. [1982] to be synthesized as a precursor which is subsequently proteolytically processed to yield the two chains (α or light one, and β or heavy one). The biosynthesis, transport and post-synthetic modifications of the lectins of Phaseolus vulgaris (PHA), one-chain lectins, has been studied in great detail by Chrispeels and coworkers [1984]. Similarly to pea lectin and favin, PHA is synthesized on polysomes attached to the RER. The signal peptide is removed cotranslationally, and the polypeptide chain is substituted with two high-mannose type oligosaccharides, which are further tailored to their mature form in the Golgi apparatus and protein bodies. Although concanavalin A, the lectin from Canavalia ensiformis, is not a glycoprotein, it is synthesized as an inactive, glycosylated precursor which is processed to maturity in a very complex way [Bowles and Pappin, 1988] involving proteolytic cleavages and reannealing of the generated fragments.

From all information available until now, it can be concluded that Leguminosae seed lectins are synthesized as prolectins on the RER, and are transported via the Golgi complex to their final destination, i.e. the protein bodies. Whether prolectins will be proteolytically cleaved or not seems to be determined by conformational restraints in the lectin precursor molecules, rather than by the lack of the processing enzyme(s) itself. This is clearly shown by the fact that in some seeds, such as those of Vicia cracca, two lectins are present which are encoded by different genes, i.e. a mannose/glucose specific two-chain lectin, and a N-acetyl-galactosamine specific one-chain lectin [Baumann et al., 1979].

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GROWTH AND SYSTEMIC IMMUNE RESPONSES IN EARLY WEANED PIGLETS FED SOYABEAN MEAL VARYING IN LECTIN, TRYPSIN INHIBITOR AND UNDENATURED GLOBULINS

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Summary

The effects of anti-nutritional factors and modified globulins on growth and diarrhoea of piglets weaned onto diets containing processed soyabean meal (SBM) were studied. There was no clear relation between weight gain and dietary content of lectin or trypsin inhibitor. Weight measurements did show a trend towards improved growth with increased denaturation of globulins. Diarrhoea was not observed in any of the piglets, but all animals produced circulatory anti-soya antibodies. Reasons for the benefical effect of processing SBM are uncertain, but may have related to alterations in protein structure.
Keywords: lectins, trypsin inhibitors, piglets, soya

Introduction

Severe diarrhoea and retarded growth is a common problem amongst early weaned piglets especially when the diet is based on non-milk protein. Both immaturity of the digestive system (Fowler, 1980) and adverse immunological reactions in the gut (Miller et al. 1984) have been implicated in the aetiology of the disorder. This, for the young pig, may limit the nutritive value of products derived from legume seeds which contain several biologically active substances such as haemagglutins and enzyme inhibitors. However, there is little understanding of the relative importance for the piglet of these anti-nutritional factors (ANFs) and of possible benefits from alterations in the structure of globulins through industrial processing. Experiments described in this paper were done to assess the effects of weaning piglets onto diets varying in their content of lectin, trypsin inhibitor and native globulins, on growth performance and occurrence of diarrhoea. Diets based on soya were used because this source of protein has been shown to cause adverse immune responses linked with disorders in gut function in piglets and young calves (Miller et al. 1984; Sissons and Smith, 1976; Kilshaw and Sissons, 1979).

Methods

Ninety six Large White x Landrace piglets from litters of 13 sows in their third parity were weaned at 3 weeks of age and assigned to one of four groups randomised for sex and weight variations. Three groups of piglets received one of three diets ('high' ANF, 'medium' ANF or 'low' ANF) containing protein (181-185g/kg DM) derived from

cereals and variously heated soyabean meal (SBM). These treatments provided soya products which varied in lectin and trypsin inhibitor activity. An 'enzyme linked immuno-sorbant assay' (ELISA) using rabbit antisera to isolated soya globulins indicated that processing with heat had altered the structures of glycinin and β-conglycinin. The products were then formulated into 'low', 'medium' and 'high' ANF diets respectively. Soya provided 45-49% of the protein. The levels of lectin (measured by an ELISA), alkali extracted trypsin inhibitor (Kakade et al. 1974) and undenatured globulins in these diets are shown in Table 1. The fourth group of piglets was weaned onto a non-soya 'control' diet containing protein (170g/kg DM) derived from cereals, cows' milk and fish. The four diets were isocaloric and balanced for available lysine.

Piglets were kept in a controlled environment house with six pigs per pen and received the weaner diet ad-libitum. Feed consumption and piglet weights were recorded weekly and the animals were inspected daily for looseness of faeces which was scored subjectively on a scale of 1-5. Samples of peripheral blood were collected shortly before weaning and at 2, 4 and 6 weeks post-weaning. Treatment differences were assessed by analyses of variance. Live weight at 4-9 weeks of age was adjusted by co-variance analysis for initial weight at weaning.

Table 1. Relative levels of anti-nutritional factors and undenatured globulins in the weaner diets.

Diet:	Lectin (g/Kg)	Trypsin inhibitor (g/Kg)	ELISA units† of glycinin	ELISA units† of β-conglycinin
'Low' ANF	1.3	0.39	1/512	1/256
'Medium' ANF	2.5	0.56	1/64	1/32
'High' ANF	4.4	0.97	1/128	1/128

† ELISA units were expressed as a proportion of the level in a defatted, but otherwise untreated, soyabean meal

Results and discussion

Piglets which received the 'control' or 'low' ANF diet showed slightly better growth than animals of the 'high' ANF group, whilst the latter group gained weight somewhat faster than the 'medium' ANF group (see Table 2). However, few differences between treatments were statistically significant ($P > 0.05$). Those that were significant occurred in the later weeks of the study i.e. comparisions between the 'control' diet and either the 'low' ANF diet (week 4) or the 'medium' ANF treatment (weeks 5 and 6).

Table 2. Comparison of growth performances of pigs weaned at 3 weeks of age.

Diet:	Mean weight (kg) of pigs						
	weaning weight	weeks post-weaning 1	2	3	4	5	6
'Control'	7.1	8.2	9.5	12.9	16.6	20.3	25.9
'Low' ANF	7.1	8.3	9.5	12.0	15.4	19.7	25.6
'Medium' ANF	7.3	8.2	9.2	11.9	15.8	19.3	24.9
'High' ANF	7.0	7.8	9.4	12.0	15.7	19.5	25.2
LSD (df12,P<0.05)	0.3	0.6	0.6	1.1	0.9	1.0	1.0

Mean feed intake was higher overall for the 'control' group compared with the ANF groups. For example, during the 6 weeks of the trial, pigs of the 'control', 'low' ANF, 'medium' ANF and 'high' ANF consummed on average 30.8, 28.2, 28.1 and 28.6 kg of feed respectively. This difference in appetite of the 'control' pigs was evident by 3 weeks post-weaning when the mean weekly feed consumption was 4.3, 3.8, 4.0 and 4.0 kg respectively. There were, however, no consistent differences in ratios expressing amounts of feed converted to live weight (Table 3).

Despite the presence of anti-nutritional constituents in the test diets containing soyabean products, throughout the trial none of the treatments led to diarrhoea (i.e. a faecal score > 3). This observation is consistent with the notion that anti-nutritional factors predispose post-weaning diarrhoea by somehow facilitating the proliferation of gut pathogens. It is plausible that had an enteric microbial infection been present in this trial the growth differences between treatments might have been larger.

Table 3. Comparison of feed conversion performances of pigs weaned at 3 weeks of age.

Diet:	Mean feed : gain ratio week post-weaning					
	1	2	3	4	5	6
'Control'	1.5	1.1	1.3	1.8	1.6	1.9
'Low' ANF	1.1	1.2	1.5	1.7	1.4	1.8
'Medium' ANF	1.4	1.1	1.4	1.6	1.5	1.7
'High' ANF	1.6	1.0	1.6	1.6	1.6	1.8

Overall differences in values for growth or feed conversion for the three ANF treatments were too small and not sufficiently consistent throughout the trial period to permit a definite conclusion to be made about a possible link between lectin or protease inhibitor and animal performance. But measurements of live weight did show a trend for pigs to gain weight at a slightly faster rate as the level of apparent undenatured glycinin and B-conglycinin in the diet was reduced.

Table 4. Measurements of circulatory anti-soya antibodies in piglets weaned at 3 weeks of age.

Diet:	Anti-soya antibody titres (log2)†							
	pre-weaning		week post-weaning 2		4		6	
	mean	SE	mean	SE	mean	SE	mean	SE
'Low' ANF	1.5	0.4	3.1	0.4	3.5	0.3	3.1	0.3
'Medium' ANF	1.7	0.4	3.9	0.4	4.0	0.3	3.6	0.3
'High' ANF	1.0	0.4	3.4	0.4	3.8	0.3	3.3	0.3

† An ELISA was used to measure levels of anti-soya antibodies by coating plates with saline extracts of defatted SBM

The small improvement in live weight gain of pigs fed the 'low' ANF diet may have been related to denaturation of soya globulins resulting from refinements in processing of the soyabean meal. The reasons for this putative benefical effect are uncertain. Alteration of globulin structure may have reduced the ability of these proteins to invoke a deleterious reaction in the gut through an immune mechanism. Some support for this idea was obtained from limited observations of circulatory antibodies against constituents of unheated SBM (see Table 4). Although anti-soya antibodies were produced in all soya fed animals, those given the 'low' ANF diet showed slightly lower titres than piglets weaned onto the 'medium' or 'high' ANF diets. However, it is possible that processing and/or partial digestion may have exposed further antigenic structures and extended the spectrum of antibodies. Some of these antibodies may have remained undetected by an assay using native rather than processed soya protein. Alternatively modifications of protein structure through processing with heat may have rendered the globulins more susceptible to proteolytic enzymes.

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DISCUSSION ON LECTINS

Chairman : R.R. Marquardt
Reported by: R.J.Hamer

Overview on ANF's

The session opened with a main paper by Liener in which an overview was given of the current state of the art regarding ANF's. Questions regarding species response, mechanism of action and determination of the different ANF were pointed out 'to set the stage' for the ANF workshop. In the short discussion which followed it was suggested not to overlook the effect of the elimination of other legume or feed ingredients on the action of ANF.

Lectins

Pusztai presented the second main paper on the biological effects of mainly Phaseolus vulgaris lectins. Based on detailed studies with rats he proposed a possible mechanism of action for lectins. The key to the harmful effect of lectins is their growth promoting effect on the proliferation of the intestine's crypt cells. This property of lectins leads - according to dr Pusztai - to a wasteful proliferation of intestinal tissue at the expense of other organs (liver, pancreas) tissues (skeletal muscles) and energy resources (glycogen). Also other effects were observed (e.g. a decreased insulin level). The discussion which followed also concentrated on the pathogenesis of lectins. Which effects are directly related to the action of lectins and what do all these effects add up to? Is the hyper-regeneration of the crypt cells a direct growth promoting effect or is it a secondary effect caused by damage of villi? No definite answers were given.

The effects observed with Phaseolus lectins appear to be general lectin effects.

It was noted that similar effects were observed when rats were fed with diets containing isolated soybean agglutin or Vicia faba beans.

In the second part of the session a series of short papers was presented. First, Hamer presented a new method of lectin analysis, named the Functional Lectin Immuno Assay (FLIA). This assay is based on the immunological detection of lectins bound to an artificial matrix of complex carbohydrates or a preparation of porcine intestinal brush border membranes. It was noted that the results of a FLIA assay, although elegantly mimicking the in vivo situation, still need validation by in vivo experiments.

In the following paper Grant presented a paper on the local and systemic response of rats to dietary soyabean proteins. His results indicated that soya contains another ANF next to the soyabean lectin and the trypsin inhibitor proteins. These ANF's have a combined negative effect on body metabolism.

The growth promoting action of lectins on the small intestine was investigated on a cellular level by Bardocz. She concluded that lectin induced hyperplastic growth is dependent on the uptake of polyamines (putrescine, spermine/spermidine). If these polyamines are derived from other internal organs this may hamper organ growth. The source of the 'sequestered' polyamines remains however to be identified. In the discussion which followed it was disputed that these polyamines can be resorbed from the diet due to their metabolism in the rats intestinal tract.

Bacterial lectins play a role in the attachment of bacteria to calve villi. Van Driessche explained that fimbriae serve as attachment factors for this lectin mediated binding. The study of the carbohydrate binding specificity of bacterial lectins will result in methods to prevent adhesion of bacteria to villi or to prevent the attachment of pathogenic plant lectins.

In the last part of the session a series of posters were presented on the effect of lectins. First, the effect of Phaseolus lectin containing diets on the intestinal mucosa of pigs was presented. It was discussed

that, apart from changes in the intestinal mucosa which are to a certain extent reversible, diarrhoea was observed. This was evidenced as a rather quick response and therefore not likely to be related to microbial colonization. A comment was made that diarrhoea was not found in rats fed with purified lectin supplemented diets.

A second poster described a study on the effect of soyprotein diets containing different levels of ANF on growth and immune response in young piglets. A comment was made that diets containing purified ANF have to be used in order to draw conclusions from this type of animal-feeding experiments.

Two posters were presented describing the effects of purified lectins on cultured human colon carcinoma (Caco-2) cells. It was commented that the use of confluent cells severely hampers the induction of mitosis by lectins. It was therefore recommended to use differentiating cells to mimic the in vivo situation. In the following poster morphological evidence was presented that soybean agglutinin affects the actin cytoskeleton of caco-2 cells. Again questions arose to the nature of this effect. No data could be presented on a direct effect of lectins on the ATP-ase involved in the actin (de-)polymerization process or on the actual penetration of the lectin into the cells.

One poster was presented on the maturation of pea lectin. It was noted that the posttranslational processing of lectins in the plant cell in the reversed order resembles the fate of lectins, internalized by epithelial cells.

Session trypsin inhibitors

PROTEIN PROTEASE INHIBITORS OF PLANT ORIGIN AND THEIR SIGNIFICANCE IN NUTRITION

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Summary

Protein protease inhibitors are widely distributed in plant seeds, and particularly in legume seeds. They differ in specificity and in potency of inhibition, which depends on the origin of the target enzyme. Feeding experiments of rats and chicks on diets supplemented with the Kunitz (STI) and Bowman-Birk (BBI) trypsin inhibitors from soybeans revealed insignificant depression of animal growth but induced enlargement of the pancreas. The latter effect was not found in the pig, the monkey, the dog or the calf. Carcinogenic changes appeared in the pancreases of male Wistar rats - but not in hamsters and mice- after long-term feeding on raw soybean protein preparations. They have been attributed to the trypsin inhibitor content of the raw soybean meal. While the trypsin inhibitory site of BBI has been found to be responsible for induction of pancreatic enlargement, the chymotrypsin inhibitory site seems to be involved in inhibition of oncogenic transformation and promotion in tissue culture systems.
Keywords: pancreatic enlargement; inhibitors and species specificity; raw soybean meal.

Introduction

A survey of the current knowledge on the distribution of protein protease inhibitors of plant origin reveals hundreds of inhibitors dispersed among different botanical families (reviewed, Liener & Kakade, 1980). The majority of the inhibitors are proteins with molecular weights of 8000-10000, with a few notable exceptions. They differ in specificity and in capacity to inhibit one or two proteases at the same time. Several different kinds of inhibitors can be present in a single tissue, as exemplified in soybeans, barley grains and potato tubers. Most of the inhibitors inhibit trypsin and many inhibit chymotrypsin. Inhibitors of elastase, kallikrein, plasmin, subtilisin, thrombin, bromelain, carboxypeptidase, cathepsin, ficin, papain and pepsin have also been found (reviewed, Birk, 1987). Recently, a number of bifunctional plant protein inhibitors of trypsin and amylases have been isolated (Richardson et al., 1987).

The physiological significance of plant protease inhibitors has been questioned for a long time. Only a few inhibitors are known to inhibit the endogenous proteases of seeds. The fact that certain seeds, such as soybeans and wheat grains, contain inhibitors of growth and of larval gut proteases of the insects Tribolium and Tenebrio, suggest the possibility that these inhibitors may have evolved as a defense mechanism against predatory insects. Although the most frequent source of the inhibitors is the seed, an "immune response" of the plant to the attack of insects has so far been established for leaves. The extensive studies of C.A. Ryan and associates revealed the expression

and rapid accumulation in potato and tomato leaves of protein protease inhibitors in response to a systemic signal. The signal - proteinase inhibitor inducing factor (PIIF) - appears in increased levels as a direct result of the wounding of plant leaves, either mechanically or following attack by insects. The latter, in turn, become starved as a result of the inhibition of their own digestive proteases by the ingested plant protease inhibitors (reviewed, Ryan, 1978, 1983; Broadway et al., 1986; Birk, 1987).

The isolation and characterization of protein protease inhibitors and the introduction of fundamental concepts associated with protease-inhibitor interactions are marked by the pioneering work of M. Kunitz during the 1930's and 1940's. The inhibitors have attracted the attention of nutritionists due to their presence in valuable plant foods and their possible, subsequent involvement in nutritive and metabolic properties. During the past decade, increasing attention has been given to protein protease inhibitors as valuable tools in medical and nutritional research because of unique properties that suggest clinical application. The widespread occurrence of protease inhibitors in important food plants and their products, and their nutritional and toxicological significance have been studied in different laboratories and summarized recently (Friedman, 1986).

Definitions and general characteristics

Of all the proteinaceous protease inhibitors studied, the inhibitors of serine proteinases received the greatest amount of attention. They are competitive inhibitors, forming stoichiometric complexes with the enzymes that they inhibit. The enzyme-inhibitor complexes are very stable (Kassoc.= 10^{8}-10^{13} M^{-1}) and enzyme activity is completely abolished. The inhibitors are classified into Inhibitor Families on the basis of sequence homology, nature of the Reactive or Inhibitory Site and interaction with the protease according to a Standard Mechanism (reviewed, Laskowski & Kato, 1980; Laskowski, 1986). X-ray crystallographic and NMR studies revealed that in enzyme-inhibitor complexes, about 10-15 residues of the inhibitor are in contact with the enzyme. Their specific nature strongly affects both the strength and the specificity of enzyme-inhibitor interaction. The species specificity of the inhibited enzyme should also be taken into consideration. A powerful inhibitor of a protease from one source may have a lesser effect on the same enzyme from a different source. Thus, it has been shown that inhibitors that strongly inhibit bovine trypsin do not inhibit human trypsin (Mallory & Travis, 1975). Since the inhibitory capacities of protease inhibitors are very often evaluated on bovine pancreatic proteases, their relevance and significance in other species should be questioned.

(a) The reactive site

The reactive site of the inhibitor is defined as the part of the inhibitor molecule that enters into direct molecular contact with the active center of the proteinase upon formation of the Proteinase-Inhibitor complex. At or near the reactive site of the inhibitor resides an amino acid residue that is specifically recognized by the primary substrate-binding site of the target proteinase. This amino acid residue is termed P_1 by the Schechter-Berger notation (Schechter & Berger, 1967) (Fig. 1). Adjacent to P_1 resides the amino acid residue P_1'. The peptide bond joining these two residues, named the reactive

site peptide bond, is susceptible to hydrolysis during complex formation between the inhibitor and its target proteinase. Most inhibitors differ from most substrates in that the reactive sites are held within disulfide bridges. After the hydrolysis of the reactive site peptide bond, the resulting modified inhibitor is still held together, practically without change in conformation.

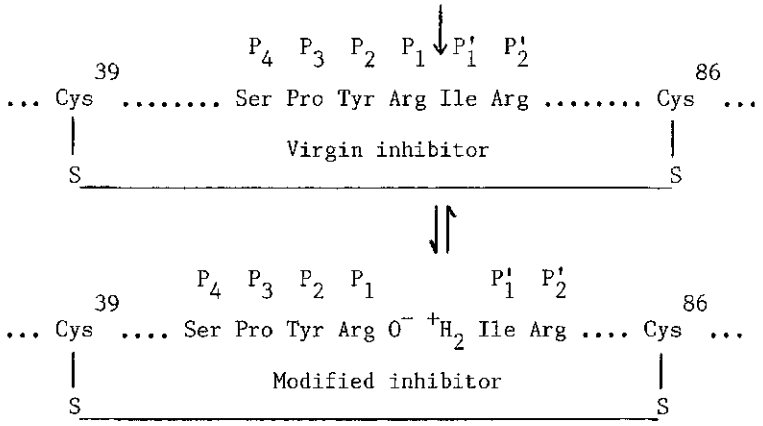

Fig. 1. Schematic diagram of the reactive site of Kunitz's soybean trypsin inhibitor. Virgin inhibitor has all of its peptide bonds intact. Modified inhibitor has the reactive site peptide bond hydrolyzed (from Laskowski, 1986).

(b) The standard mechanism

Most of the well-studied inhibitors of serine proteinases interact with the enzymes by the Standard Mechanism. The latter has been derived from studies with inhibitors that were isolated from plant, mammalian and avian sources. Inhibitors obeying the standard mechanism are highly specific substrates for limited proteolysis by their target enzymes. The standard mechanism has been primarily proposed by M. Laskowski Jr. for Kunitz's soybean trypsin inhibitor (STI). Treatment of STI (Fig. 2) with catalytic levels of trypsin under acidic

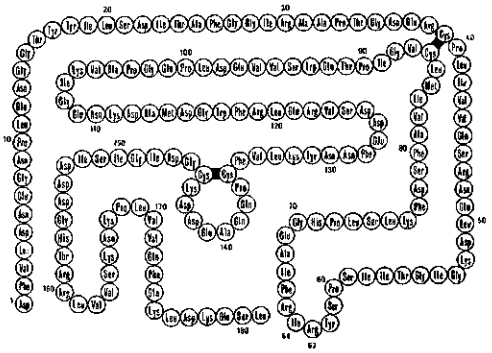

Fig. 2. Covalent structure of Kunitz soybean trypsin inhibitor (STI), (from Koide & Ikenaka, 1973).

conditions led to specific hydrolysis of a single peptide bond, the reactive site peptide bond, identified as Arg63-Ile64 in the amino acid sequence of the inhibitor. The resulting modified inhibitor (Fig. 1) is fully active and forms the same stable enzyme-inhibitor complex. Removal of either of the newly-formed COOH- or NH_2-terminal amino acid residue from the modified inhibitor (namely, P_1 or P'_1 in Fig. 1) results in loss of inhibitory activity. Exchange of Arg for Lys at the P_1-position of STI, by either semisynthetic replacement or by mutation, fully retains the specificity and potency of the inhibitor. Replacement of Lys or Arg at P_1 for a chymotrypsin specific residue, such as Trp or Tyr, changes the trypsin inhibitor to a chymotrypsin inhibitor (reviewed, Laskowski & Kato, 1980).

Inhibitor families: classification and characterization

A list of families of plant protein inhibitors of serine proteinases is given in Table 1. The major inhibitor families and groups found in important plant foods and feeds will be discussed herewith.

Table 1. Families of plant protein inhibitors of serine proteinases.

Soybean trypsin inhibitor (Kunitz) family
Soybean proteinase inhibitor (Bowman-Birk) family
Potato I inhibitor family
Potato II inhibitor family
Squash inhibitor family
Barley trypsin inhibitor family
Other families

(a) Kunitz soybean trypsin inhibitor (STI) family

The first plant proteinase inhibitor to be isolated and characterized was STI (Fig.2). Its purification, crystallization, kinetics of interaction and complex formation with trypsin (Kunitz, 1947a,b) comprise a major landmark in the study of protein proteinase inhibitors. STI has a molecular weight of about 21000 and includes two disulphide bridges. It is primarily an inhibitor of trypsin but also weakly inhibits chymotrypsin. It is inactivated by heat and by gastric juice. However, only a few homologous inhibitors to STI have been found in common legume and other edible seeds. The numerous studies on STI regarding specificity, stability, physical, kinetic and other properties have been compiled (Kassell, 1970; Birk, 1976) and recently summarized (Birk, 1987).

(b) Bowman-Birk proteinase inhibitor (BBI) family

The Bowman-Birk inhibitor from soybeans (BBI) serves as the prototype for a family of inhibitors that are predominant in legume seeds (reviewed, Birk, 1985, 1987). BBI has a molecular weight of about 8000 with a high content of cystine, forming seven disulphide bridges. The inhibitor consists of two tandem homology regions on the same polypeptide chain, each with a reactive, inhibitory site (Fig. 3). It

forms a 1:1 complex with either trypsin or chymotrypsin and a ternary

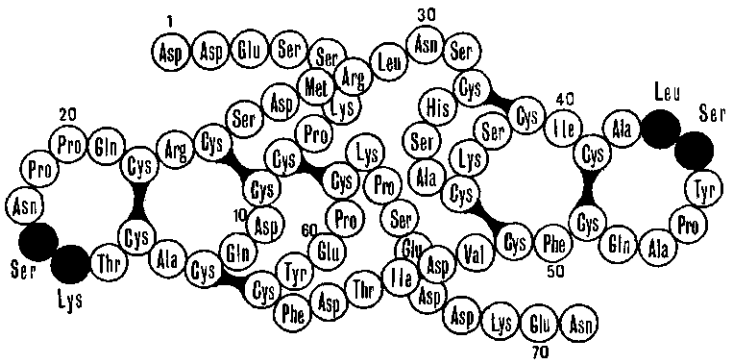

Fig.3. Covalent structure of Bowman-Birk soybean trypsin-chymotrypsin inhibitor (BBI). Residues at the two reactive sites are shown as solid black circles (from Odani & Ikenaka, 1973a).

complex with both enzymes. In aqueous solutions, BBI undergoes self-association which is concentration dependent. BBI inhibits human trypsin and chymotrypsin, it is highly active against dog trypsin and chymotrypsin and it inhibits carp trypsin and chymotrypsin with a 1:2 stoichiometry of inhibition, indicating the binding of two molecules of either trypsin or chymotrypsin to one molecule of inhibitor. It also is a potent inhibitor of trypsins and chymotrypsins from the digestive tracts of insects, such as Tenebrio molitor and Locusta migratoria and has shown a strong interaction (K_i = 10 8-10 $^{-9}$ M) with elastases from human and dog granulocytes. In vitro synthesis of BBI has been achieved with the aid of mRNA isolated from immature soybean embryos (Hammond et al., 1984). BBI has an unusual resistance to various proteolytic enzymes including pepsin and pronase. Limited proteolysis or chemical modifications at either of the inhibitory sites may be utilized to modify the 'double-headed' BBI into a 'single-headed' inhibitor that inhibits solely either trypsin or chymotrypsin, or to alter the specificity of inhibition (Birk, 1985; Kurokawa et al., 1987a,b). The scission of BBI with cyanogen bromide followed by pepsin, resulted in two active fragments, one with trypsin inhibitory activity and the other, with chymotrypsin inhibitory activity (Fig. 4).

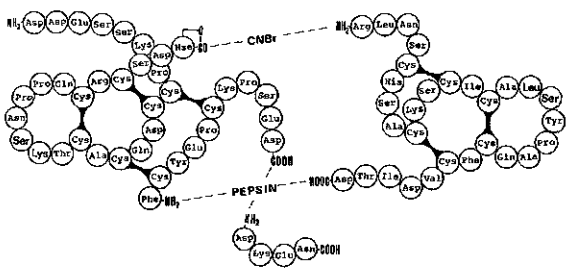

Fig.4. Sites of peptide bond cleavage by cyangen bromide followed by pepsin and the structure of BBI fragments (from Odani & Ikenaka, 1973b)

Most of the members of the BBI family exhibit numerous isoinhibitor forms. Homologous inhibitors have been found in lima beans, garden beans, azuki beans, mung beans, ground nuts, chick peas and recently, also in plant sources other than legumes seeds, such as wheat germ and rice (Odani et al., 1986; Tashiro et al., 1987). Highly homologous inhibitors may possess inhibitory sites with different steric orientations. Thus, in CI, the double-headed inhibitor from chick peas, the two inhibitory sites are close to each other, allowing the formation of stoichiometric complexes with either trypsin or chymotrypsin, but not with both. However, similarly to BBI from soybeans, scission of CI with cyanogen bromide followed by pepsin yields two active, independent fragments (Smirnoff et al., 1979).

(c) Potato I inhibitor family.

This major family includes protein inhibitors that are highly homologous to chymotrypsin inhibitor I from potato tubers, which is also a weak inhibitor of trypsin. The inhibitors are noncovalent tetrameres of four highly homologous subunits with a molecular weight of about 10000 each. The subunits comprise of single chains with one intrachain disulphide bridge each and a major chymotrypsin-reactive site at either Met47-Asp48 or Leu47-Asp48. Members of this family have been identified in sources other than the Solanaceae (reviewed, Richardson et al., 1977).

Potato tubers contain also a polypeptide of molecular weight 4300 that is a potent inhibitor of mammalian pancreatic carboxypeptidase A and B from various animals. Due to its unusual heat stability and solubility in ethanol, a large scale purification of the inhibitor can be easily accomplished (Pearce & Ryan, 1983).

(d) Squash inhibitor family

The newly-defined squash inhibitor family consists of protein inhibitors of trypsin (Kassoc. $5.9 \times 10^{10} - 9.5 \times 10^{11}$ M^{-1}) that were isolated from squash, zucchini, summer squash and cucumber seeds. The striking characteristic of this family is that its member inhibitors are very small. Their molecular weight is about 3000, they are crosslinked by 3 disulphide bridges and their reactive site is the Arg5-Ile6 peptide bond (Wieczorek et al., 1985).

(e) Protease inhibitors in cereals

Numerous protease inhibitors have been found in cereal grains, such as barley, rye, wheat, maize (corn), rice, sorghum and oats. However, only few reports have been given on their nutritional influence, probably due to the significantly lower inhibitor activity in cereal seeds compared with legume seeds (surveyed, Boisen, 1983). Homologous proteins, including proteinase inhibitors, alpha-amylase inhibitors and bifunctional proteinase-amylase inhibitors, have been isolated and many of them - recently sequenced. The highly homologous trypsin inhibitors from barley, rye and maize, as well as the bifunctional amylase-trypsin inhibitor from seeds of ragi, contain an Arg-Leu reactive site peptide bond in positions corresponding to the Arg33-Leu34 in the sequence of the barley inhibitor (Lyons et al., 1987). The latter, a single polypeptide chain of molecular weight about 13000 with 5 disulphide bridges, serves as a prototype for this group (Odani et al., 1983). A corn inhibitor for trypsin and activated Hageman Factor with

a reactive site peptide bond at Arg36-Leu37 also belongs to this group (Mahoney et al., 1984). Recently, single-headed and double-headed trypsin inhibitors of the BBI family have been isolated from wheat germ (Odani et al., 1986) and the presence, in rice bran, of a double-headed trypsin inhibitor that has a duplicated structure of the BBI type inhibitor has been reported (Tashiro et al., 1987). A thermostable, multifunctional inhibitor of trypsin, chymotrypsin and Tribolium midgut proteinases, isolated from the seeds of amaranth, appears not to belong to any of the established inhibitor families (Tamir et al., 1988).

Nutritional, metabolic and physiological effects

Most of the information on the effects of ingested plant protease inhibitors has come from experiments with animals. The inhibitors have attracted the attention of nutritionists because of their possible effect on the nutritive value of plant proteins in general, and legume seeds in particular. Of these, the soybeans have assumed a major role in animal and human nutrition. The discovery of the heat-labile protein STI in raw soybeans, and experiments showing that the nutritive quality of soybean flour heated at various temperatures increased in proportion to the destruction of the trypsin inhibitor, led to the conclusion that the inhibitor is the major cause of the lower than expected nutritional value of raw soybean meal in rats and chicks, and for the consequent pancreatic enlargement. The availability in pure form of the two distinct soybean trypsin inhibitors, STI and BBI, enabled the elucidation of the effect of the inhibitors per se on growth rate, pancreatic enlargement and intestinal proteolysis of living organisms. Since then, numerous investigations on short- and long-term effects of the ingested inhibitors have been published (reviewed, Birk, 1985, 1987).

(a) Effect on animal growth

Feeding experiments of rats and chicks carried out on properly heated soybean meal diets supplemented with STI, BBI or with both, resulted in an insignificant depression of animal growth rate, but the inhibitors were responsible for pancreatic enlargement. The proteolytic activity in the small intestine was decreased by addition of BBI to the diet but not by STI. This was expected since the latter possesses negligible activity against chymotrypsin and is presumably inactivated during the preceding peptic digestion (Gertler et al., 1967; Konijn et al., 1970). However, feeding experiments of rats on raw soybean protein isolates with very low trypsin-inhibitor content, resulted in growth depression (Naim et al., 1982). The failure of soybean trypsin inhibitors to cause growth depression was also demonstrated in calves (Kakade et al., 1976). Attention has been recently drawn to winged beans - a potential food source comparable to soybeans. The antinutritional effects of raw winged beans on rats, such as growth inhibition, pancreatic hypertrophy, and ultimately, death have been found to be more deleterious than those of raw soybeans. However, when the isolated winged bean trypsin inhibitors were fed with casein, only slight growth inhibition, pancreatic and spleen hypertrophy were noted (summarized, de Lumen & Chan, 1986). It is of interest to point out that the thermostable pancreatic carboxypeptidase inhibitor from potatoes had no significant effect on growth when ingested by chicks (Pearce et al., 1983).

(b) Effects on the pancreas

Short-term feeding studies have shown that raw soybean meal and purified trypsin inhibitors cause pancreatic enlargement in the rat, the chick, the mouse and the young guinea pig. However, pancreatic enlargement failed to occur in the adult guinea pig, the dog, the growing swine, the calf, and presumably, the human (reviewed, Gallaher & Schneeman, 1986), and in primates - even after five years of feeding on soybean-based protein diets containing trypsin inhibitors (Harwood et al., 1985). The pancreases of rats and chicks adapted to raw soybean meals synthesized more trypsinogen and chymotrypsinogen and less amylase than pancreases of rats adapted to heated soybean meal (reviewed, Birk, 1985). Ingestion of diets supplemented with BBI modified by maleylation or succinilation, or with the complex of BBI plus trypsin (in which the trypsin-inhibitory site is masked) did not cause pancreatic enlargement and had no significant effect on the amount of pancreatic proteinases. This indicates that the trypsin-inhibitory site, rather than the chymotrypsin-inhibitory site of BBI is involved in the enlargement of the pancreas and in the increase of pancreatic proteolytic activity (Madar et al., 1974).

The pancreatic enlargement and increased proteolytic enzyme concentrations caused by trypsin inhibitors are explained in terms of the mechanism of regulation of pancreatic secretion. The free intestinal trypsin and chymotrypsin regulate the level of pancreatic secretions and pancreas size by a negative feedback inhibition mediated by the humoral agent, cholecystokinin-pancreozymin (CCK-PZ), which is known for its ability to stimulate pancreatic secretion and cause both pancreatic hypertrophy and hyperplasia. The free enzymes present in the upper intestine suppress release of CCK-PZ whereas the trypsin inhibitors, which form the "inert" enzyme-inhibitor, complexes, stimulate the release of CCK-PZ-like activity. The negative feedback mechanism of pancreatic enzyme secretion found in the rat exists also in the pig and calf, which do not develop pancreatic enlargement (reviewed, Gallaher & Schneeman, 1986). A recent study has confirmed the existence of feedback control in humans (Liener et al., 1988).

The finding that prolonged feeding of male Wistar rats on raw soybean meal enhanced the action of a pancreatic carcinogen azaserine (Morgan et al., 1977) triggered a series of investigations on the effects of ingested trypsin inhibitors, as unheated soy protein, on the pancreas of various animal species. In the "USDA trypsin inhibitor study", male Wistar rats fed raw soybean meal or experimental unheated soy protein isolates for two years, developed pancreatic nodular hyperplasia and acinar adenoma in a dose-dependent manner (Gumbmann et al., 1985). However, similar long-term feeding of mice and hamsters on raw soybean meal, in the presence or absence of chemical carcinogens, failed to induce carcinogenic changes in their pancreases. Moreover, the raw soybean meal seems to have exerted a protective effect on the chemical induction of tumors in the hamster (Liener & Hasdai, 1986).

(c) Therapeutic potential of protease inhibitors

Epidemiological studies have identified legumes as possible protective agents in the decreased occurrence of breast, colon and prostatic cancers in vegetarian populations. Synthetic and natural protease inhibitors have been shown to inhibit tumor promotion in vivo and in vitro. It is suggested that the mechanism of anticarcinogenesis of ingested protease inhibitors may involve the indirect effect of

partially blocking protein absorption (Yavelow et al., 1983). In addition to the possible anticarcinogenic effects of dietary protease inhibitors, the inhibitory effects of plant protease inhibitors on oncogenic transformation and promotion have been observed in tissue culture systems. In a series of experiments on transformation of C3H/10T1/2 cells, it has been shown that nanomolar concentrations of BBI suppress the X-ray-induced transformation in vitro, and that the chymotrypsin-inhibitory domain of BBI is responsible for this effect (Yavelow et al., 1985). A similar inhibition of radiation-induced transformation of C3H/10T1/2 cells was achieved by chymotrypsin inhibitor I from potatoes (Billings et al., 1987). Several successful attempts have been made recently to identify potential intracellular target enzymes and proteins of the anticarcinogenic BBI (summarized, Billings et al., 1988).

The continued search for therapeutic applications of plant protease inhibitors in treatment of ailments associated with enhanced proteolytic activity, such as pancreatitis and emphysema, has yet to achieve conclusive results.

In conclusion - plant protease inhibitors comprise a significant component of animal and human diets. Their alleged antinutritional properties should be weighed against their potential in protecting valuable crops during storage and also as possible therapeutic and cancer-chemopreventive agents. Further insight into cellular aspects of the inhibitors on a molecular level and of their target enzyme(s) is needed to better understand the nutritional and metabolic effects of plant protease inhibitors and whether or not they should pose a concern for humans.

Acknowlegement

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LONG TERM DIETARY EFFECTS OF SOY PROTEIN IN CEBUS MONKEYS

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Summary

Since adverse pancreatic effects have been observed in a variety of rodents when fed diets containing soybean trypsin inhibitor, this study was designed to evaluate whether long-term exposure of primates to such diets has any adverse nutritional impact. Twenty-six Cebus monkeys were supplied from infancy with semisynthetic diets containing as their sole protein source casein, lactalbumin, soy isolate or soy concentrate. Each batch of the four diets was assayed for trypsin inhibitor content. After three years a pancreatic biopsy was obtained by surgical section and examined histologically. No evidence of pancreatic hypertrophy or hyperplasia was noted. After five years of continuous exposure to the diets (about 25% of the estimated lifespan) a full autopsy was performed together with hematology and blood chemistry. Examination of major organ systems at autopsy and histological examination of the tissues revealed no adverse pathology related to ingestion of trypsin inhibitor. Overall health, body weight and blood parameters were comparable between the groups. Under these experimental conditions, the findings suggest that long-term exposure (five years) of the Cebus monkey to trypsin inhibitor-containing diets does not cause any adverse pancreatic effects, and that primates may not therefore be susceptible to the adverse effects observed when such diets are fed to rodents.
Keywords: trypsin inhibitor, soy-based diets, Cebus monkey, chronic toxicity, nutritional safety.

Introduction

Soy products are used in the United States Armed Forces, school lunch programs and in numerous institutions. Soy protein products used in human nutrition are heat treated at a level to preserve protein quality yet destroy about 90% of the trypsin inhibitor present in the soy. The safety of the residual content of trypsin inhibitor has recently been questioned as a result of studies in rats in which high doses of trypsin inhibitor over an 18-month period produced pancreatic neoplasms (Gumbman et al, 1985). The widespread exposure of the human population to soy products and other trypsin inhibitor-containing foods (Doell et al, 1981) makes it important to know whether similar effects occur in non-human primates and in humans. Thus, a study was undertaken in Cebus monkeys to determine the effects of long-term feeding of soy protein products on pancreatic tissue. In 1983 a pancreatic biopsy was taken and pancreatic RNA, DNA, protein, trypsin and chymotrypsin, as well as pancreatic histology were evaluated (Ausman et al, 1985). The findings of the biopsy will be summarized and the results of the final necropsy of the animals after completion of the 5-year feeding study will be described.

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Study Design

This study used 26 Cebus monkeys (Cebus albifrons) divided into four groups fed semisynthetic diets with the sole source of protein as shown below:

GROUP	MALES	FEMALES	DIETARY PROTEIN	TRYPSIN INHIBITOR[1]
1	5	3	Lactalbumin	0.12
2	5	5	Soy Isolate	0.54
3	3	3	Casein	0.08
4	1	1	Soy Concentrate	2.41

1. Trypsin inhibitor activity is expressed as mg/g of diet as assayed by the procedure of Kakade et al, 1974.

The 26 Cebus monkeys were fed from infancy on semisynthetic diets in which the primary source of protein was casein or lactalbumin for two control groups, and soy isolate and soy concentrate. The four diets were prepared every three months, stored in a cold room and assayed for trypsin inhibitor activity using standard techniques (Kakade et al, 1974). Each monkey was supplied every day with 100 g of the diet pellets in two portions, monitored daily for general health, and weighed monthly.

The monkeys in this study were originally used for an FDA contract to evaluate the protein efficiency of soy-based formula. These animals were continued on their respective diets and adopted into the present study for evaluation of the effects of trypsin inhibitor in 1982. Hence the distribution of the monkeys between the different groups reflects the number of animals on the respective diets in 1982. It was considered critical that there should be no change in the diet of a particular animal and hence no redistribution of the animals was attempted to optimize the numbers in each group. The soy concentrate group was included in the study to provide an indication of the effects of a higher dose of trypsin inhibitor although it was recognized that any results from a group of only two animals would not be statistically valid.

Results and Discussion

A common finding in rodents fed trypsin-inhibitor containing diets for six weeks is pancreatic hypertrophy and hyperplasia. To observe whether similar effects occurred in monkeys, the animals were biopsied surgically under anesthesia at about three years of age. Visual inspection of the pancreas in situ showed no gross macroscopic changes. A small section removed from the head and tail region of the pancreas was assayed for DNA, RNA, protein, trypsin and chymotrypsin, and was examined histologically by two independent veterinary pathology laboratories. No remarkable changes in the pancreas of the soy isolate fed monkeys occurred, although one of the animals fed the soy concentrate diet had diffuse interstitial fibrosis associated with mild to moderate atrophy of acinar tissue. These changes are thought to be unrelated to hypertrophy or proliferative changes. For a full report on this part of the study see Ausman et al, 1985.

Although the biopsy showed little evidence of pancreatic change after three years of exposure to the trypsin inhibitor-containing diets, it was decided to continue feeding the animals for a period that corresponded to not less than 25% (five years) of their estimated life span. The weight of the monkeys was monitored monthly which served as an index of overall health, adequacy of the semisynthetic diets to support growth, as well as an indicator of any adverse nutritional effect of the soy diets. All animals showed a consistent growth pattern without differences between those on the soy diets and those on the two control diets of lactalbumin and casein.

Blood samples drawn prior to necropsy showed no significant differences between the groups of animals in hematology and blood chemistry. At necropsy, all animals were euthanized with ketamine and exsanguinated. External and internal gross pathologic examinations were performed for each animal, and the liver, kidneys, thymus, testis, ovaries, thyroid, adrenals, pancreas and pituitary were weighed. Since the pancreas was the focus of attention, it was removed, mounted on a piece of cardboard, head and tail ends identified and photographed. Although rodents fed soy diets have increased ratios of pancreatic weight to whole body weight which is indicative of pancreatic hypertrophy (Hasdai and Liener, 1983), no such change was observed in the pancreas of the Cebus monkeys.

Tissues from the following organs were collected from each monkey, fixed in 10% neutral buffered formalin, stained with hematoxylin and eosin, and evaluated histologically: adrenal gland, aorta, rib, femoral bone marrow, brain, eyes, esophagus, gallbladder, liver, lungs, heart, kidneys, stomach, duodenum, jejenum, ileum, colon, cecum, rectum, mammary gland, ovary, fallopian tube, uterus, testis, epididymis, pancreas (head, body and tail), sciatic nerve, pituitary, prostate gland, lymph nodes, salivary gland, seminal vesicle, skeletal muscle, diaphragm, spinal cord, spleen, sternum, thymus, thyroid, tonsil, trachea, and urinary bladder.

The pancreas was carefully examined after fixation for any morphological alterations. Representative sections were made from the head, body and tail regions to ensure adequate sampling of all regions of the organ. No gross lesions were noted during this re-examination. The diagostic criteria of the National Toxicology Program (Boorman and Eustis, 1984) were used to evaluate proliferative lesions of the pancreas.

No gross or microscopic evidence of pancreatic hypertrophy, nodules or neoplasia was seen in any of the monkeys. When the ratio of pancreatic to body weight was calculated, no differences in the weights or ratios were noted between the groups. In the single male monkey fed the soy concentrate diet, moderate diffuse acinar atrophy, moderate diffuse interstitial fibrosis and moderate chronic pancreatitis were seen in all three sections examined. These lesions were characterized by small, variable sized acinar cells, strands of mature collagen in the interstitial areas and scattered mononuclear inflammatory cells. The lesions were similar to those noted in the biopsy specimen from this monkey and

reported previously (Ausman et al, 1985). No conclusions can be drawn from such a small group with respect to the relationship of this lesion to diet. However, it should be noted that the atrophic and fibrotic changes were not of the type associated with the adverse effects of trypsin inhibitor diets observed in rodents.

In one male monkey fed the soy isolate diet, a single small focus of acinar atrophy with focal chronic pancreatitis was noted in the body of the pancreas. Minimal vacuolated acinar cells were observed in various sections of the pancreas in three males and two females and all four groups were represented. Similar lesions had been noted in the biopsy specimens taken previously. Minimal foci of mononuclear cells were noted in various sections of the pancreas of three males and in the tail pancreatic section of two females. These foci were seen in the monkeys fed the lactalbumin diet and the soy isolate diet and were considered to be incidental. Minimal lymphoid hyperplasia was noted in the body of the pancreas of the single female fed the soy concentrate diet. In other organs, various spontaneous lesions were observed occasionally without respect to treatment group.

Conclusion

In a chronic feeding study in which Cebus monkeys were exposed to soy diets for a minimum of five years, histopathological examination of all major organ systems, with particular emphasis on the pancreas, revealed no adverse effects. Under the conditions of this study and with inadequate numbers of animals on the high dose of trypsin inhibitor, the results suggest that the primate species as represented by the Cebus monkey may not be susceptible to the adverse pancreatic effects of soy as observed in rodents.

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SOYBEAN PROTEINASE INHIBITORS AFFECT NUTRIENT DIGESTION IN RAINBOW TROUT

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Summary

The results of the present experiment showed similar dependency of temperature in bovine and Rainbow trout trypsin. Kunitz soybean trypsin inhibitor showed a greater effect on the trout trypsin activity, most likely due to a higher specific activity of Rainbow trout trypsin. Soybean proteinase inhibitors reduced intestinal trypsin activity markedly and increased excretion of protein and lipids.
Keywords: trypsin, bovine, trout, soy inhibitor, digestion

Introduction

Synthetic and natural proteinase inhibitors have shown inhibition of salmonid trypsins that might appear more severe than the inhibition of bovine trypsin. The differences in inhibition might be due to a higher specific activity of the salmonid enzymes (Kitamikado & Tachino, 1960, Croston, 1960, Krogdahl & Holm, 1983, Uchida et al., 1984).

Raw and improperly heated soybeans cause growth retardation, enlarged pancreas, reduc.. protein utilization, impaired fat absorption, lowered energy utilization and reduced availability of minerals in rats. A main cause is the content of proteinase inhibitors (Rackis, 1974). These effects have also been found in other monogastric animals. The effects are generally more severe in the young animals than in the older. Rats, pigs, and possibly humans appear to increase pancreatic enzyme secretion upon ingestion of raw soybeans. In calves, unaffected or reduced secretion have been found. (Review by Krogdahl & Holm, 1979, Holm et al., 1988).

The aims of the present investigations were

- *to study in vitro effects of purified soybean proteinase inhibitors on Rainbow trout trypsin at different temperatures and to compare the effects with effects on bovine trypsin
- *to study in vivo effects on nutrient digestibility and intestinal enzyme acitivities in Rainbow trout.

Materials and methods

A preparation of proteolytic enzymes were made from Rainbow trout pyloric caeca by homogenization with buffer (0.2 M Tris-HCl, 0.05 M CaCl , pH 8.0, 1:36 w/v) and centrifugation at 15,000 g for 15 min. Volumes corresponding to 2.8 mg wet weight of caeca were used in

the assay. Bovine trypsin (Sigma type III, no T-8253, 0.125 mg/ml 0.001 HCl) was also used, 12.5 ug per analysis. The mounts of enzyme preparations chosen were hydrolyzing the substrate at similar rates. Purified Kunitz soybean trypsin inhibitor (Sigma type I-S, no T-9003, 0.020 mg/ml 0.001 HCl) was added; 3.0 ug in samples with bovine trypsin and 0.4 ug with Rainbow trout enzymes. The amount of inhibitors chosen were causing trypsin inhibitions of similar magnitude. N-benzoyl-L-arginine-p-nitro anilide was used as trypsin substrate. The analyses were carried out according to Kakade et al. (1969)(n=5). Incubation time was 20 min.. The effects of temperature on trypsin activity and inhibition were studied as shown in Fig. 1. Trypsin acitivities are given per 12.5 ug bovine trypsin and 2.8 mg wet pyloric caeca.

Effects of proteinase inhibitors on nutrient digestion and intestinal trypsin activity were studied with Rainbow trout in fresh water, 10 kg fish per tank, fish weight about 300 g. The fish was fed a reference diet containing 83 % fish meal, 11 % fish oil, 2 % binder, 2 % mineral mix, 1 % vitamin mix, and 1 % chromic oxide. Diets for the other treatments were obtained by adding a purified soybean proteinase inhibitor (Sigma type-II, no T-9128) in replacement of fish meal at the following levels: 0.37, 0.73, 1.10, and 1.47 % in the diets (n=1). The inhibitor levels chosen corresponded to inhibitor levels that would have been obtained with 15, 30, 45, and 60 % raw soybean meal in the diets. Feces were stripped from the large intestine of the fish after about 10 days of adaptation to the diet. Contents of the proximal small intestine were collected for measurement of trypsin activity. Enzymes were extracted from freeze dried feces with saline (1:10 w/v). Enzyme activities are given per mg dry matter of feces. Proximate analysis were carried out according to standard procedures.

The results were evaluated using regression analysis.

Results and discussion

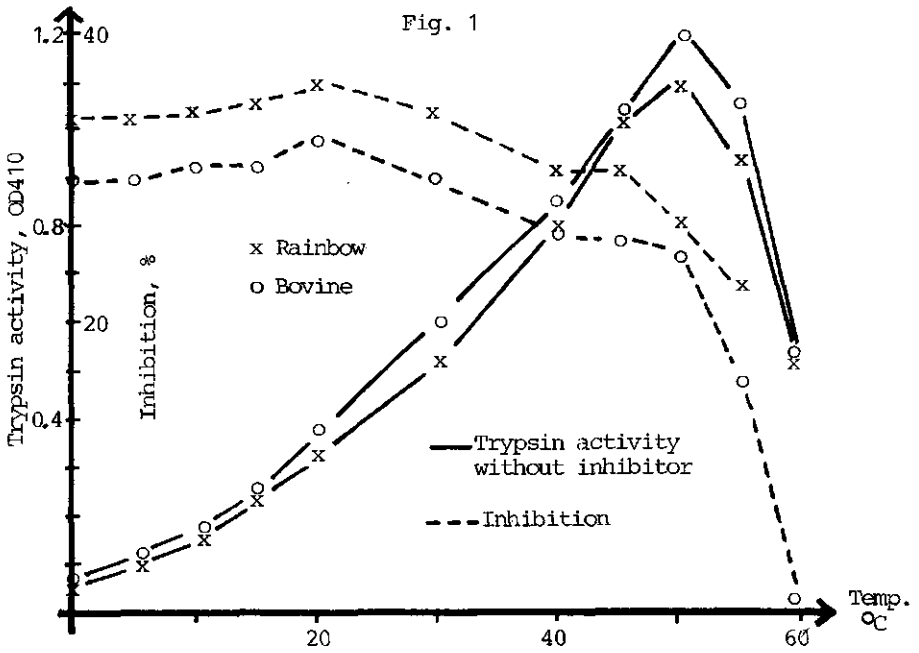
The effect of temperature on the acitivities of bovine and Rainbow trout trypsin were almost identical under the present condition, both regarding effects below temperature optimum and the temperature optimum itself (Fig.1).

The inhibitions found for the two enzymes were fairly stable over a wide temperature range. A decreasing tendency was apparent when the temperature approached 50 °C.

The Kunitz soybean inhibitor preparation used in the present experiment affected the Rainbow trout trypsin more efficiently than the bovine trypsin. The ratio of amounts of inhibitor needed for similar inhibition was 9:1. Soybean proteinase inhibitors are competitive and the Kunitz inhibitor has very high association/dissociation ratio with bovine trypsin. It is, therefore, likely that the difference in inhibitory effect was due to higher specific acitivity of the Rainbow trout trypsin (Krogdahl & Holm, 1983).

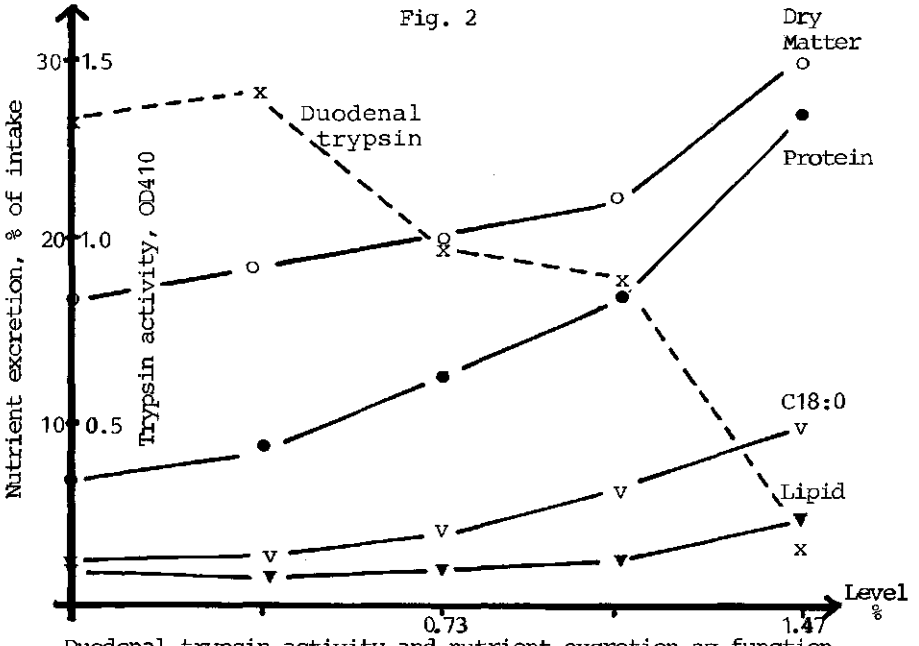
The results of the feeding experiment, given in fig. 2,

Fig. 1



Trypsin activity and inhibition as function of incubation temperature

Fig. 2



Duodenal trypsin activity and nutrient excretion as function of level of inhibitors in the diet

indicated that inclusion of soybean proteinase inhibitors or ingredients containing inhibitors in diets for Rainbow trout may cause severe reduction in intestinal proteolytic activity.

The decrease appeared to intensify with increasing level of inhibitors. This might indicate that the Rainbow trout, to a certain degree, is able to compensate for the effects of inhibitors by increased enzyme secretion. However, the capacity seemed to be limited.

The consequence of the reduction in proteolytic activity was a marked increase in fecal excretion of protein. The decrease in proteolysis in the small intestine would affect both the dietary proteins and the endogenous proteins. Analysis of excretion of amino acids indicated similar effects on all the essential acids.

A slight increase in excretion of lipids was also apparent. The effect was most pronounced for the long saturated fatty acids. The decrease in protein digestion would cause unfavourable conditions in the intestine that might explain the effects on the fat excretion. However, the increased amounts of protein that are present in the intestine might possibly also affect fat digestion specificly by adsorbing bile salts and other emulsifiers necessary for efficient absorption of lipids (Krogdahl, 1986).

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RELATIONSHIP BETWEEN TRYPSIN-INHIBITOR CONTENT OF PEA SEEDS AND PEA PROTEIN DIGESTIBILITY IN POULTRY

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Summary

Two balance experiments were conducted in poultry using several pea cultivars varying in trypsin-inhibitor contents. Pea seeds were either untreated or treated. Treatments consisted in autoclaving and steam-pelleting in the first and second experiments respectively. Pea meals were included in diets at 40 % and 47 % levels in experiments 1 and 2, respectively. The remaining part of diets consisted of a maize/soya-bean meal mixture. The first experiment was carried out with young chicks (3 weeks) and the second one with young chicks (3 weeks) and adult birds. The apparent digestibility of protein of the basal diet measured in young chick were similar in experiment 1 (81.7 %) and 2 (82.5 %). Pooling data obtained in the young chicks in experiments 1 and 2 revealed no significant correlation between apparent digestibility of protein of untreated peas and trypsin-inhibitor level. In young chicks, increase in apparent digestibility of pea protein induced by treatments was not significantly correlated with decrease in trypsin inhibitor level. In adult cockerels (experiment 2), pelleted peas with low level of trypsin-inhibitor had a higher protein digestibility than pelleted peas with high level of trypsin-inhibitor ; but this was not observed for unpelleted peas. Both experiments tended to show that trypsin-inhibitor activity was not the only factor responsible for the variation in pea protein digestibility in poultry. At least 50 % of the variation in pea protein digestibility observed in poultry remained unexplained.

Introduction

The low nutritional value of smooth peas (Pisum sativum) has often been attributed to their content of anti-nutritional factors (ANFs) (Huyghebaert and de Groote, 1979). Correlatively, the beneficial effect of heat treatment on pea nutritional value is often believed to be due to inactivation of ANFs (Huyghebaert and de Groote, 1979). However, it has already been suggested (Liener, 1976 ; Marquardt et al., 1976) that reduction in the activity of ANFs induced by heat treatment could only explain a part of the improvement of the nutritional value of legume seeds. Present experiments were undertaken with the aim to assess the effect of trypsin inhibitors of peas on pea protein digestibility in poultry by testing several pea samples varying in trypsin inhibitor level. Pea seeds were either untreated or treated by thermo-mechanical processes.

Materials and methods

Experiment 1

The spring sample of peas (Amino + Finale) was a fifty percent blend of each spring cultivar Amino and Finale. P_{146}, P_{147} and Frisson pea samples, classified as winter cultivars, were provided by the C.N.R.A.-I.N.R.A. (Versailles).

Seeds were ground in a hammer mill using a 3 mm screen. The half parts of the 4 pea meals were processed together in an autoclave at 130°C for 3 min with steam under 170 kpa pressure. Pea meals were not soaked in water before or during treatment.

Eight pea diets were prepared by mixing 40 % peas (treated or untreated) with 60 % maize-soya bean basal diet.

Pea diets and basal diet were given to 72 three week old chickens with 8 chickens per diets and 2 birds per cage for measurement of apparent protein digestibility. Total collection method was used as described previously (Lacassagne et al., 1988).

Experiment 2

Two pea samples were tested, one from the spring cultivar Finale and the other one from the winter cultivar Frisson. The pea seeds were either ground (2 mm screen) or steam pelleted after grinding (2 mm) ; pellets were reground (2.5 mm screen) before feeding. Pea flours were introduced at 47 % level in diets with the other part being composed of a maize-soya bean basal fraction and minerals. Pea diets and basal diet were given to 70 three week old chicken and 35 adult cockerels with 14 young and 7 adult birds per diets. Adults were placed in individual cages, whereas cages for young birds contained two individuals. Total collection method was used for measurement of apparent protein digestibility as described previously (Lacassagne et al., 1988 ; Carré et al., 1987). The number of Trypsin Unit Inhibited (TUI) was measured using the method of Kakade et al. (1974) modified by Valdebouze et al. (1980). Apparent digestibility values of proteins assigned to pea fractions were calculated assuming that digestibility of protein of basal diet does not change when given alone or mixed with peas.

Table 1. Composition of basal diets (%).

	Experiment 1	Experiment 2
Maize	66.8	58.5
Soya-bean meal	27.0	32.5
Soya-bean oil	-	4.0
DL-methionine	0.24	0.20
Sodium chloride	0.46	0.40
Dicalcium phosphate	3.41	2.00
Calcium carbonate	1.16	1.50
Mineral mixture	0.15	0.10
Vitamin mixture	0.78	0.80

Table 2. Proximate analysis of basal diets and pea seeds (%/dry matter).

	Experiment 1					Experiment 2		
	Basal diet	Peas				Basal diet	Peas	
		Amino + Finale	P146	P147	Frisson		Finale	Frisson
N x 6.25	21.02	22.7	27.0	26.8	26.6	24.1	25.5	24.9
Starch	45.8	49.9	45.3	44.7	43.8	43.3	48.1	46.3
TUI/mg :								
Untreated	-	3.2	11.7	15.1	11.9	-	3.5	9.7
Treated	-	1.9	2.2	2.2	2.2	-	3.0	9.7

Results and discussion

TUI levels (Table 2) of the untreated peas used in the 1st experiment varied from 3.2 TUI/mg dry matter (Amino + Finale) to 15.1 (P147). Autoclaving reduced TUI levels in all samples to about 2 TUI/mg DM. In the second experiment, spring and winter pea samples contained respectively 3.47 and 9.72 TUI/mg DM. Steam pelleting did not change the TUI level of winter peas and reduced slightly that of spring peas to 2.98 TUI/mg DM.

Pooling results of both experiments, it appeared that correlation between TUI level of untreated peas and pea protein digestibility measured in young birds ($r = -0.507$; $n = 6$) was not significant. Higher correlation ($r = 0.790$; $n = 6$) was found between TUI decrease induced by treatments and corresponding increase in pea protein digestibility measured in young birds, but this still remained not significant.

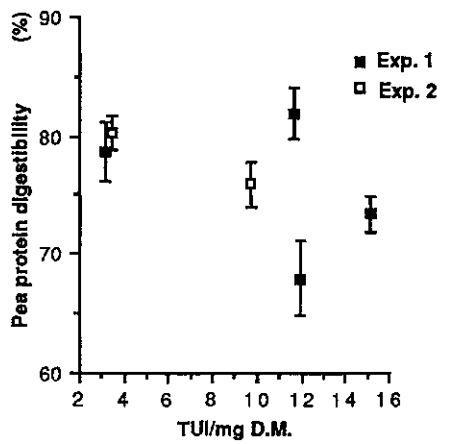

Figure 1. Protein digestibility of untreated peas related to their trypsin-inhibitor level (means and S.E., young chicks)

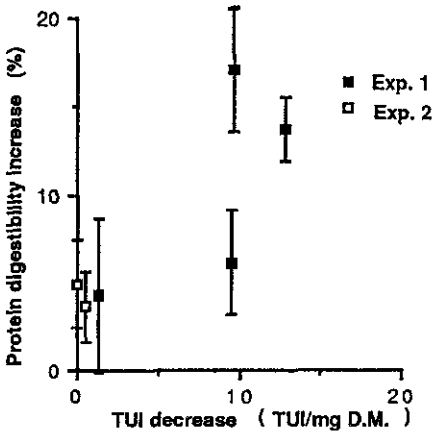

Figure 2. Increase in digestibility of pea protein (means and S.E., young chicks) related to pea TUI decrease, induced by thermo-mechanical processes

In adult birds (experiment 2), no difference between cultivars appeared in the apparent digestibility of pea proteins when untreated (75.3 and 74.6 for spring and winter seeds, respectively) whereas, when pelleted, higher digestibility values of pea proteins (81.7 % against 71.9 %) was observed on peas with low TUI level.

Both experiments tend to show that trypsin inhibitor of peas are not the major factors which could explain the variation in pea protein digestibility observed in poultry. Researches are needed to investigate other factors which could be responsible for the variation in pea protein digestibility.

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THE EFFECT OF FEEDING RAW SOYFLOUR ON THE GROWTH AND ON THE PANCREAS OF GUINEA PIGS

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Summary

Guinea pigs fed a diet containing raw soyflour (R) grew 1.1 g/d as compared with a daily growth of 3.1 g for their counterparts fed a diet containing heated soyflour (H). Food inake, food utilization and protein digestibility were 30, 50 and 27% lower, respectively, in the R-than in the H-fed groups. The relative weight of the pancreas was 5% less (non significant) in the R group than in the H group. The activities of trypsin, chymotrypsin and amylase were lower in the pancreas and in the small intestinal chyme in the R as compared with the H group. Increasing the level of R in the diet accentuated the negative effects.
Keywords: Guinea pigs, raw soyflour, growth, pancreas, digestive enzymes

Introduction

The feeding of raw soyflour (R) to experimental animals such as the rat, mouse and chick leads to growth depression, hypertrophy and/or hyperplasia of the pancreas, and hypersecretion of digestive enzymes (Liener & Kakade, 1980). No change or reduced pancreas weight and hyposecretion of pancreatic enzymes was reported for calves, dogs and pigs (Nitsan & Nir, 1986). The response of guinea pigs to consumption of a raw vs heated (H) soyflour diet at two levels of dietary protein (12% and 19%) is reported herein.

Results and discussion

Experiment 1

Sixteen 3-5 d-old guinea pigs were fed semisynthetic diets containing 12% protein from R or H for 21 d. The diets contained 4.40 and 0.42 mg trypsin inhibitors/g for R and H diets respectively. Body weight gain, food intake and food utilization were reduced in the R group by 65, 30 and 50%, respectively, of that found in the H group (Table 1). Digestibility of the protein was 27% lower in the R than in the H group. The relative weight of the pancreas of the R group was 95% of that recorded in the H group but the differences were non significant.

Contribution No. 2489-E Series, of the Agricultural Research Organization.

Table 1. Body weight gain, food intake and utilization, protein digestibility and pancreas weight of guinea pigs fed diets containing 12% protein from raw (R) or heated (H) soyflour.

	R	H	R/H
Initial body weight (g)	136	138	1.00
Final body weight (g)	160[b]	204[a]	0.78
Body weight gain (g/d)	1.1[b]	3.1[a]	0.35
Food intake	199[b]	283[a]	0.70
Food utilization (gain/feed)	0.12[b]	0.24[a]	0.50
Protein digestibility (%)	49.2[b]	67.1[a]	0.73
Pancreas (g/100g body weight)	0.40[a]	0.42[a]	0.95

[ab]Means within lines not followed by the same letter are significantly different (P<0.05).

Table 2. Activities[1] of trypsin, chymotrypsin and amylase in the pancreas, and contents of the jejunum and ileum of guinea pigs fed diets contaning raw (R) or heated (H) soyflour.

	Trypsin			Chymotrypsin			Amylase		
	R	H	R/H	R	H	R/H	R	H	R/H
Pancreas	79[b]	191[a]	0.41	6.30[b]	23.5[a]	0.27	190[b]	876[a]	0.22
Jejunum	1.20[b]	4.61[a]	0.26	1.61	2.31	0.70	7.81[b]	25.2[a]	0.31
Ileum	4.69	4.91	0.96	1.71	2.12	0.81	12.7	15.4	0.82

[1]Units of activity as defined by Nitsan & Liener (1976).
[ab]Means within lines (for each enzyme separately) not followed by the same letter are significantly different (P<0.05).

Trypsin, chymotrypsin and amylase were lower in the pancreas, jejunum and ileum of the guinea pigs fed the R diet than in their counterparts fed the H diet. The differences between the two groups were significant for the three enzymes in the pancreas, for trypsin and amylase in the jejunum, and non-significant in the ileum.

Diets containing 12% protein are marginal in protein supply, and in the present experiment due to low food intake and protein digestibility, deficiency of protein could be one of the reasons for the markedly lower performance of the R group. Increasing the dietary protein content by raising the level of R resulted in better performance of rats (Gertler et al. 1967).

The second experiment was planned to examine the use of a higher level of R in the diet - thereby raising the protein level from 12 to 19%.

Experiment 2

Sixteen 3-5 d old guinea pigs were fed semisynthetic diets containing 19% protein from H or R. The diets contained 6.60 and 0.55 mg trypsin inhibitors/g for R and H diets respectively. The experiment was terminated after 15 d, since from 10 d on the experiment some of the animals started to lose weight (Table 3).

Table 3. Body weight gain, food intake and utilization and pancreas weight of guinea pigs fed diets containing 19% protein from raw (R) or heated (H) soyflour

	R	H	R/H
Initial body weight (g)	127	127	1.00
Final body weight (g)	141[b]	217[a]	0.65
Body weight gain (g/d)	0.95[b]	6.0[a]	0.16
Food intake (g)	118[b]	210[a]	0.56
Food utilization (gain/feed)	0.12[b]	0.42[a]	0.29
Pancreas (g/100g body weight)	0.39	0.41	0.95

[ab]Means within lines not followed by the same letter are significantly different ($P<0.05$).

Increasing the dietary protein level improved body weight gain and food utilization in the H-fed group by 94% and 75%, respectively. However, in the R-fed animals growth rate was reduced by 14% and food utilization remained the same.

Contrary to the situation in rats or chicks, there was no pancreatic enlargement in guinea pigs consuming the R diet. Pancreas relative weight was 5% lower in the R group in the two experiments as compared with the H group. It was suggested by several authors (Liener & Kakade, 1980; Nitsan & Nir, 1986) that pancreatic enlargement and hypersecretion are associated with a loss of essential amino acids which is responsible at least in part for reduced growth.

In guinea pigs, consumption of R caused some pancreatic reduction and hyposynthesis of digestive enzymes, and still the growth reduction was very marked and was further reduced by increasing the level of R in the diet. It is suggested that pancreatic enlargement and hypersecretion of digestive enzymes (rats, chicks), although accountable for at least part of the growth inhibition, may contribute in overcoming the deleterious effects of R. The latter is feasible, since in those species (guinea pigs, calves, pigs) which do not respond to R feeding in the same manner, growth retardation is more pronounced.

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The trypsin inhibitor assay: Improvement of an existing method

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Abstract

In this paper an improved trypsin inhibitor (TI) assay is presented which is based on the Kakade assay (Kakade et al. 1974). The new assay is simplified and miniaturized and is very accurate and sensitive. With this assay also low amounts of TI activity can be measured reproducibly.

Introduction

Trypsin inhibitors (TI) constitute at least 6 % of the proteins of soybeans (Ryan,1973) and are known to have anti-nutritional effects (reviewed by Gallagher and Schneeman, 1984). Therefore, the use of legumes as soybeans is restricted. Reduction of anti-nutritional factors is usually achieved by treating the soy flour with heat (e.g. toasting). Due to this treatment, the protease inhibitors are largely inactivated but 10-15 % residual activity remains (Rackis et al, 1986). To control the efficiency of the heat treatment, treated products have to be tested for this activity. A common procedure for determining the TI activity is the method of Kakade et al. (1974). However, this method has some drawbacks in terms of linearity, accuracy and sensitivity. Therefore, an improved TI assay, based on the Kakade assay, is developed in which the TI proteins are determined more accurately and sensitively. This paper describes the improved assay and compares both methods. Furthermore, the reproducibility of the new method, the influence of trypsin purity and the influence of simplifications as centrifugation instead of filtration are investigated.

Materials and Methods.

Procedure for TI activity measurement. Trypsin samples were obtained from Worthington USA (Cooper Biomedical, 1974), from Merck (Germany) and from Boehringer (Germany). Soy Bean Trypsin Inhibitor (SBTI) was obtained from Merck (Germany). Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA) was a product from Sigma (USA).
Soy bean samples were ground to 100-200 mesh and 1 gram sample was suspended in 50 ml 0.01 N NaOH. The suspension was stirred magnetically for 3 hr. After this period the mixture was left for 30 minutes without stirring and then 0.5 ml of the supernatant was mixed with 0.5 ml 0.01 N NaOH in an Eppendorf vial. This solution was centrifuged 5 minutes at 10,000 rpm. After aspiration of the fat layer, 0.1 ml of this solution was mixed at 37 °C in a cuvet with 1.5 ml buffer (0.05 M

Tris-HCl, pH 8.2 containing 0.02 M $CaCl_2$), with 0.1 ml trypsin solution (from a stock solution of 40 μg in 10 ml 0.001 N HCl) and after mixing (5 minutes) with 0.1 ml of a BAPNA solution (of a stock solution of 10 mg/ml in 0.01 NaOH). The mixture was incubated for 45 minutes and then 0.2 ml 30 % acetic acid solution was added. The cuvet was centrifuged and the extinction in the supernatant was measured at 410 nm against a blank. At least three different dilutions of the sample extract are measured. The extinctions are plotted against the volume of the sample. From the plot the volume (X) of sample necessary to obtain a decrease in extinction of 50 % is calculated.
The amount of trypsin inhibitor present is read from a calibration curve of trypsin inhibition with purified SBTI. From this curve the volume (Y) of SBTI solution (of a known concentration) necessary to obtain 50 % inhibition is calculated, and, from the volumes X and Y, the TI activity in the sample is calculated.

Results and discussion

Improved TI assay. The differences between the TNO assay and the Kakade assay are summarized as follows: First, the TNO assay is miniaturized, i.e. only small volumes are necessary which allows the assay to be carried out in sample cuvettes. In the TNO procedure, a small aliquot of the sample suspension is centrifuged after which the fatty layer which is found on top of the clear solution is aspirated off. This procedure is cheaper and less time consuming than extraction with an organic solvent. Furthermore, instead of filtrating the solution after the incubation as is done in the Kakade assay, in the TNO method the solution is centrifuged in the cuvet. As is found by turbidity measurements, centrifugation is sufficient to obtain clear solutions. The ratio of absorbance at 620 and 410 nm never exceeded 0.02.

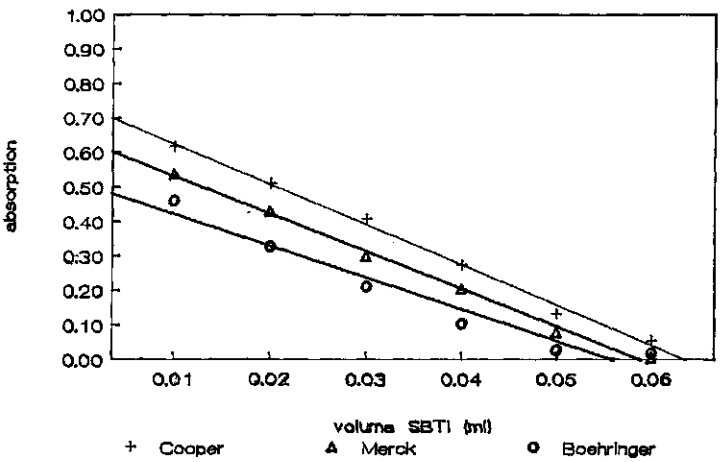

Figure 1. Calibration curves of inhibition of three different trypsin samples by SBTI (the SBTI concentration is 0.04 mg/ml). (+ = Cooper trypsin, Δ = Merck trypsin, 0 = Boehringer trypsin)

Since a high quality trypsin will improve the assay in terms of sensitivity and accuracy, the influence of the trypsin source is investigated (see figure 1). In this assay it was investigated how much SBTI was needed to obtain 50 % inhibition of trypsin samples from three different suppliers. It appeared that the highest amount of SBTI was needed (1.28 μg) for 50 % inhibition of Cooper trypsin. For 50 % inhibition of Merck trypsin and Boehringer trypsin, 1.16 and 1.12 μg SBTI was needed respectively. The curves obtained for trypsin inhibition with different amounts of SBTI solution are plotted in figure 1.
Since in all cases the same trypsin concentration is used, it is calculated from these results that the purity of the trypsin samples is 64 % for the Cooper trypsin, 58 % for the Merck trypsin and 56 % for the Boehringer trypsin. Therefore, Cooper trypsin is the best suited trypsin for this purpose. For these calculations, the SBTI had to be assumed to be 100 % pure. Since it is almost impossible to obtain this, always the same SBTI must be used to allow comparisons of results from different laboratories.

The results of TI activity measurements with the TNO method in 5 soy samples varying in TI contents are shown in Table 1. The results are compared with the Kakade method. For reasons of comparison the values given in table 1 for the Kakade assay have been translated to give the values in mg inhibited trypsin per gram sample.

Table 1. Reproducibility of the new TNO TI activity assay and comparison with the Kakade assay.

	TI activity (mg inhibited trypsin/gram sample)	
Sample	TNO	Kakade method
1	0.29 ± 0.00	0.36 ± 0.02
2	5.90 ± 0.01	9.9 ± 1
3	8.64 ± 0.09	8.5 ± 0.7
4	12.38 ± 0.03	9.4 ± 0.1
5	15.93 ± 0.06	15.9 ± 0.5

From these data it is obvious that the TNO method gives highly reproducible results with standard deviations equal or below 1%. This is found for low (0.29 mg/g sample, sample 1) as well as high (15.9 mg/g sample, sample 5) TI contents.
With the Kakade assay different results are obtained. Apart from the lower reproducibility (5-10 %) for two samples clearly different results are obtained. These differences may be due to a number of factors. Already in 1974 Rackis et al. (5) reported problems encountered in measuring Trypsin inhibitor activity of soy flour. These problems led to serious underestimation or overestimation of the true TI content of the sample.

In his paper Rackis et al. suggested that these problems can be minimized by using 0.01 N sodiumhydroxide as the extracting solvent, by diluting the extracts within narrow limits of the 50 % inhibition level, and by selecting conditions to eliminate cloudiness or reduce absorbance values after the reaction was terminated. From the material and methods section of this paper it is clear that all of these suggestions have been incorporated in the TNO improved TI assay. In view of the importance of the TI assay and the long standing experience with problems encountered with this assay, we are convinced of the necessity to also validate the improved TNO TI assay in an extensive international ring-test.
Finally, the TNO method is very sensitive. This is shown in Table 2.

Table 2. Reproducibility of TI activity measurements on samples with low TI activity using the TNO and the Kakade assay. Activities are given in mg inhibited trypsin per gram sample.

Sample	TNO TI assay	Kakade assay
35	0.79 ± 0.02	0.67 ± 0.05
36	0.08 ± 0.01	0.30 ± 0.03
37	0.04 ± 0.01	0.10 ± 0.02
40	0.09 ± 0.01	0.12 ± 0.01
41	0.11 ± 0.02	0.11 ± 0.04
42	0.14 ± 0.01	0.16 ± 0.02

From these results it appears that the TNO method gives also reproducible results for samples with low contents of TI activity although small errors will lead to relatively large standard deviations. The results of the Kakade assay are in good agreement (except sample 36) with the TNO method.
For the TNO method it is clear that miniaturation of the procedure and centrifugation of the sample in the cuvet has no influence on the accuracy and the sensitivity.

Literature

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ANALYSIS OF RESIDUAL TRYPSIN INHIBITOR ACTIVITY IN FEED FLOUR

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Abstract

A method has been developed for the analysis of low concentrations of trypsin inhibitor in seeds and flour. The samples are treated with 0.5 M NaCl solution and the inhibitor in the extract is purified by trypsin-sepharose 4B affinity chromatography. The amounts of protein obtained are determined by the Coomassie protein assay with the Kunitz inhibitor as a standard. The data are compared with results of activity measurement methods. Big differences have been established probably because of differences in the nature of the inhibitors.

Introduction

Utilization of legume seeds for animal feed is limited by undesirable attributes including enzyme inhibitors, lectins, tannins, bad flavours and others (Sathe & Salunkhe, 1984). Some of them are known as antinutritional factors (ANF), of which proteinase inhibitors have been thoroughly studied (Koide et al, 1973; Whitaker & Sgarbieri, 1981; Birk, 1985).

The search for trypsin inhibitor (TI) assays can be divided into methods for TI activity (Smith et al, 1980), TI units (Hamerstrand et al, 1981), TI affinity chromatography (Roozen & de Groot, 1987), TI spot test (Kourteva et al, 1987), and TI immunoassay (Brandon et al, 1988). In the present work the affinity method has been chosen because it determines only protein-type inhibitors forming complexes with trypsin. These inhibitors are generally considered as carriers of inhibitor activity to be removed by heating or sifting of raw materials.

Materials & Methods

Smooth-seeded (Imposante & Finale) and wrinkled-seeded (C_{306}) pea flours were obtained from the Department of Animal Nutrition of the Agricultural University, Wageningen. The pea (Solara & Finale) and broad bean seeds were obtained from the Institute of Preservation and Processing of Agricultural Products, Wageningen. Kunitz inhibitor, trypsin and benzoyl-DL-arginine-p-nitroanilide hydrochlorid (BAPA) were purchased from Merck. CNBr activated- sepharose 4B was from Pharmacia, Coomassie Brilliant Blue G-250, bovine serum albumin and ovalbumin were from Sigma and lysozyme was from Worthington. Other reagents were Merck analytical grade.

Extraction, isolation and determination of the protein-type trypsin inhibitors were performed as reported before (Roozen & de Groot, 1987 and 1988). Sodium dodecyl sulphate-electrophoresis and silverstaining of the isolated protein-type trypsin inhibitors were carried out according to Laemmli (1970) and Morrissey (1981). The presence of polyphenols in eluates pH 3 from the affinity column was detected by a modified Lowry method (Potty, 1969).

Results & Discussion

Table 1. 0.5 M NaCl extraction of 25 g pea flour (var. Finale) and trypsin-sepharose 4B chromatography: recovery of trypsin inhibitor units (TIU) determined according to Hamerstrand et al. (1981).

	Extract	Affinity chromatography	
		eluate pH 8	35854 ± 1670
		eluate pH 5.2	1005 ± 144
		eluate pH 3	2850 ± 688
TIU:	41736		39709

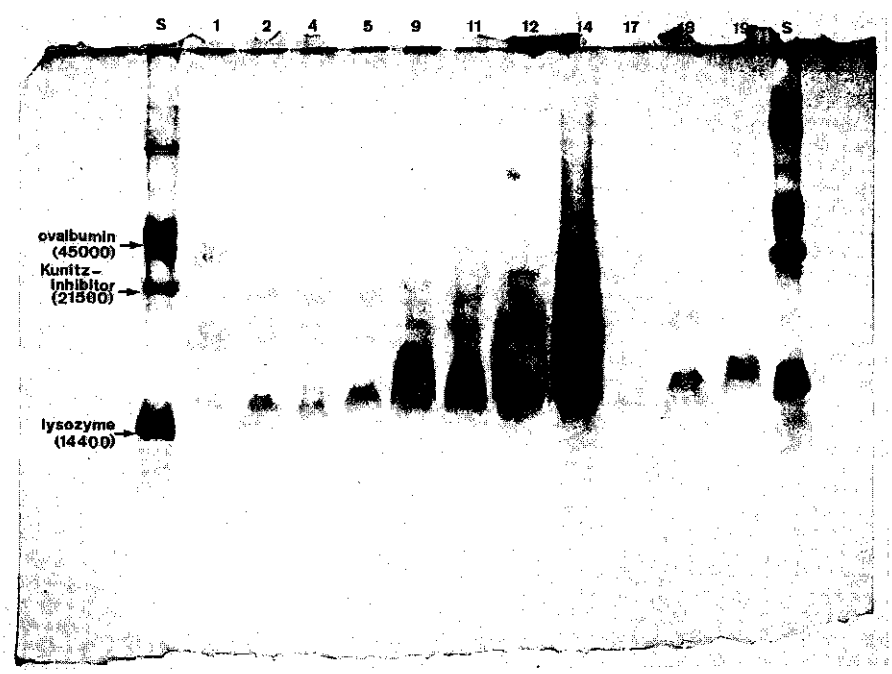

Figure 1. Sodium dodecyl sulphate slab gel electrophoretic patterns of a S(tandard) and eluate pH 3 preparations (dialyzed), of which the numbers correspond with the ones in table 2. S = m.w. 45000 (ovalbumin), m.w. 21500 (Kunitz inhibitor), m.w. 14400 (lysozyme).

Table 1 shows the distribution of trypsin inhibitor activity among the eluates of the affinity column: only 7% of the extracted activity is present in eluate pH 3. Unexpectedly the inhibitor activities of eluates pH 3 remain at 2850, when different amounts of extract are applied to the column. However, the protein contents of these eluates correlate very well with the amounts of extract applied. These results indicate that the protein-type inhibitors are preferably bound to the column and that other-type inhibitors (polyphenols were detected in eluates pH 3) fill up the residual places and in majority leave the column. Consequently the protein-type inhibitors contribute only for 7% or less to the total inhibitor activity of pea flour (Roozen & de Groot, 1988). It also explains the large differences between method A and B in table 2. Small sample sizes of wrinkled-seeded pea flour resulted in very low amounts of protein-type inhibitor isolated in method A and so the protein assay came to its lower limit giving less reliable results.

The proteins obtained in eluates pH 3 were subjected to sodium dodecyl sulphate-electrophoresis (figure 1). The largest protein fraction has a molecular weight of about 15000, and matches with results published for peas and beans elsewhere (Belew et al, 1975; Whitaker & Sgarbieri, 1981).

Table 2. Trypsin inhibitor (TI) content of peas and beans. Comparison of method A (Roozen & de Groot, 1987) with method B (Smith et al., 1981).

Sample (# in fig.1)	Method A (mg protein TI /g product)	Method B (mg inhibited trypsin/g product)
SMOOTH-SEEDED PEA FLOUR		
1 imposante	0.054	1.55*
2 finale	0.031	0.81*
3 finale (heated)	0.021	1.68*
4 finale (heated)	0.011	0.08*
5 finale (sift fraction)[1]	0.63	2.73*
6 finale (sift fraction)[1]	0.73	3.02*
7 finale (sift fraction)[2]	0.033	0.60*
8 finale (sift fraction)[2]	0.005	0.18*
WRINKLED-SEEDED PEA FLOUR		
9 C_{306}	0.015	5.07
10 C_{306} (heated)	0.025	3.16*
11 C_{306} (heated)	0.011	0.32*
12 C_{306} (sift fraction)[1]	0.022	6.91
13 C_{306} (sift fraction)[1]	0.060	5.78
14 C_{306} (sift fraction)[1]	0.036	12.96
15 C_{306} (sift fraction)[2]	0.023	1.95
16 C_{306} (sift fraction)[2]	0.018	3.85
SEEDS		
17 pea (Solara)	0.007	0.39
18 pea (Finale)	0.029	0.87
19 broad bean	0.19	0.89

* = data obtained from Department of Animal Nutrition
[1] = protein enriched fraction ; [2] = starch fraction

Using Kunitz inhibitor (m.w. 21500) as a standard for the protein assay means also that the activity of the legume protein-type trypsin inhibitors (m.w. ± 15000) is underestimated, that is to say (on molar basis) 1 mg legume protein-type inhibitor is as active as ± 1.5 mg Kunitz inhibitor. Caution should be taken for overloading the columns callibrated with Kunitz inhibitor (maximum load is equivalent to the activity of ± 0.5 mg Kunitz inhibitor per ml gel).

The method introduced here for analysis of trypsin inhibitors in feed flour shows that inhibitor activity can only partly be ascribed to proteins.

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COMPARATIVE STUDIES ON TRYPSIN INHIBITORS IN LEGUMES AND CEREALS

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Summary

The activity of trypsin inhibitors from the seeds of legumes and cereals was measured. The inhibitory activity against trypsin preparations from ox, pig and rat was compared. Furthermore, the influence of extraction pH and pepsin on the measured activity was investigated. Based on the obtained results, an extraction procedure at pH 2 with added pepsin followed by an incubation of the extract with porcine trypsin (instead of the normally used bovine trypsin) was concluded to give the best evaluation of the antinutritional effect of trypsin inhibitors in pig feeds.
Keywords: porcine trypsin, bovine trypsin, pepsin, pig feed.

Introduction

Legume seeds and cereal grains contain varying amounts of proteinase inhibitors which may reduce the utilization of protein from these sources in monogastrics. The inhibitor activity can effectively be reduced by proper heat treatments. The most important proteinase inhibitors appears to be the trypsin inhibitors. The activity of these inhibitors is normally measured by inhibiting bovine trypsin, after extraction at optimal conditions. The inhibiting capacity is typically obtained with saline solutions at pH 5 for cereal grains (Boisen 1983), or with neutral or basic solutions for legumes. Processed soybean products are recommended to be extracted at pH 9.6 (Smith et al. 1980). However, the antinutritional effect of trypsin inhibitors in pig feeds may be dependent on their degree of extraction and stability at pH 2 in the presence of pepsin.

The purpose of the present work was to measure the trypsin inhibitor activity in seeds of legumes and cereals as influenced by varying extraction conditions including pepsin incubation and compare the resulting inhibiting capacity on bovine and porcine trypsin, respectively.

Results and discussion

The inhibition of trypsin from ox, rat and pig was compared by justifying the concentrations of the different trypsins after active site titration with bovine pancreatic trypsin inhibitor. One inhibitor unit (1 U) was defined as the calculated amount of inhibitor which inhibits 1 mg trypsin at pH 8.2 using DL-DAPA as substrate. All results are mean values from at least two measurements made in duplicate. No double determinations varied more than 5 % from each other. The trypsin inhibitor activity in extracts from seeds of selected legumes and cereals is shown in Table 1. Trypsin inhibitor activity in legume seeds varied from 0 to approximately 20 U/g sample (50 U/g protein) whereas the range in grains was from 0 to approximately 1 U/g sample (10 U/g protein). The variation in inhibition of trypsin from the three investigated animals was similar. However, compared to bovine trypsin, rat trypsin was generally inhibited more by legume inhibitors but less by cereal inhibitors, whereas porcine trypsin was inhibited less by both legume and cereal inhibitors.

Table 1. Trypsin inhibitor activity (A: U/g sample; B: U/g protein) in extracts from different feedstuffs after extraction at pH 5.

Feedstuff	Origin of trypsin					
	bovine		rat		porcine	
	A	B	A	B	A	B
Legumes:						
Soybean	19.5	49.2	26.0	65.7	20.8	52.5
Pea (Progreta)	6.0	23.9	6.6	26.3	4.5	17.9
Pea (Stehgolt)	1.9	8.8	1.9	8.8	1.2	5.6
Faba bean	1.9	5.8	1.3	4.0	1.0	3.1
Lupin	0.1	0.3	0.0	0.0	0.0	0.0
Cereals:						
Rye	1.0	8.8	0.3	2.6	0.9	7.9
Triticale	0.9	6.9	0.4	3.1	0.8	6.2
Wheat	0.4	2.7	0.2	1.4	0.4	1.4
Barley	0.7	4.0	0.5	3.2	0.6	3.2
Oats	0.1	0.7	0.0	0.0	0.0	0.0

The effect of extraction pH and pepsin on the extracted trypsin inhibitor activity was also investigated (Table 2). A slurry with 1 g finely ground (d<1mm) sample in 25 ml 0.1 M sodium acetate buffer, pH 5.0 was initially made. The samples were then extracted directly (pH 5) or after adjusting to pH 2 with HCl (pH 2) or after further addition of 1 mg pepsin (pH 2 + pepsin). All extractions were performed with continuously stirring at room temperature for 1 hour. The results indicate that legume inhibitors are slightly more, while cereal inhibitors are slightly less effectively extracted at pH 2 compared to pH 5. The increasing effect of pepsin on the measured inhibitor activity in some legumes may be due to a more effective extraction after degradation of associated proteins. Cereal trypsin inhibitors were found to be pepsin labile in rye, partially pepsin stable in barley and triticale and pepsin stable in wheat. Porcine trypsin was in nearly all cases inhibited less than bovine trypsin.

Different commercial soybean products were extracted at pH 9.6 and measured against bovine trypsin according to Smith et al. 1980. The extracted inhibiting capacity was compared with those obtained after extraction at

Table 2. Trypsin inhibitor activity (U/g sample) in extracts from legumes and cereals after different extraction conditions and measured against bovine trypsin (b) or porcine trypsin (p).

Feedstuffs	Extraction conditions					
	pH 5		pH 2		pH 2 + pepsin	
	b	p	b	p	b	p
Legumes:						
Soybean	19.5	20.8	24.2	22.7	26.1	23.4
Pea (Progreta)	6.0	4.5	6.9	4.7	7.1	5.2
Pea (Stehgolt)	1.9	1.1	1.2	1.0	1.7	1.3
Faba bean	1.9	1.0	2.5	1.2	3.0	1.4
Cereals:						
Rye	1.0	0.9	0.9	0.8	0.2	0.1
Triticale	0.9	0.8	0.9	0.8	0.2	0.1
Barley	0.7	0.6	0.7	0.5	0.3	0.3
Wheat	0.4	0.4	0.4	0.4	0.4	0.4

Table 3. Trypsin inhibitor activity (U/g sample) in extracts from commercial soybean products after different extraction conditions and measured against bovine trypsin (b) or porcine trypsin (p).

Soybean product	Extraction conditions					
	pH 9.6	pH 5	pH 2		pH 2 + pepsin	
	b	b	b	p	b	p
A	23.6	29.8	25.6	22.3	28.7	25.0
B	21.7	16.5	16.8	16.2	13.3	13.9
C	15.7	9.1	11.8	9.5	15.4	9.7
D	13.3	12.6	16.2	13.8	18.5	13.4
E	10.1	6.3	11.1	8.9	14.3	11.6
F	2.1	0.0	0.2	0.1	0.3	0.2
G	1.1	0.0	0.2	0.2	0.4	0.3

pH 5, pH 2 and pH 2 + pepsin, respectively (Table 3). The inhibition against bovine trypsin was generally lower after extraction at pH 5 compared to pH 9.6 or pH 2. In samples that were properly heat treated (sample F and G) some residual activity could still be extracted at pH 9.6, whereas very little activity could be detected after extraction at pH 5 or pH 2. The extracted activity was generally increased by pepsin but still much lower activity was measured than after extraction at pH 9.6. Nearly all extracts inhibited porcine trypsin less than bovine trypsin. The results indicate that the measured activity in heat treated products after extraction at pH 9.6 may be higher than the actual activity at physiological conditions. Furthermore, extracts from soybean products seems generally to inhibit bovine trypsin more effectively than porcine trypsin.

Based on the obtained results an extraction procedure at pH 2 with added pepsin followed by an incubation of the extract with porcine trypsin is proposed to give the best evaluation of the antinutritional effect of trypsin inhibitors in all types of pig feeds.

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INTER- AND INTRAVARIETAL VARIABILITY OF THE TRYPSIN INHIBITORS CONTENT OF PEAS AND HIS INFLUENCE ON APPARENT DIGESTIBILITY OF CRUDE PROTEINS BY GROWING PIGS

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Summary

The trypsin-inhibiting activity (TIA) of 33 European spring pea varieties ranged from 1.69 to 7.56 TIU/mg DM with an average of 2.46 TIU/mg DM and a coefficient of variation of 56 %. The TIA of winter peas were 3 or 4 times higher than those of the spring ones (7.34 to 11.24 TIU/mg DM). The peas imported from outside E.E.C. and 4 of the 6 other legume seeds studied had an intermediate TIA. The growth conditions influenced the TIA of peas. The increase of the TIA in a pig diet from 1.2 to 3.8 TIU/mg DM induced a 5 % decrease ($P< 0.05$) of the apparent digestibility of the diet crude proteins.

Key words : peas, Pisum sativum, trypsin inhibitors, pigs.

Introduction

The depressive effect of the trypsin-inhibiting activity (TIA) of pea (*Pisum sativum L.*) seeds on the pigs growth performances is well documented (Fekete *et al.*,1984; Grosjean *et al.*, 1986). The TIA varies with the pea type : the winter varieties have higher TIA than the spring ones, and the smooth cultivars higher than the wrinkled ones (Valdebouze *et al.*, 1980). But the variation among varieties or among samples of the same variety is largely unknown. Moreover, there are few data about the influence of pea TIA on the apparent digestibility of the diet by pigs. The purpose of this study was to obtain more precise informations about these subjects.

Materials and methods

Experiment 1

The TIA of 33 European spring pea varieties, 6 winter pea varieties, peas imported from outside E.E.C. and 6 others legume seeds was determined by the method of Valdebouze *et al.* (1980). The samples had been ground with a Dangoumeau mill. For the spring variety Finale, the samples originated from 2 different fields situated at 2 different agricultural locations. The results were expressed in trypsin inhibited units per mg of dry matter (TIU/mg DM).

Experiment 2

The apparent digestibility of 2 spring pea varieties, with the same chemical composition (25 % crude proteins (C.P.), 42 % starch and 18.4 MJ crude energy (C.E.)/ kg DM) except for the TIA (Finale : 2.35 and Progreta : 7.48 TIU/mg DM), was determined by the difference method according to 3x3 latin square design using 3 belgian Landrace pigs (initial weight : 45 kg). The basal diet (2/3 wheat- 1/3 soybean meal) had the same chemical composition than the 2 pea varieties. In the 2 other diets,

the 2 pea varieties were substituted at the 0.40 level for the basal diet.The animals were fed 100 g fresh matter/kg $P^{0.75}$ as a liquid diet (2.5 l/kg) in 2 identical meals at 8 h and 17 h. A 8-day adaptation period was followed by a 10-day collection period on each diet, in which faeces were quantitatively collected once a day.

Results and discussion

Experiment 1.

Table 1. Trypsin-inhibited activity of different pea varieties and 6 other legume seeds

Variety	n	Mean (TIU/mgDM)	C.V. (%)	Variety	n	Mean (TIU/mg DM)	C.V. (%)
Spring peas				Winter peas			
Finale	15	2,02	19,7	Frijaune	1	10,54	-
Amino	11	2,13	18,6	Frilène	2	11,18	0,2
Maxi	4	1,82	7,8	Frisson	1	11,24	-
Progreta	5	7,56	6,8	Amac	2	7,35	2,3
Solara	7	2,10	7,7	Laser	2	7,54	0,3
Countess	3	3,18	9,7	Santon	1	8,65	-
Consort	1	4,88	-				
Radley	1	7,46	-				
Katrijn	3	2,06	11,5				
Danto	1	1,94	-	Imported peas			
Thérèse	2	2,23	0,4				
Miranda	1	2,03	-	Canada	15	3,20	12,8
Bodil	2	2,10	12,2	-Trapper	3	5,83	21,2
Belinda	1	1,75	-	-Century	1	2,78	-
Filby	1	2,46	-	China	3	4,44	12,6
Belman	1	2,28	-	Hungary	4	2,14	15,6
Sentinel	1	1,69	-	Poland			
Bruant	1	2,53	-	-Fidelia	2	2,86	11,0
Mike	1	2,26	-	-Peluschken	1	3,98	-
Birte	1	1,83	-	Australia &			
Stehgolt	1	1,69	-	New Zealand			
CM 91	1	1,85	-	-Dun	4	2,41	17,5
CM 92	1	1,91	-	-Maple	4	2,79	25,0
CM 93	1	2,33	-				
CM 94	1	2,11	-				
CM 95	1	2,10	-	Legume seeds			
MJASI 845	1	2,27	-				
MJASI 893	1	3,09	-	*Vicia faba L.*		4,51	-
NRPB 338	1	1,96	-	*Vicia sativa L.*		5,21	-
NRPB 412	1	1,96	-	*Vicia lens*		5,38	-
LD 8954	1	2,17	-	*Cajanus cajan*		6,65	-
FR-57	1	1,88	-	*Cicer arietinum*		1,68	-
FR-RAM	1	1,71	-	*Phaseolus vulgaris L.*		16,56	-

The spring pea varieties revealed very low TIA (< 3 TIU/mg DM) except for 3 British varieties : Consort (4.88), Progreta (7.56) and Radley (7.46). Radley, for instance, is a crossbreed between spring and winter varieties (Cors, personal communication). This explains the high values observed for this variety. The 6 French winter varieties had higher TIA; they may be classified in 2 groups, according to their TIA. The first group includes Amac, Laser and Santon with a moderate activity (mean = 7.69 TIU/mg DM) and a coefficient of variation (C.V.) of 6,5 %, and the second one Frijaune, Frilène and Frisson with a high activity (mean = 11.24 TIU/mg DM) and a C.V. of 2.6 %.

For the peas imported from outside E.E.C., only the Chinese peas and the Canadian Trapper cultivar exhibited much higher TIA than the European spring varieties, but they never reached the winter variety values, except for one sample of Trapper (7.57 TIU/mg DM). The 15 samples of Canadian peas originating from the livestock food industry averaged 3.20 TIU/mg DM, with a C.V. of 13 %.

All the legumes experimented exhibited higher TIA values than the spring pea varieties. The winter peas had higher values than faba beans (*Vicia faba*), lentils (*Vicia lens*), vetches (*Vicia sativa*) and pigeonpeas (*Cajanus cajan*), about the same values than chickpeas (*Cicer arietinum*), but lower than haricot beans (*Phaseolus vulgaris*).

Eight samples of the variety Finale were collected at random in each field. The TIU values observed for the 2 fields were 1.58 and 2.21 TIU/mg DM, the C.V. values observed within the fields being very low (4.1 and 6 %). Other samples of Finale, originating from other fields or other regions gave also very different values : from 1.93 to 2.94 TIU/mg DM. The growth conditions (climate, soil, sowing date) influence thus the pea TIA. This observation confirms the results of Valdebouze & Gaborit (1985).

Experiment 2

The apparent digestibility of the 2 pea varieties by growing pigs did not differ significantly ($P<0.05$) for the O.M. and the C.E., but the apparent digestibility of C.P. was significantly lower for the variety Progreta than for Finale (table 2). This difference may be attributed to the TIA of Progreta which is 3 times as high as Finale. The presence of trypsin inhibitors in the small intestine would induce the formation of an enzyme-inhibitor complex and the hypersecretory activity of the pancreas, with an increase in endogenous nitrogen fecal excretion as a result (Liener, 1979). The apparent digestibility of the crude protein is thus lower.

Table 2. Apparent digestibility of the organic matter, the crude protein and the crude energy of 2 pea varieties by pigs.

		Digestibility (%)		
		Organic Matter	Crude Protein	Crude Energy
Diet	Progreta	89,1^a	85,0^a	87,2^a
	Finale	90,0^a	89,0^b	88,2^a
Variety	Progreta	88,9^a	81,8^a	86,8^a
	Finale	91,0^a	91,0^b	89,4^a

a,b : means with different superscripts differ significantly ($P< 0.05$)

As the pancreatic enzymes content in the sulfur containing amino acids is high, the enhanced excretion would reduce dramatically the intestinal availability of these amino acids which are already deficient in pea. In this trial, the TIA of the Finale diet was 1.23 TIU/mg DM *vs* 3.77 TIU/mg DM for the Progreta diet. This difference was sufficient to induce a 5 % decrease ($P< 0.05$) in the apparent digestibility of the diet crude proteins, corresponding to a 10 % decrease ($P< 0.05$) of the pea proteins (table 2). However, the protein quality may also be incriminated, but this was not checked in this study.

Conclusions

Our results show that the spring pea varieties (except Consort, Progreta and Radley) and most of the peas imported from outside E.E.C. have low TIA as compared to winter varieties. Moreover, the growth conditions can influence the pea TIA. A content of 3.8 TIU/mg DM in a pig diet reduces the apparent digestibility of the crude protein as compared to a diet with a content of 1.2 TIU/mg DM.

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INHIBITOR RESISTANT TRYPSIN AND CHYMOTRYPSIN DUE TO FEEDING RAW SOY, THE BOWMAN-BIRK INHIBITOR AND THE KUNITZ TRYPSIN INHIBITOR IN THE RAT

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Introduction

Pancreatic proteolytic enzymes of humans and various species of animals are inhibited by soybean and other plant proteinase inhibitors. Reduction in duodenal enzyme activity in humans by infusion of extracts of raw soybeans (RS) was demonstrated in a previous study. The chymotryptic activity was severely inhibited, and the total proteolytic activity was reduced while the tryptic activity showed a transitory reduction, and finally increased above basal levels (H. Holm et al, 1988). It was further shown that this tryptic activity, demonstrated both by esterolytic activity with Benzoyl-Arginine p-nitroanilide (BAPNA), and by proteolytic activity (casein), was present in spite of surplus amounts of inhibitors from the raw soy. This activity was further extremely resistant to other well known serine proteinases as Phenylmethylsulfonyl fluoride (PMSF), α_1-antitrypsin, Aprotinin and the Lima bean inhibitor (H.Holm, A.Krogdahl & L.E.Hansen, 1988). In three of the eleven persons participating in the tube feeding experiments, some samples of duodenal juice obtained during RS installation gave some chymotryptic activity with Benzoyl-L-Tyrosine ethyl ester (BTEE) as substrate. Resent experiments have revealed that this chymotryptic activity resembles the tryptic activity by being resistant to a number of known chymotrypsin inhibitors (to be published). Blood samples taken during the 2 hours of tube feeding did not give any increase in cholecystokinin (CCK), secretin or somatostatin levels. These results strongly suggest large differences between man and the rat - the animal most frequently used in proteinase/inhibitor studies. In the study described below, rats were therefore tube fed the same test proteins as in the human study described above.

Experimental

Thirty six male Wistar rats, average weight 250 g were randomly divided in six groups of six and housed individually in metabolic cages. After an overnight fast they were tube fed one of the following test meals (A-F) suspended in 2 ml of water.

Table 1. Composition of diets.

A: Bovine serum albumin (BSA)[1]: 300mg
B: Soybean protein isolate (SPI)[2]: 300mg

[1] Sigma Chemicals St.Louis, MO. [2] Purina Protein 710 Ralston, St.Louis.

C: Raw soy bean extract (RS)[3]: 300mg
D: BSA : 300mg, and Bowman-Birk inhibitor (BBI)[4]: 2mg
E: BSA : 300mg, and Kunitz inhibitor (SBTI)[1]: 4mg
F: BSA : 300mg, and Lima bean inhibitor (LBI)[1]: 2mg

The BSA, SPI and RS were from the same batches as used in the human experiments.

Enzyme preparation

After 30 min the animals were sacrified and the pancreatic enzymes obtained by flushing the isolated duodenum and proximal part of the intestine (approximately 30 cm) with 2 ml of ice cold buffer (Tris-HCl, BAPNA assay). The supernatant after centrifugation at 20000xg for 30 min. was stored in aliquots at -70°C. Enzyme activities were analysed as described earlier (A. Krogdahl & H. Holm 1979).

Inhibitors

Phenylmethylsulfonylfluoride[1] (PMSF) was used a 10^{-1} M solution (20% acetonitrile in water). Human α_1-Antitrypsin[1] (α_1-A): 1mg/ml, and LBI[1]:
0,16mg/ml was dissolved in assay buffer, Aprotinin[2] : 2000 KIE.

Statistical analysis

All results are mean ± SEM from 6 animals. The significance of differences between groups was assessed by a Student's test. A value of P of less than 0.05 was judged significant.

Results and discussion

The duodenal/intestinal enzyme activities measured after different test meals are given in Table 2.

Table 2. Enzyme activities in duodenal/intestinal chyme (25µl) and correlation between tryptic and chymotryptic activity (r).

Test meal*:	BSA	SPI	RS	BBI	SBTI	LBI
Trypsin**	1.11	0.93	0.24	0.23	0.31	0.25
	±0.10	±0.11	±0.04	±0.03	±0.08	±0.05
Chymotr***	0.83	0.84	0.45	0.42	1.46	-
	±0.15	±0.16	±0.06	±0.06	±0.43	-
r	0.981	0.970	0.872	0.092	0.076	-

* Test meal as in Table 1. **: BAPNA - $OD_{410/20min}$. ***:BTEE-OD_{253}-/min.

[1] Sigma Chemicals St.Louis, MO. [2] BAYER LEVERKUSEN.FRG.
[3] (H.Holm et al, 1988), [4] Kindly supplied as a gift from Prof. Yehudith Birk, The Hebrew University of Jerusalem, Israel.

The raw soy bean extract is seen to reduce the tryptic activity to 1/4 of that "normally" found with BSA. BBI and LBI gave very similar activities and not different from the ones obtained with RS. This is in accordance with their similar structure (Y.Birk, 1985). SBTI, of an entirely different structure, gives a slightly higher tryptic activity, though not statistically different from the other inhibitors. The chymotryptic activity was reduced to about 50%, both by RS and BBI. The value obtained with SBTI was not significantly higher than with BSA and SPI. As with the human duodenal juices, a surplus of inhibitors from the meals was demonstrated by adding minor amounts (2-5µl) of either RS-, BBI-, SBTI- or LBI-enzyme preparations to a standard incubation containing BSA-enzyme preparation as a source of rat trypsin or chymotrypsin. The inhibition obtained indicated that 3-40% of the tubefed inhibitors were present in the isolated enzyme preparations. As with the human enzymes both rat trypsin and chymotrypsin in BSA and SPI preparations may be severely inhibited as shown in Fig. 1.

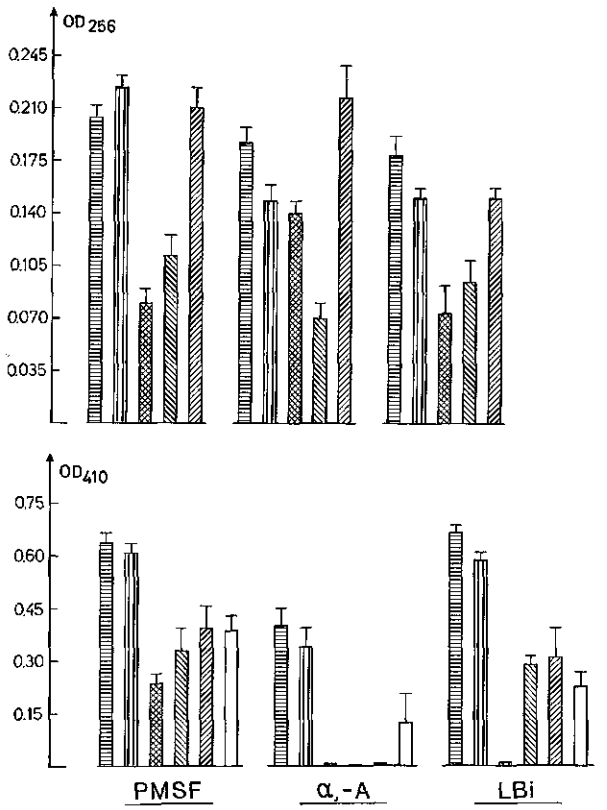
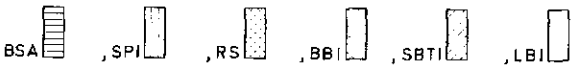


Fig. 1 Reduction in tryptic activity, (OD_{410}) and chymotryptic activity, (OD_{256}) by purified inhibitors, amounts as in Table 3.



The trypsin and chymotrypsin in RS- BBI- and LBI- preparations demonstrate inhibitor resistance similar to the human enzymes obtained when RS was given intraduodenally. The SBTI-enzyme preparation, and to some extent also the LBI-preparation, gave enzymes with resistance more like the "normal" enzymes in BSA and SPI-preparations, particular with respect to chymotyptic activity. To verify that the "resistant" enzymes posessed protolytic activity as well, incubation with casein as substrate were carried out as shown in Table 3.

Table 3. Inhibition of total proteolytic activity (OD_{280}), in duodenal/
intestinal enzyme preparations.

Enzyme preparation:	BSA	RS
Addition		
None	0.59±0.05	0.59±0.09
PMSF, 100 µl	0.28±0.06	0.54±0.08
α_1-A, 100 µl	0.23±0.07	0.42±0.10
LBI, 100 µl	0.21±0.04	0.53±0.11
Aprotinin, 100 µl	0.32±0.09	0.45±0.06

Conclusion

The ingestion of raw soy in the rat appears to result in an inhibitor resistant trypsin similar to that previously found in man. In contrast to the low chymotryptic activity in the human study, considerable amount of chymotryptic activity were found in the rat gut after this treatment. The chymotrypsin is also resistant to low as well as high molecular weight inhibitors. Of the two common inhibitors in soybeans, BBI seems to be far more potent than SBTI in making the enzymes inhibitor resistant. This may have practical implications in animal and human nutrition as BBI is assumed to be the most resistant one towards heat, acid, and food processing in general (A.Krogdahl & H.Holm 1981).

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EFFECT OF SOYBEAN TRYPSIN INHIBITOR [BOWMAN-BIRK] ON THE EXOCRINE SECRETION OF THE HUMAN PANCREAS

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Summary

This study was undertaken to clarify the issue whether feedback regulation of pancreatic enzyme secretion occurs in human beings. Bowman-Birk inhibitor from soybean was used to inhibit the activities of trypsin and chymotrypsin. Pure pancreatic juice was collected by endoscopic retrograde cholangiopancreatography. Levels of enzymes studied, trypsin, chymotrypsin, elastase, and amylase, were increased in output and concentration two to threefolds. The results confirmed the existence of feedback control of pancreas in humans.
Keywords: Bowman-Birk inhibitor, feedback regulation, endoscopic retrograde cholangiopancreatography.

Introduction

When pancreatobiliary secretion from the proximal region of the small intestine was diverted, it produced sharp increase in pancreatic secretion in the rat (Green & Lyman, 1972). When rats were fed a diet containing raw soyflour (rich in trypsin inhibitors), a similar response was observed (Nitsan & Liener, 1976). These effects have been attributed to the feedback inhibition of enzyme secretion by the pancreas. Such mechanism is operative in rat, chicken, pig and calf, but not in dog (Diaz et al., 1982).

The manner in which pancreatic secretion is controlled by intestinal proteases in humans is uncertain. Conflicting findings regarding the presence (Ihse et al., 1977) and absence (Hotz et al., 1983; Dlugosz et al., 1983) of a feedback mechanism of pancreatic secretion in man are reported in the literature. The reasons for discrepancy could be due to: the use of duodenal aspirates for measuring the pancreatic secretory products, failure to obtain a quantitative collection of duodenal contents and their dilution by gastric and biliary secretions, perfusion of wrong part of the intestine, and use of different protease inhibitors.

To understand the regulation of pancreatic secretion, the present study was undertaken in which the secretory activity in man is based on the measurements of enzymes in pure pancreatic juice (PJ), collected by direct cannulation of the pancreatic duct using endoscopic retrograde cholangiopancreatography (ERCP) technique. The Bowman-Birk inhibitor (BBI) from soybeans was used to inhibit trypsin and chymotrypsin.

Materials and Methods

BBI preparation, free from Kunitz inhibitor, used in this study was kindly provided by Nestec Ltd. (Vevey, Switzerland). Heat inactivated BBI (HBBI) was prepared by autoclaving a 0.75% solution of BBI in normal saline at 125°C for 3 h.

Healthy human volunteers, between ages 19 and 37, were selected for the study. The individuals were fasted overnight, and they were subjected to

one of the four treatments. Length of periods, sequence of treatments, and number of subjects in each treatment are presented in Table 1. This protocol served a very useful purpose in that each individual served his/her own control, thus avoiding variation among individuals.

Table 1. Protocol for studying influence of Bowman-Birk inhibitor on the secretory activity of the pancreas.

Periods	Time (min)	Sequence of treatments			
		A	B	C	D
1	15	SBB	SBB	SBB	SBB
2	35	PJ+SBB	PJ+HBBI	PJ+SBB	PJ+BBI
3	55	PJ+BBI	PJ+BBI	PJ+SBB	PJ+HBBI
No. of subjects		3	5	3	1

BBI = Bowman-Birk inhibitor; HBBI = Heat inactivated BBI; PJ = Pure pancreatic juice; SBB = Saline-bicarbonate buffer.

A small 8F Keofeed polyvinyl tube was attached "piggy-back" style by tape to the side of the tip of an Olympus model, JF-B2 fiberoptic duodenoscope. The scope was then positioned in the duodenum, and the cannula was then inserted into the main pancreatic duct to a depth of 2-3 cm using a standard Teflon cannula (ID 1.7mm). After successful placement of the cannula into the pancreatic duct the individual was given secretin (Kabi) intravenously at a rate of 1 U/kg body wt. per hour and 1% amino acid solution at the rate of 2 ml/min through piggy-back tube (Slaff et al., 1984). Colorless PJ was collected by aspiration every 5-min interval. About 10-15% of the PJ was retained for further analysis, and remaining portion was mixed with BBI, HBBI or SBB, according to the protocol, and introduced back into the duodenum.

Pancreatic juice was activated with enterokinase and analyzed for trypsin, chymotrypsin, elastase and amylase. Enzyme activities were expressed in terms of arbitrary units, where 1 unit is defined as an absorbance change of 0.01 under the conditions of each assay system. Statistical analysis was conducted by the non-parametric Mann-Whitney rank sum test (Conover, 1971) and Student t-test (Snedecor & Cochran, 1967).

Results and Discussion

About 95% inhibition of trypsin and chymotrypsin and 50% inhibition of elastase activities were obtained (*in vitro*) when ≥ 4 mg of BBI was added per ml. of PJ. To ensure complete inactivation of trypsin and chymotrypsin, a solution containing 7.5 mg of BBI per ml was routinely added to equal volume of PJ.

The rates of enzyme production in terms of output (units per min) during periods 2 and 3 in the various treatments in this study are presented in Table 2. The addition of BBI caused a significant ($p \leq 0.05$) increase in output in all four enzymes, regardless of the sequence of addition. No significant changes between periods 2 and 3 were observed in sequence C.

Table 3 shows that, when enzyme secretion was expressed in terms of concentration (units/ml PJ), the same general pattern of response to the various treatments was observed. This parameter is not influenced by the volume of PJ that is collected during each period. When compared with values in Table 2, no significant differences in enzyme production were observed in sequence D.

Table 2. Enzyme output (units/min) of pancreas as influenced by the addition of BBI to PJ before duodenal infusion.

Enzymes (units/ml PJ)	Period	Sequence of treatments A	B	C	D
Trypsin (X 10^4)	2	5.23^b	6.11^b	11.11^a	12.27^a
	3	14.10^a	11.47^a	10.14^a	5.27^b
Chymotrypsin (X 10^4)	2	2.05^b	1.53^b	3.88^a	2.77^a
	3	5.46^a	2.82^a	3.29^a	1.38^b
Elastase (X 10^3)	2	2.91^b	2.45^b	5.31^a	1.73^a
	3	9.21^a	4.68^a	4.49^a	0.75^b
Amylase (X 10^5)	2	3.44^b	2.82^b	17.05^a	6.73^a
	3	12.82^a	5.75^a	15.25^a	2.91^b

^{a,b}Values within columns for each enzyme not sharing common superscript differ significantly ($p \leq 0.05$) when analyzed by the Mann-Whitney test.

Table 3. Enzyme concentration (units/ml PJ) in pancreatic juice in response to the intraduodenal infusion of pancreatic juice treated with BBI.

Enzymes (units/min)	Period	Sequence of treatments A	B	C	D
Trypsin (X 10^4)	2	5.09^b	3.95^b	7.82^a	5.50^a
	3	7.01^a	9.15^a	5.33^a	5.01^a
Chymotrypsin (X 10^4)	2	1.69^b	1.09^b	2.60^a	1.24^a
	3	2.77^a	2.19^a	1.85^a	1.26^a
Elastase (X 10^3)	2	3.17^b	1.67^b	4.06^a	0.76^a
	3	4.65^a	3.47^a	2.83^a	0.67^a
Amylase (X 10^5)	2	2.79^b	3.01^b	11.65^a	3.00^a
	3	4.84^a	5.78^a	8.59^a	2.83^a

^{a,b}Values within columns for each enzyme not sharing common superscript differ significantly ($p \leq 0.05$) when analyzed by the Mann-Whitney test.

When the ratio of enzyme output and concentration in period 3 to that in period 2 was calculated, it became apparent that there was about a threefold increase in output and twofold increase in concentration. These increases were significant ($p \leq 0.05$). These ratios are presented in Table 4.

The stimulatory effect of BBI on the secretory activity of the pancreas demonstrated here indicates that the human pancreas is responsive to such inhibitor. Two important features of this study were, the analysis of

Table 4. Comparison of the ratios of enzyme activities in period 3 with period 2, based on combined data obtained with 3 subjects in Sequence A and 5 subjects in Sequence B.

Enzyme	Ratios of period 3 to period 2	
	Output (units/min)	Concentration (units/ml PJ)
Trypsin	3.5	2.4
Chymotrypsin	3.3	2.0
Elastase	3.1	2.0
Amylase	2.7	1.7

pure PJ obtained by the cannulation of the pancreatic duct and the use of an inhibitor of trypsin and chymotrypsin. The possibility of leakage during cannulation, as discussed by Robberecht et al. (1977), was minimum as the correlation between enzyme output and concentration was highly significant ($p \leq 0.01$).

Therefore, the results of this study using pancreatic duct cannulation lend supporting evidence that the intraduodenal infusion of a trypsin and chymotrypsin inhibitor, such as BBI, increased pancreatic exocrine secretion. The data supported the concept of negative feedback regulation of pancreatic secretion in man.

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HYDROTHERMICALLY TREATED FULLFAT SOYBEANS IN RAINBOW TROUT (Salmo Gairdnerii R.) DIETS

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Summary

Trial 1: 4 diets were fed for 83 days to rainbow trout initially weighing 27 g per fish. Each diet was fed to triplicate groups of 20 trout. On DM basis the diets contained per kg 300, 200, 100 or 0 g fish meal (FM) and 0, 200, 400 or 600 g hydrothermically treated soybeans (SB) respectively. The diets had similar concentrations of protein, lysine, methionine and fat. Intake and weight gain were highest in groups fed diets containing 200 g FM and 200 SB per kg. Feed conversion ratio was lowest for diets containing 0 or 200 g SB and increased significantly with greater proportions of SB.
Trial 2: Supplementing inorganic phosphate to a diet containing 600 g SB per kg significantly increased intake and weight gain and reduced feed conversion ratio from 1,53 to 1,14 in a trial lasting 42 days.
Trial 3: During 42 days, a diet comprised of SB, gluten, wheat and søybean oil was compared to diet containing 325 g fishmeal and 120 g fish oil per kg. Feed conversion ratio was 1.19 in the vegetable diet and 0,88 in the diet containing fish meal and fish oil.

Introduction

Solvent extracted soybeans may be used as a source of dietary protein for salmonids on the condition that the trypsin inhibitor activity has been reduced by heat and pressure treatment. Results of Reinitz et al. (1978): indicate that hydrothermically treated fullfat soybeans may be used as an ingredient in trout diets. More information on possible limitations will be reported here.

Material and Methods

The fullfat soybean used in the trials described here was treated by a special pressure cooking method of the Meelfabriek Weert (Netherlands). A detailed description of the processing method and parameters of the used fullfat soybean were not available. The TI activity was determined by the method of Kakade et al. (1974).
In trial 1 the trout were reared in a tank system with water recirculation, whereas trout of trial 2 and 3 were reared in a tank system with aeriated water and no water recirculation.

Results and discussion

Trial 1:
The composition of the four diets which were used is shown in Table 1. From diet S0 to S6, the proportion of hydrothermically treated fullfat soybeans was increased from 0 to 600 g per kg diet on a dry matter

basis replacing fishmeal, heat treated wheat and fish oil. The diets were isonitrogenous (46%) and had the same level of fat (16%), lysine and methionine. The level of TI in the different diets was increased by increasing amounts of fullfat soybeans. The heat treatment of the soybean caused a TI level of 2,5 TIU/mg in the soybeans.

Table 1. Composition of the Test Diets and results of Trial 1.

Compounds	g/kg feed DM S0	S2	S4	S6
Fish meal	300	200	100	0
Soybeans	0	200	400	600
Wheat (heat treated)	370	283	198	112
Feather meal (hydrol.)	200	199	196.7	194.4
Wheat gluten	0	12	24	36
Fish oil	110	85	58	32
Vit. Premix	11	11	11	11
Cholinchloride	9	9	9	9
HCl-Lysin	0	0	1.3	2.6
DI-Methionin	0	1	2	3
Initital weight g/fish	27	27	27	27
Weight gain g/fish	148^{b}	156^{a}	134^{c}	82^{d}
Feed intake g/fish	141^{b}	154^{a}	144^{c}	106^{d}
Feed / gain	0.95^{a}	0.98^{a}	1.08^{b}	1.29^{c}

Means followed by the same letter are not significantly different (P=0.05).

For 83 days, each of the diets was fed to three parallel groups of 20 trout. Average initial weight was 27 g per fish. Diets were fed to satiation two times daily. Feed intake and weight gain were highest for diet S2 and lowest in diet S6 (Table 1). No significant difference of feed conversion ratios was found between the two diets containing either 0 or 200 g per kg soybeans. Increasing the proportion of soybeans to 400 and 600 g per kg significantly increased feed conversion ratio. The level of TI in the diet must have been reduced sufficiently by the hydrothermical treatment as no fish died during the whole experiment.

Table 2. Body composition, Productive protein value (PPV) and Gross energy efficiency (k-tot.) of the trout.

Crude protein	g/kg	149^{b}	155^{a}	153^{a}	160^{c}
Ether extract	g/kg	139^{a}	131^{b}	125^{c}	109^{d}
PPV	%	34^{a}	33^{a}	30^{b}	26^{c}
k-tot.	%	43^{a}	45^{a}	39^{b}	31^{c}

Means followed by the same letter are not significantly different (P=0.05).

Increasing proportions of soybeans in the diet caused proportional declines in fat content and a tendency towards higher protein contents in the body composition of the trout (Table 2). Corresponding to feed conversion ratio, efficiencies of utilization of dietary protein (PPV) and energy (k-tot.) were similar for diets S0 and S2, but lower for S4 and

even more for S6.

Trial 2:

In diet S6 of trial 1 most of the dietary P must have been phytate-P. This poorer availability of this source of P may have contributed substantially to the poorer performance of fish fed diets containing high proportions of soybeans. In a trial lasting 42 days, duplicate groups of 15 trout weighing on average 24 g, were fed diet S6 either unsupplemented or after addition of 33.3 g dicalciumphosphate per kg dry matter. Table 3 shows that the addition of inorganic phosphate increased intake and weight gain and decreased feed conversion ratio substantially.

Table 3. Results after P-supplementation to diet S6.

		S6+P	S6
Initial weight	g/fish	24.3	24.4
Weight gain	g/fish	27.1	15.8
Feed intake	g/fish	30.4	23.8
Feed / gain		1.14	1.53

Trial 3:

A diet composed exclusively from ingredients of plant, inorganic or synthetic origin was compared to a diet containing 325 g fishmeal and 120 g fish oil per kg dry matter. The composition of both diets is presented in Table 4. The two diets were fed to triplicate groups of 15 trout of an average initial weight of 19.4-19.9 g/fish for 42 days.

Table 4. Composition of test diets and results by using only vegetable ingredients in diets for rainbow trout.

Compounds g/kg Feed DM		vegetable diet	control
Soybeans		600	0
Fish meal		0	325
Wheat gluten		200	165
Wheat (heat treated)		98	370
Soybeanoil		40	0
Fish oil		0	120
Vit. Premix		20	20
Dicalcium phosphate		34	0
HCl-Lysine		5	0
DL-Methionine		3	0
Initial weight	g/fish	19.4[a]	19.9[a]
Weight gain	g/fish	29.4[b]	50.7[a]
Feed intake	g/fish	35.4[b]	44.8[a]
Feed / gain		1.19[b]	0.88[a]

Means followed by the same letter are not significantly different (P=0.05).

Trout fed the vegetable diet showed lower weight gain, feed intake and increasing feed conversion ratio. The performance level of the fish meal diet was not reached, although the vegetable diet was supplemented with amino acids and inorganic phosphate. Digestibility trials which were conducted after these experiments showed lower digestibility of protein and fat in fullfat soybeans compared to fish meal which was affected to some extent by the rest of the TI activity in the soybean.

Conclusions

Sufficiently hydrothermically treated fullfat soybeans may replace a substantial part of fish meal in diets for rainbow trout. A supplementation of inorganic phosphate may become necessary if plant materials are the predominant source of dietary P. Diets containing no ingredients of animal origin may produce a respectable performance in trout, though still inferior to diets containing fish meal.

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DISCUSSION ON TRYPSIN INHIBITORS

Chairman: B.O. Eggum
Reported by: I.E. Liener

This session of the workshop was opened by the presentation of a main paper which served to give an overview of the chemical properties and nutritional significance of protease inhibitors. A point in this paper which served to generate the most discussion was the carcinogenic effect which the long-term feeding of raw soy flour has on the rat pancreas. The speaker emphasized the fact that this effect has only been observed with the male Wistar rat and not in the mouse and hamster, and even then only under the most rigorous condition, namely the feeding of raw soyflour over a very long period of time (15-18 months), which is equivalent to almost the entire lifetime of the rat. These are conditions which are not likely to obtain under the more normal situation where processed rather than raw soy protein is consumed for much shorter periods of time. Furthermore, there is no conclusive evidence that the trypsin inhibitor is in fact the causative agent. Paradoxically, the Bowman-Birk inhibitor (BBI) has been shown to inhibit the transformation of tissue cell cultures exposed to x-ray irradiation. BBI has also been found to reduce the growth of x-ray induced mammary tumors in mice. The question was raised as to how this in vivo effect can be explained since BBI has been shown to be very poorly absorbed into circulatory system. The speaker could only surmize that BBI may be acting in some indirect fashion similar to the negative feedback mechanism which controls pancreatic function of animal consuming diets containing trypsin inhibitors.

The four short papers and several of the posters dealt with the variation in response to protease inhibitor by different animal species. The trypsin of the rainbow trout appears to be particularly sensitive to the trypsin inhibitors of soybeans which cautions against the use of improperly processed soybean meal for feeding these fish. In response to a question as to whether the pancreas of these fish showed any signs of enlargement, the speaker pointed out the technical difficulties in obtaining this kind of information, but efforts are continuing to overcome this problem. Allthough the feeding of raw soyflour to young guinea pigs markedly depresses growth, there was no evidence of any pancreatic hypertrophy or enhanced secretion of enzymes. In the discussion that followed, it was suggested that this failure of the pancreas to adapt to trypsin inhibitor may account for the fact that the guinea pig is more sensitive to trypsin inhibitors compared to the rat and chick where pancreatic adaptation does occur.

Unlike the rat and chick, the feeding of soybean preparations containing trypsin inhibitor to monkeys for as long as five years produces no adverse effects on the pancreas, other major organs, or the blood chemistry. These findings were compared with the results of short term experiments with human subjects where it was found that the introductions of BBI into the duodenum caused an increase in the enzyme content of the pancreatic juice. This observation provides evidence that a negative feed back mechanism exists in humans. In discussing what appeared to be a difference in pancreatic response between monkeys and man, it was pointed out that the negative feedback mechanism does not necessarily result in pancreatic

hypertrophy, as exemplified by the pig and calf.

The several posters on the trypsin inhibitor in peas illustrate the difficulty in attempting to elucidate its nutritional significance. In experiments with chicks, no significant correlation could be found between the trypsin inhibitor content of different cultivars of peas and apparent protein digestibility. Although heat treatment increased protein digestibility, the latter was not correlated with a decrease in trypsin inhibitor activity. In a more limited study with pigs involving only two varieties of peas having high and low trypsin inhibitor content, apparent digestibility was reduced with diets containing the variety with the higher activity. The significance of these findings in the pig experiment was questioned since only two samples of peas were compared. It was generally agreed that factors other than trypsin inhibitor content may account for the differences in digestibility observed with different varieties of peas.

Several posters dealt with attempts to improve the assay for trypsin inhibitor activity. Several modifications of the Kakade procedure were proposed to simplify this assay and increase its sensitivity, including an extension of the incubation period from 10 to 45 minutes. A question was raised as to whether a linear response of enzyme activity was still obtained despite a long incubation period of 45 minutes; the answer was in the affirmative. The posible interference by tannins was also questioned, but the speaker indicated that this point has not yet been investigated. In an investigation involving various extraction methods of legumes and cereals and the effect of such extracts on ox, porcine, and rat trypsins, it was concluded that extraction at pH 2 with added pepsin followed by incubation with porcine trypsin may give the best evaluation of the antinutritional effects of trypsin inhibitors in pig diets. It was pointed out in the discussion that followed that this conclusion needs experimental verification. Also questioned was the possible effect which pepsin digestion might have on the Kunitz trypsin inhibitor which is known to be inactivated by gastric juice. To meet the need for accurately measuring residual trypsin inhibitor activity, a method was described which involves the application of affinity chromatography for the selective determination of protein-type trypsin inhibitors. Application of this method to the determination of trypsin inhibitor content of pea flour revealed that only 7% of the extracted trypsin inhibitor activity was due to protein-type inhibitors. These results provide a further indication of the problem associated with an evaluation of the nutritional significance of the trypsin inhibitors occuring in peas. To what extent a similar problem may exist with respect to other legumes remains to be determined.

Session tannins

DIETARY EFFECTS OF TANNINS, VICINE AND CONVICINE

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Introduction

Faba beans (Vicia faba L.) have been used for centuries as a foodstuff for man and animals without evidence of adverse effects. While this observation indicates that this protein-rich, grain legume is free of significant amounts of nutritional inhibitors, several inhibitors are known to be present. Some of these factors may affect attainment of maximum efficiency of feed utilization under intensive animal production. Antinutritive factors that are present in faba beans include condensed tannins, vicine and convicine, phytates, protease inhibitors, lectins (hemagglutinins), hypocholesterolemic factor (saponins) and unavailable carbohydrates. Condensed tannins, and vicine and convicine appear to be the two groups of antinutritional factors of greatest concern in faba beans (Marquardt & Bell, 1988). Condensed tannins are also found in other legume crops such as beans (Phaseolus vulgaris) and certain dark pigmented cultivars of peas (Pisum sativum) (Price & Butler, 1980; Marquardt & Bell, 1988) and cereal crops including barley (Hardeum vulgare) and sorghum (Sorghum bicolor) (Price & Butler, 1980).

Condensed tannins (proanthocyanidins) in faba beans appear to be structurally similar but not identical to those in sorghum (Martin-Tanguy et al., 1977; Cansfield et al., 1980) and therefore their biological effects should also be similar. In this review, a considerable amount of information is presented on sorghum tannins as much of the basic work on tannins has been with this grain. Nevertheless, relevant information on faba bean condensed tannins is also presented. A review of tannins in general has been made by Haslam, (1977); tannins in sorghums by Butler, (1982); Butler et al., (1986), Butler (1989), Gupta & Haslam 1979, Mehansho et al., (1987), and Price & Butler, 1980; and in the faba beans by Marquardt & Bell (1989).

Vicine and convicine are glycosides that are found primarily in faba beans. These compounds have been strongly implicated as causative agents in favism, a hemolytic disease in humans and have also been shown to adversely affect metabolism in the laying hen. See Mager et al. (1980) and Marquardt (1989) for detailed reviews.

Definition and nomenclature of tannins

The generally accepted definition of tannins is that they are water-soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins. (Gupta & Haslam, 1979).

Tannins have been grouped into two classes, the hydrolyzable and the

non-hydrolyzable or condensed. Treatment of hydrolyzable tannins with acid or alkali and in certain cases with hydrolytic enzymes (tannase) splits them into sugars and some recognizable phenolic carboxylic acids (eg, gallic acid or ellagic acid). Condensed tannins do not readily break down in this manner, nor do sugars contribute to their overall structure. Both groups of compounds, however, have sufficient phenolic groups to form multiple hydrogen bonds with the substrate.

The condensed tannins are the polyphenols found in "high tannin" sorghum grain or faba beans. Gupta and Haslam (1979) proposed that sorghum tannins is an oligomer of five to seven flavan-3-ol units (Fig. 1). In strong acids flavan-3-ol oligomers depolymerize to yield monomeric anthocyanidin pigments (cyanidin in the case of sorghum tannins) so they, like faba bean tannins (Cansfield et al., 1980), are designated as proanthocyanidins. In addition to these oligomers, many monomeric and dimeric flavanols have been reported in sorghum and some of these form anthocyanidins in acid.

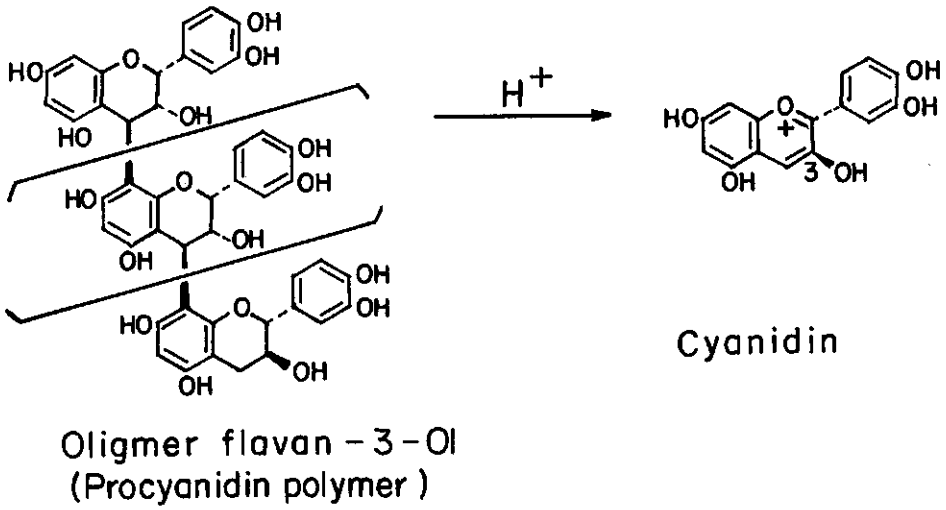

Fig. 1. Structure and decomposition product of procyanidin polymer from sorghum.

Assays for tannins

Many different assays have been utilized to estimate tannins, none of which are completely satisfactory. Assays such as the Folin-Denis (Folin & Denis, 1912) and Prussian blue (Price & Butler, 1977), which detect phenolic groups and other reductants such as ascorbate, have been used. These assays give little qualitative information about tannin content unless combined with selective extraction procedures (Price & Butler, 1977).

Condensed tannins can be assayed as proanthocyanidins by their oxidative depolymerization to anthocyanidin pigments, promoted by Fe^{+3} in hot acid (Porter et al., 1986). The most widely used assay for sorghum and faba bean tannins is the vanillin method (Burns, 1971). Its

main advantage is that only plant phenolics which give a positive test are the condensed tannins and some flavanoids. For convenience, catechin, a monomeric flavan-3-ol unit of condensed tannins, is often used to standardize the assay rather than purified condensed tannin, although this leads to a considerable overestimation of tannin content (Price et al., 1978). Revised procedures for the vanillin assay as proposed by Price et al. (1978) gave excellent reproducibility. A second modification of the method which involves carrying out the reaction of flavanols with vanillin in the presence of glacial acetic acid rather than methanol provides a basis for measuring the molar concentration of proanthocyanidin oligomers rather than the weight percent (Butler et al., 1982). Therefore, for estimation of the tannin content of a sample the vanillin assay is the most useful of several methods tested (Earp, 1981) whereas a combination of this method and that of Butler et al. (1982) provides a basis for estimating degree of polymerization of tannins.

Protein precipitation assays have been developed which are dependent on tannin binding to protein. Generally these assays do not distinguish between condensed and hydrolyzable tannins but give good correlation with nutritional quality of sorghum (Bullard et al., 1981). Protein precipitation assays are facilitated by use of a protein labeled with either a radioisotope (Hagerman & Butler, 1980) or a blue dye (Asquith & Butler, 1985) which minimizes interference from plant pigments.

Mehansho et al. (1987) concluded that no assay for tannins provides an unequivocal value for tannin content. Most procedures only measure tannins indirectly in an extract of plant material and only those tannins that are extractable. Often the amount of unextractable tannins is greater than the amount of readily extractable tannins (Gupta & Haslam, 1980). Even with optimized procedures, there is no assurance that all of the tannin has been extracted.

Effect of water and protein on the extractability of tannins

Water appears to have a profound effect on the extractability of sorghum grains. Butler (1982) reported that germinating grain had virtually no extractable tannins but after drying, the amount of extractable tannins was much greater. Price et al. (1979) reported that the assayable tannin content of several high-tannin, high-moisture (>16% water) sorghums increase as much as 190% in drying. Butler (1982), however, reported that the addition of a limited amount of water (15% of seed weight) to whole grain just before grinding, to ground grain just after grinding, or to the methanol extract had no effect on assayable tannins. Larger amounts of water added to ground grain, as in preparation of a batter for cooking, extract protein and tannin and permit formation of insoluble tannin-protein complexes from which tannin can not be extracted (Price et al., 1980).

Studies in our laboratory have demonstrated that tannins can not be detected in ground whole faba beans using the Burns (1971) assay but are readily detected in the testa (hull) of the bean. These results would suggest that tannins from the hull of faba beans interact preferentially with protein from the endosperm of the bean during the extraction procedure, forming a protein-tannin complex which can not be detected (unpublished results). Additional research with faba beans should be carried out to establish the nature of interaction between protein,

moisture and condensed tannins and the corresponding effects on the recovery and yield of assayable tannins.

Characteristics of tannin-binding proteins

The relative affinity of condensed tannins for different proteins vary by almost four orders of magnitude (Hagerman & Butler, 1981). A protein with high affinity such as gelatin is selectively bound by tannin in the presence of a 100-fold excess of another protein such as lysozyme. Proteins with high affinity for tannins generally have a high molecular weight, an open loose structure, high proportion of hydrophobic amino acids, and a high proline content (Hagerman & Butler, 1981). Proteins with low affinity for tannins are generally small, compact, and cross-linked with disulfide bonds. The characteristic that best correlated with affinity for condensed tannin is its content of proline. The importance of proline is presumably due to its inability to fit into the α-helix, which leads to a loose, open structure readily accessible to tannins and the formation of hydrogen bonds with the phenolic groups of tannins (Hagerman & Butler, 1981).

Dietary effects of sorghum polyphenolics

Condensed tannins in sorghum grains affect the palatability and the nutritional value of the diet and may have a direct toxic effect on the animal. Tannins are noticeably astringent, a characteristic which has been cited for the repellent properties of high tannin sorghums. However, food consumption in ruminants does not appear to be altered by high tannin sorghums. It has also been suggested that humans have a unique "taste for tannins" (Butler, 1989).

Nutritional studies have consistently shown reduced weight gains and feed efficiencies in rats (Featherstone and Rogler, 1975; Mehansho et al., 1983), mice (Asquith et al., 1985) and hamsters (Mehansho et al., 1985) fed increasing concentrations of high-tannin sorghum. Similar results were obtained with livestock such as poultry (Rostagno et al., 1973; Armstrong et al., 1974) including diminished egg production (Sell and Rogler, 1984) and swine (Cousins et al., 1981).

Polyphenols have a somewhat more complex effect on ruminant nutrition (Kumar & Singh, 1983). Sorghum tannin may adversely depress weight gains, feed efficiencies, and metabolizable energy values in the ruminant but the effects appear to be relatively less intense than in non-ruminants. In some cases dietary tannins can actually have a beneficial effect on ruminant nutrition by complexing with dietary protein and protecting it from degradation by the rumen (Zelter et al., 1970) or by reducing the potential for bloat when pasturing on legumes (Reid et al., 1974). These effects have been reviewed for ruminants (Kumar & Singh, 1983) and monogastrics (Butler et al., 1986).

In addition, sorghum polyphenols exhibit certain toxic effects which seem to require absorption from the digestive tract. Chicks fed on high-tannin diets develop leg abnormalities, especially an outward bowing of the legs with swelling at the hock joints (Elkin et al., 1978). This condition appears to affect the organic matrix rather than the mineral component of the bone (Armstrong et al., 1974). Other examples of metabolic effects that require absorption of tannins include the observation that chicks, but not rats, fed sorghum diets develop

elevated levels of liver microsomal UDP-glucouronyltransferase, an enzyme involved in metabolic detoxification of phenolic materials (Sell & Roglers, 1983).

Studies with 125-I labelled sorghums tannins have demonstrated that 20% of the radioactivity was recovered in the urine, 60% in the feces and 20% remained in the blood and other tissues. It is not clear, however, if the active components are associated with the polymer tannins or the low molecular weight flavonoids (Butler, 1989).

Mechanism of the antinutritive effects of sorghum polyphenols

Although tannins are known to inhibit almost every enzyme tested, in vitro experimental results would suggest that they have relatively little effects on enzymes in vivo (Butler, 1989). Dietary condensed tannins have little effect on the morphology of the rat gut or on mucin excretion with no evidence for interference with nutrient uptake (Sell et al., 1985). Butler et al. (1986), suggested that since the dietary effects of tannins can be overcome by supplementation with additional dietary protein, particularly protein that has a high affinity for tannins, or with a non-nutritive tannin-binding agent, the major dietary effect of condensed tannin within the digestive tract must be the formation of less digestible complexes with dietary proteins rather than direct inhibition of digestive enzymes. This conclusion is in agreement with the many reports that protein digestibility is reduced in animals fed a tannin-containing diet.

Adaptive response of animals to tannins

Weanling rats when fed a diet containing a high tannin sorghum grain undergo an adaptation period in which they initially lose weight (Butler, 1986). After approximately four days they begin to gain weight but not as fast as rats fed the tannin-free sorghum. During this adaptation period, the parotid glands of the rat undergo a dramatic hypertrophy accompanied by a large increase in their content of unique proline-rich proteins (Mehansho et al., 1983). Maximal (3 to 5 fold) stimulation of gland weight and proline-rich protein synthesis (approximately 12 fold) coincides with initiation of body weight gain. Dietary tannins induce the synthesis of four proteins. The protein with a molecular weight of 38,000 daltons has a high molar content of proline (32%), glutamate (25%) and glycine (24%), (Mehansho, et al., 1983). These proteins have a very high relative affinity for condensed tannins which suggest they function as tannin-binding proteins (Mehansho et al., 1983). Their rapid and specific induction by dietary tannin suggest they serve to defend the animal against tannins (Butler et al., 1986).

The parotid glands in hamsters in contrast to rats and mice do not undergo hypertrophy when they are fed a diet containing high tannin sorghum (Mehansho et al., 1987). The failure to induce defensive secretion of salivary proteins is probably the basis for the high sensitivity of hamsters to dietary tannins. Chicks also appear to have a somewhat different defense mechanism against tannins than that of rats or mice as tannins do not seem to induce salivary secretions (Butler, 1989).

Detoxification of sorghum condensed tannins

Several methods have been employed to reduce the toxic effect of sorghum tannins including dehulling, different chemical treatment, water soaking, and supplementation of the diet with specific nutrients.

Dehulling is an effective means of reducing the tannin content of sorghum (Eggum et al., 1983) as these compounds and related polyphenols are deposited in the testa layer just under the seed coat (Morrall et al., 1981).

Soaking the grain in aqueous sodium hydroxide followed by washing with water reduced the assayable tannins, improves the nutritional value of the grain and its in vitro protein digestibility. Similarly, imbibing whole grain with aqueous solutions of alkalies particularly dilute ammonia (0.1M) for 12 hours followed by drying of the grain improves the nutritional value of the grain (Butler, 1989).

The simplest detoxification treatment, although inconvenient, is "reconstitution"; addition of water to bring the moisture content up to near 40% followed by anaeratic storage for two to three weeks (Reichert et al., 1980). This procedure has been shown to reduce the assayable tannin content of the grain and improve its nutritional value when fed to rats (Reichert et al., 1980), chicks (Pearson-Priller, 1985) and pigs (Mitaru et al., 1984).

Several heat treatments, in contrast to those obtained with faba bean condensed tannins, appear to be rather ineffective at improving the nutritional value of sorghum grains. This includes boiling high-tannin sorghum (whole) grain, treatment of the whole grain with steam or dry heat, autoclaving ground sorghum grain and cooking ground high-tannin sorghum grain as a batter (Butler, 1989).

The most effective treatment appears to be that associated with soaking of the seeds with a dilute solution of formaldehyde (Daiber and Taylor, 1982). Formaldehyde is well known to react with phenols such as tannins to produce high molecular weight resins.

Chemical treatments to "detoxify" the tannin in sorghum grain are more effective when carried out on whole rather than ground grain. In ground grain, the tannins become accessible to the protein of the seed so the formation of tannin-protein complexes occurs to a much greater extent than is possible in the intact seed (Price et al., 1980).

The additions of compounds to the diet that have a high affinity for tannins overcomes the effect of tannins in sorghum grain. Included in the group are polyvinylpyrrolidone (Armstrong et al., 1973), polyethylene glycol (Ford & Hewitt, 1979b) and proteins, particularly gelatin (Mehansho et al., 1985). The beneficial effects are due to the binding of dietary tannin so that it has little effect in the digestive tract. In contrast, to the above compounds, crystalline amino acids when added at the same concentration as gelatin were not helpful in overcoming the effect of tannin. Methionine supplementation of a sorghum-soybean meal diet completely overcomes the growth depression due to tannin. It does not, however, alter the reduced protein digestibility observed in chicks fed high tannin sorghum or the tannin induced leg problems (Elkin et al., 1978).

Influence of condensed-tannins on the nutritional value of faba beans

Although tannins from faba beans are generally similar to those from

sorghum (Martin-Tanguy et al., 1977; Cansfield et al., 1980) they appear to be differentially affected by heat. Autoclaving and steam treatment of sorghum, as discussed previously, has no effect on the nutritional value of sorghum whereas heat treatment of faba beans or faba bean tannins as discussed subsequently, completely neutralizes their effect. It is not clear, however, if these differences are attributable to real differences between tannins from the two types of grains or if experimental conditions were sufficiently different to account for these effects. An important difference between sorghum and faba beans is that the concentration of tannins is much greater in sorghum than in faba beans and therefore the corresponding effects on digestibility of protein is much greater (Ford & Hewitt, 1979 a & b). General reviews of the nutritive value of faba beans as a feedstuff (Blair, 1977) and the use of peas, lentils, faba beans and chick peas as a human food and an animal feedstuff (Marquardt and Bell, 1988) have been presented.

The principal thermolabile antinutritional factor in faba beans has been shown to be condensed tannins (Marquardt et al., 1977; Ward et al., 1977). Picard (1975) and Bond (1976) were the first to demonstrate that improved in vitro digestibility of nutrients could be obtained through the use of tannin-free faba beans and that the tannins were located in the seed-coat (hulls on testa) of the bean. They also reported that the seed-coat of all white-flowered varieties of faba beans was free of tannin. More recent studies on the variation of tannin content of faba beans have been carried out by Carbrera and Martin (1986). Some of the limitations of these assays have been discussed. Marquardt et al. (1978 a & b) reported that the elution profiles on Sephadex LH-20 of polyphenolics from tannin-free and tannin-containing cultivars were markedly different. Also the hull content of the seed from the tannin-free cultivar was greater than that from the tannin-containing cultivar as were the percent content of tannins and lignins.

Marquardt et al. (1977) and Ward et al. (1977) were able to produce a concentrated form of faba bean condensed tannins by freeze-drying a water-extract of faba bean hulls. This material was further purified by fractionating an acetone-water solution using LH-20 chromatography. This procedure yields large quantities of relatively pure condensed tannins that can be readily utilized for nutritional studies. Incorporation of the isolated tannins into a chick diet, depressed growth rate, efficiency of feed utilization and liver size. The retention of dry matter, protein and amino acids but not fat were also decreased. Crude fiber, in contrast, yielded negative retention values with increasing concentration of dietary tannins indicating the formation of a tannin-protein complex which was included in the crude fiber fraction. In another study, Marquardt and Ward (1979) demonstrated that the correlation (r) between the amount of tannins added to the diet and chick performance were : -0.70 for feed intake,-0.90 for weight gain, -0.97 for efficiency of feed utilization, -0.98 for dry matter retention, -0.99 for protein retention and 0.96 for fat retention. Tannins also depressed in vitro digestion by rumen microorganisms of both dry matter and protein (Buckley et al., 1983). The composition of faba bean condensed-tannins have been reported (Martin-Tanguy et al., 1977; Cansfield et al., 1980).

Neutralization of the effects of faba bean tannins

The effects of tannins in faba beans can be overcome by the use of tannin-free cultivars of faba beans (Bond, 1976; Martin-Tanguy et al., 1977; Marquardt et al., 1978a; Marquardt and Ward, 1979), the addition to the diet of specific tannin-binding agents such as polyvinylpyrrolidone (Marquardt et al., 1977) or polyethylene glycol (Ford and Hewitt, 1979b), treatment of faba beans with an ammonia solution (Ford and Hewitt, 1979b), dehulling of faba beans (Edwards and Duthie, 1973; Bond, 1976; Edwards, 1977; Ward et al., 1977; Marquardt et al., 1978a) and various heat-treatment procedures.

Heat treatment procedures that improve the nutritional value of faba beans included autoclaving of the ground beans (Wilson and McNab, 1972; Edwards and Duthie, 1973; Edwards, 1977; Marquardt et al., 1976; Campbell and Marquardt, 1977), extrusion (Marquardt et al., 1976), infrared radiation (McNab and Wilson, 1974), to some extent, steam pelleting (Marquardt et al., 1976), soaking ground beans with an equal volume of water for 24h (Rodriguez-Castanon, 1988) and microwave treatment of ground grain containing 18% added water (Marquardt et al., 1976). Microwave treatment of dry faba beans (90% dry matter) in contrast, did not improve the nutritional value of faba beans.

Heat treatment of purified tannins in the absence of other nutrients, of ground faba beans or of a diet containing added tannins, resulted in improved animal performance and an increased retention of protein and amino acids (Marquardt et al., 1977; Marquardt and Ward, 1979). The results suggest that heat treatment even in the presence of protein neutralizes the effects of faba bean tannins and thereby prevents their interaction with the protein component of the diet. The net effect is an increased degree of digestion and absorption of protein and amino acids.

Performance of swine, rats, chicks and ruminants as affected by the consumption of tannin-containing faba beans

In a general review on the utilization of faba beans by hogs Blair (1977) concluded that at least ten percent faba beans can be included in the diet of growing and finishing hogs with a lower level being advised for breeding swine. Aherene et al. (1979) reported progressive decreases in weight gain and efficiency of feed utilization as the dietary concentration of faba beans was increased from 0 to 30% whereas Mateos and Puchal (1981) reported that the use of up to 20% faba beans as a source of protein for starting pigs did not impair pig performance. Liebert and Gebhardt (1983) reported that the digestibility of crude protein of a tannin-free cultivar of faba beans was approximately 3 to 4 percentage units (83.5%) higher than that of a tannin-containing cultivar. Presumably tannins in faba beans are partially responsible for a reduced level of performance in pigs fed faba beans.

Comparative studies with the rat and the chick have demonstrated that heat treatment improved weight gain and feed utilization in both species with the later showing the greatest response. (Marquardt et al., 1974). Ford and Hewitt (1979b) fed both sorghum and faba bean containing diets to both rats and chicks. They concluded that the effect of sorghum was much greater than that of faba beans and that the chick was much more sensitive to the effects of tannins than the rat. It might be

hypothesized that chicks, because of the more elementary nature of their digestive tract, would also be more sensitive to the effects of tannins than swine. Also, they may secrete different amounts of the tannin-binding protein.

Ingalls et al. (1980) studies the nutritive value of faba beans in the diet of young Holstein calves and lactating dairy cattle. Replacement of soybean meal (10% in the diet) with faba beans (24% in the diet) had no effect on feed intake, milk yield or milk composition. Similar results were also obtained with calves fed either faba beans or soybean meal. Although, Ingalls et al. (1980) was not able to detect the presence of an antimetabolite in faba beans in feeding trials with young ruminants, in vitro studies have indicated that tannins markedly depress the digestibility of the hull portion of faba beans but not the cotyledon portion of the bean (Bond, 1976; Marquardt et al., 1978a; and Buckley et al., 1983).

Effect of faba bean tannins on energy and amino acid availability values in poultry.

Numerous studies have been carried out on the feeding value of faba beans for poultry. In an extensive study, Campbell and Marquardt (1977) reported that chicks responded to increasing levels of raw faba beans (up to 84% of the diet) by a marked increase in feed consumption but that the increase in consumption was not sufficient to maintain a constant body weight. Heat treatment (autoclaving 121°C for 20 min) markedly altered this response presumably in part due to neutralization of the effects of tannins. Marquardt and Ward (1979) reported that the retention of protein for chicks fed tannin-free and tannin-containing faba beans was 83.7 and 73.2%, respectively, a difference of 10%. Heat treatment improved the retention of protein obtained with the tannin-containing cultivars by 12% and that of the tannin-free cultivars by 7%. These results indicate that both tannins and some other factor affects the digestibility of amino acids in faba beans.

The metabolizable energy (ME) value of faba beans also appears to be affected by antimetabolites in faba beans, particularly tannins. Edwards and Duthie (1973) reported that autoclaving faba beans for 30 min at 108°C improved ME values from 2350 to 2440 kcal/kg. Edwards and Duthie (1973) further found that MEn value of a sample of hulled beans was 2280 kcal/kg, while the cotyledon had values of 3033 (kcal/kg). Heat treatment further improved the MEn from 3070 to 3330 kcal/kg. Rodrigues-Castanon (1988) using a modification of Sibbalds (1986) method obtained the following TMEn (kcal/kg dry matter) values, 2540, raw faba beans; 2890, autoclaved faba beans; 3284, dehulled, autoclaved faba beans and 0, autoclaved hulls. Tannins seem to greatly delay the passage of digesta as raw hulls were still being excreted after 96h whereas autoclaved hulls were essentially cleared within a 48h period. These results would suggest that caution should be used when the Sibbald (1986) method is utilized to study the effect of tannins in chickens and that excreta collection periods should be increased from 48 to 96 h.

Faba beans in the laying hen - effect of vicine and convicine

Campbell et al. (1980) demonstrated that egg weight declined whereas Haugh units increased as the level of faba beans in the diet increased.

Further studies by Olaboro et al. (1981) and Muduuli et al. (1982) have demonstrated that egg size depression in laying hens was caused by two thermostable compounds (vicine and convicine) which are present in the cotyledon of the bean. These compounds also increase yolk fragility, increase the number of blood spots in the egg, decrease the fertility and hatchability of the eggs, elevate the concentration of plasma lipids and lipid peroxides and increase the degree of erythrocyte hemolyses.

Vicine and convicine are hydrolyzed by intestinal microflora (Frohlich & Marquardt, 1983) to highly reactive free-radical generating compounds divicine and isouramil (Albano et al., 1984). These compounds have been strongly implicated as the causative agents in favism (Mager et al., 1980; Arbid and Marquardt, 1988; Marquardt, 1989) a hemolytic disease in humans (Mager et al., 1980).

These free-radical generators may also cause other adverse effects including lipid peroxidation, altered fat and mitochondrial metabolism and possibly diabetes (Marquardt, 1989). Some of the adverse effects may be neutralized by increasing the concentration of free-radical scavenging compounds in the diet such as vitamin A, C and E, and through the use of chelating agents such as EDTA or desferrioxamine (Muduuli et al., 1982; Marquardt & Arbid, 1988). The adverse effects of these compounds can also be reduced or eliminated by developing glycoside-free cultivars of faba beans or by using processing techniques that selectively extract or hydrolyze the compounds (Marquardt, 1989).

Vicine and convicine also appear to have certain beneficial properties including the prevention of cardiac arrythmia and under certain conditions are able to inhibit the growth of the malaria parasite, Plasmodium falciparum . More detailed reviews have been made (Mager et al., 1980; Marquardt, 1989).

Suggested research on tannins

1. Carry out further nutritional studies in swine and poultry with tannin-free and tannin-containing faba beans to more precisely determine effect of tannins on the performance of domestic livestock particularly with regard to altered amino acid availabilities.
2. Establish if preconditioning of whole ground faba beans at a high temperature and pressure, as occurs in the newer pelleting systems, effectively neutralizes tannins.
3. Carry out additional studies on means by which the effects of tannins can be neutralized. Previous study with both faba beans and sorghum can serve as a basis for these tests.
4. Determine if tannins from faba beans and sorghum differentially respond to heat treatment.
5. Use purified tannins to standardized chemical assays and for in vivo animal studies. These can be readily prepared from faba bean hulls but the similarity of tannins in faba bean hulls and those isolated from hulls would have to be established.
6. Further characterize the physical and chemical properties of faba bean tannins and to compare these with sorghum tannins. Also does moisture content, age or maturity of the bean and extracting conditions affect assay values. Recovery studies with purified tannins should be carried out.
7. Establish if swine, poultry and other domestic animals are able to

adapt to the effects of tannins in a manner similar to that of the rat. Also can animals be prestimulated to secrete the hydrophobic secretary proteins from the parotid glands. The fecal assay procedures as developed by Butlers group may be of value.

8. Determine if faba bean tannins primarily interact with exogenous or endogenous proteins in the adapted and non-adapted animal. Nitrogen prelabelling techniques similar to those used by Dr. W. Sauer, University of Alberta, should assist in solving this problem.
9. Establish more clearly the effects that tannins have on the reliability of the Sibbald TME and TAAA method. Do tannins greatly alter transit time of digesta in the gastrointestinal tract and are longer collection times required.
10. Establish more clearly the relationship between the different assays for tannins and the biological effects of tannins. Which in vitro test best predicts in vivo results?

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EFFECT OF TANNIFEROUS PLANT MATERIAL ON PROTEIN AND CARBOHYDRATE DEGRADATION IN RUMEN FLUID *IN VITRO*

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Summary

Air dried material of 46 different African fodder trees and shrubs (browse) was investigated for various tannin fractions and for its effect on carbohydrate and protein degradation in rumen fluid *in vitro*. Some species were shown to reduce degradation of cellulose, starch, pectin and glucose, and of protein of soybean meal, sesame seed meal, cottonseed meal and peas. The extent of inhibition decreased with increasing incubation time with all these substrates. Maximal inhibition was little affected by the type of carbohydrate, whereas the degradation of protein was dependent on its source. It was found that degradation of browse protein, added soybean meal protein and of cellulose was negatively correlated with total soluble phenolics and with soluble condensed tannins. A negative correlation was also obtained between the content in total soluble tannins and degradation of soybean meal protein and of cellulose, as well as between the content in insoluble condensed tannins and degradation of browse protein.
Key-words: browse, tannins, carbohydrates, protein, *in vitro* degradation, inhibition.

Introduction

The nutritional value of African browse may be reduced due to plant phenolics including tannins. So far, chemical and *in vitro* parameters allowing the prediction of the nutritional value of tanniferous feedstuffs are not available. Plant species from East and West Africa were investigated for their content in phenolics and their effect on protein and carbohydrate degradation in an artificial rumen.

Material and Methods

Chemical analyses: Air dried plant material (46 species) was examined. Total soluble phenolics including tannins were assayed gravimetrically after extraction with aqueous acetone as described by Reed et al.(1985). Condensed tannins were determined photometrically in the same extract (soluble) and in the neutral detergent fibre fraction (NDF, insoluble) after heating with n-butanol/HCl according to Reed (1986). As a standard a condensed tannin fraction was prepared from *Combretum collinum* by affinity chromatography. Total soluble tannins were determined photometrically with Folin-Ciocalteu reagent in an extract before and after treatment with hide powder as described by Diagayete & Huss (1982). The extract was prepared by incubating plant material in a buffer solution described by Steingass & Menke (1986) at 39°C for 1 h. Gallic acid was used as a standard.

In vitro studies: An artificial rumen system described by Steingass & Menke (1986) was used for the determination of carbohydrate degradation. In this system, gas production is used as a measure for the digestibility of organic matter. Degradation of browse protein and of an added protein source was determined according to Thomsen (1985) (modified). In this nitrogen limited system the gas production is a function of the nitrogen available for rumen microbes. Degradation of added protein was also determined according to Raab et al. (1983). This system is not limited in nitrogen, and protein degradation is calculated from gas and ammonia production.

Results and Discussion

The effect of browse (seven species) on carbohydrate degradation was studied. A marked reduction of gas production from cellulose, starch, pectin and glucose was observed only with *Combretum collinum*, *Acacia nilotica* and *Acacia decurrens*, when 300 mg of plant material and 100 mg of carbohydrate were added per incubation tube. Inhibition decreased with increasing incubation time, suggesting an adaptation of rumen microorganisms to tannins. Inhibition decreased much more rapidly with starch, pectin and glucose compared to cellulose. Maximal inhibition occured at the time of maximal rate of gas production obtained with carbohydrate without browse. There was only a slight effect of type of carbohydrate on maximal inhibition (Fig.1). When 300 mg of the other four species, *Combretum fragrans*, *Cassia sieberiana*, *Diplorhynchus condylocarpon* and *Alchornea cordifolia*, or 100 mg of all seven species were added per tube no or almost no effect on gas production was observed with all four carbohydrates.

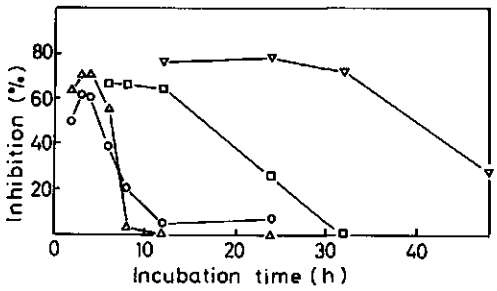

Fig. 1. Effect of incubation time on inhibition of carbohydrate (100 mg/tube) degradation by *Acacia nilotica* (300 mg/tube).
▽: cellulose □: starch ○: pectin △: glucose.

Degradation of protein of soybean, sesame seed, cottonseed meal, and peas was examined with four tanniferous species. With *Acacia sieberiana*, *A.seyal* and *C.fragrans* protein degradation was between 78 and 20% of the control after an incubation time of 24 h (Table 1). During further incubation inhibition decreased. As suggested above, this effect may be due to adaptation processes in rumen microbes. In the case of *C.collinum*, which showed the strongest inhibition of protein

breakdown, there was no decline of inhibition with increasing incubation time.

With C.collinum and A.sieberiana, degradation of pea protein was considerably less inhibited than the degradation of the other three protein sources. With A.seyal and C.fragrans, the effect of protein source was less pronounced. Breakdown of gelatin was affected only by C.collinum, but again only to a slight extent (Table 1).

Table 1. In vitro degradation of protein of different sources after 24 h in the presence of various tanniferous plant species expressed as percent of protein degradation without tanniferous material (control). Numbers with different letters within columns are significantly different ($P < 0.05$).

Protein source	Tanniferous species			
	Combretum collinum	Acacia sieberiana	Acacia seyal	Combretum fragrans
Soybean meal	$17.1^{b} \pm 3.7$	$29.4^{b} \pm 1.4$	$60.5^{bc} \pm 7.3$	$52.4^{ac} \pm 2.0$
Peas	$49.9^{a} \pm 2.0$	$51.0^{c} \pm 6.2$	$78.1^{a} \pm 10.0$	$59.5^{bc} \pm 5.7$
Cottonseed meal	$14.1^{b} \pm 4.2$	$29.7^{b} \pm 0.4$	$65.5^{ab} \pm 6.4$	$66.0^{b} \pm 3.6$
Sesame seed meal	$12.8^{b} \pm 5.1$	$19.8^{b} \pm 8.8$	$51.4^{c} \pm 5.8$	$42.1^{a} \pm 7.4$
Gelatin	82	96	129	110

A negative correlation between both total soluble phenolics and soluble condensed tannins, and in vitro parameters such as degradation of plant protein, added soybean protein and cellulose was found for all 46 browse species. Total soluble tannins were negatively correlated with degradation of soybean protein and cellulose. Insoluble condensed tannins had a negative correlation only with degradation of browse protein (Table 2).

Inhibition of carbohydrate and protein breakdown may be due to several modes of action: Complexation of tannins with polysaccharides, substrate proteins, enzymes involved in carbohydrate or protein degradation, and/or with macromolecules of the bacterial cell e.g. of membranes (Konishi et al., 1987).

The results presented in Tables 1 and 2 are in accordance with the general view of specific tannin-protein interactions. The observation that insoluble condensed tannins correlate negatively with degradation of browse protein may be explained by complexation of the two fractions within the plant.

Although significant correlations were obtained with all 46 species there are some species and varieties which show considerable discrepancy between tannin and in vitro parameters. As an example two varieties of Acacia nilotica may be mentioned which are almost identical in their

content in the four tannin fractions and in tannin patterns determined by HPLC but differ markedly in their inhibition of protein and cellulose breakdown. This difference may be due to different contents in secondary metabolites other than tannins.

Table 2. Correlation coefficients (r) between in vitro parameters and tannin fractions of 46 African browse species.
+++ $P < 0.001$; ++ $P < 0.01$, n.s. not significant.

	In vitro degradation of			
	protein of tanniferous plant Thomsen('85)	soybean meal protein Thomsen('85)	Raab et al.('83)	cellulose
Total soluble phenols (gravim.)	- 0.47 +++	- 0.60 +++	- 0.60 +++	- 0.56 +++
Total soluble tannins (photom.)	- 0.24 n.s.	- 0.50 +++	- 0.66 +++	- 0.60 +++
Soluble condensed tannins	- 0.52 +++	- 0.60 +++	- 0.43 ++	- 0.43 ++
Insoluble condensed tannins	- 0.47 +++	- 0.21 n.s.	- 0.09 n.s.	+ 0.04 n.s.

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RELATIONSHIP BETWEEN TANNIN CONTENT AND "IN VITRO" NUTRITIVE VALUE IN SEEDS OF 24 STRAINS OF Vicia faba L.

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Summary

The tannin content of 24 strains of V. faba was determined by the Folin-C. or TI methods proposed by Sjödin et al. (1981) and Ford & Hewitt (1979), respectively. Acid detergent fiber and lignin content, soluble nitrogen and "in vitro" digestibility of dry matter and crude protein were also determined. A high correlation (r=0,93) between the tannin values obtained with both methods was found. The negative effect of tannin content on nutritive value was demonstrated by the correlation of -0.84 (Folin-C. method) and -0.94 (TI method) with "in vitro" dry matter digestibility.
Keywords: Vicia faba L., tannin, analysis, "in vitro" nutritive value.

Introduction

The purification and characterization of tannin present in the grain of faba beans has been widely studied (Marquardt et al., 1977, 1978; Martin-Tanguy et al., 1977; Cansfield et al., 1980). However, there are very few references on the standardization and optimization of the analytical methods in the literature, contrary to other grains like sorghum (Maxon & Rooney, 1972; Price et al., 1978). The need for these types of studies is obvious. Moreover, they should, as Desphande & Cheryan (1985) point out, include a large number of lines as even within the same species the type of tannin may vary.

Between the different methods used for the tannin analysis, we have chosen two for the present study, both methods have been used previously on faba bean and they differ in two important aspects: in their ease and rapidity of application. For these reason, we were particularly interested in comparing and determining their validity to evaluate the nutritional effect of tannin.

Material and Methods

The faba beans were obtained from a genetic breeding program designed to produce lines with low tannin content. Eight randomly chosen lines of white flower with no pigments (WF), 8 of normal flower with yellow spots on the wing petals (NF) and 8 of diffuse pigmentation completely covering the flower (CF), were used.

The methods for acid detergent fiber (ADF) and acid detergent lignin (ADL) were those proposed by Robertson & Van Soest (1977). Soluble nitrogen (SN) was determinated by solution in a buffer pH 6,9 (Verité & Demarquilly, 1978). "In vitro" digestibility of the protein (IVDP) by using HCl-pepsin (AOAC, 1975) and "in vitro" digestibility of dry

matter (IVDDM) by incubating of the sample with rumen juice and HCl-pepsin (Tilley & Terry, 1963). The methods proposed by Sjödin et al. (1981) and Ford and Hewitt (1979) were used for the tannin analysis. The first, uses the Folin-Ciocalteu as specific test for total phenols, but it has been adapted for condensed tannins by using polyvynylpyrrolidone. The second is founded, in the spectrophotometric mesure of the colour produced by treatment of the sample with HCl-Methanol. The results obtained with these two methods will be referred to as Folin-C. and Tannins Index (TI) respectively. The statistical analysis was done with the SAS software package (SAS User's Guide Statistics, 1982).

Results and discussion

Table 1 shows the absorbance values found for each line with the Folin-C. and TI methods together with the chemical and "in vitro" characteristics studied.

Table 1. Chemical and 'in vitro' characteristics of the lines studied.

	Folin-C A_{730nm}	TI A_{535nm}	ADF %MS	ADL %MS	SN %NT	IVDP %	IVDDM %	
VF-4	0,00	0,03	12,9	0,2	75,8	96,7	94,6	
VF-14	0,00	0,03	9,5	0,2	-	97,1	94,0	
VF-15	0,00	0,02	9,7	0,2	78,8	98,1	93,7	
VF-83	0,00	0,02	10,1	0,2	-	97,8	92,8	WHITE
VF-100	0,00	0,03	10,4	0,2	72,2	96,8	93,3	FLOWER (WF)
VF-110	0,00	0,02	10,5	0,3	-	97,1	93,7	
VF-112	0,00	0,02	11,0	0,3	-	96,6	92,7	
VF-164	0,00	0,02	10,3	0,1	78,5	97,5	95,7	
X ± SE	0,00±0,0[a]	0,02±0,0[a]	10,5±0,4[a]	0,2±0,0[a]	76,3±1,5[b]	97,3±0,2[a]	93,9±0,3[a]	
VF-16	0,40	0,58	13,6	0,4	-	94,8	87,2	
VF-23	0,12	0,37	9,9	0,3	-	95,7	90,3	
VF-27	0,19	0,78	15,2	0,5	-	94,5	80,7	
VF-34	0,43	0,70	-	-	61,3	93,8	-	NORMAL
VF-41	0,29	0,58	12,6	0,3	70,7	95,1	86,4	FLOWER (NF)
VF-44	0,26	0,48	-	-	-	94,0	-	
VF-45	0,31	0,79	-	-	59,9	94,3	-	
VF-50	0,22	0,57	11,9	0,4	73,1	94,7	86,5	
X ± SE	0,27±0,0[b]	0,60±0,1[b]	12,6±0,9[b]	0,4±0,0[b]	66,2±3,3[b]	94,6±0,2[a]	86,2±1,6[b]	
VF-59	0,42	0,81	10,9	0,5	-	95,4	85,6	
VF-91	0,41	0,78	12,0	0,4	-	94,5	84,4	
VF-114	0,52	0,90	12,3	0,6	63,2	93,9	85,6	
VF-116	0,46	0,93	12,8	0,4	57,2	95,5	84,1	
VF-117	0,46	0,80	12,0	0,5	63,8	94,8	83,8	DIFFUSED
VF-118	0,31	0,81	11,9	0,6	-	94,9	81,8	FLOWER (DF)
VF-121	0,43	1,13	13,7	0,4	-	93,6	83,4	
VF-127	0,34	0,85	11,9	0,4	64,2	94,9	85,1	
X ± SE	0,42±0,0[a]	0,87±0,1[a]	12,2±0,3[b]	0,5±0,0[b]	62,1±1,7[b]	94,7±0,2[b]	84,2±0,5[b]	

Means in the same column with different letters are statistically different, p<0,05

Both methods show the absence of tannin in the WF lines and the variable presence in the NF and DF lines. The DF lines had the highest tannin

content which is in accord with previous results found by other authors (Cabrera & Martin, 1986). A variance analysis applied to the Folin-C. and TI values demonstrates that both allow the differentiation between the flower colour groups ($p<0.001$) but that it is only possible to differentiate between lines in a given group with TI. The smaller percentage of error in the variance obtained with the TI method (1.35%) when compared to the Folin-C. one (23.16%) shows that TI is more reproducible. This is partly due to the fact that Folin-C. is a more elaborated method and requires a very careful control of the analytical conditions, which give rise to errors. Moreover, TI is a colorimetric method in which visible absorbance takes place without the use of reagents which may react with other components of the sample analysed.

The correlation between tannin content obtained with both methods and characteristics studied (Table 2) confirm the hypothesis that the tannin levels detected affect the chemical and "in vitro" characteristics of faba bean seeds.

Table 2. Correlation coefficients, significance level (p) and number of observations (n).

	Folin-C.	TI	ADF	ADL	SN	IVDP	IVDDM
Folin-C.	1,00	0,93	0,57	0,78	-0,88	-0,84	-0,84
p	-	***	**	***	***	***	***
n	24	24	21	21	12	24	21
TI	0,93	1,00	0,66	0,81	-0,89	-0,87	-0,94
p	***	-	***	***	***	***	***
n	24	24	21	21	12	24	21

** = p(0,01; *** = p(0,001;

Both methods are highly correlated indicating a similar capacity to quantify the same type of tannin. However, the highest correlations are always obtained with TI. The closest correlations were found with IVDDM, being $r=-0.84$ and $r=-0.94$ for Folin-C. and TI, respectively. This is not surprising as the incubation medium has microorganisms and enzymes which have demonstrated the negative role of faba bean tannins (Griffiths & Jones, 1977; Larwence, 1979). Other authors have found a smaller correlation of IVDDM with the Vanillin method (Marquardt et al., 1978; Buckley et al., 1983). Larwence (1979) found a very similar correlation ($r=-0.92$) although he obtained it by taking tannin content, expressed as V/LA (vanillin/leucoanthocyanidin) as the independent variable. It should be pointed out that this measurement entails a much more complex analysis than the one used here.

When the WF lines were excluded, we only found a significant correlation between TI and SN ($r=-0.81$, $p<0.05$) and IVDDM ($r=-0.68$, $p<0.01$). This again confirms the inability of Folin-C. to differentiate between lines within a given group.

We may conclude by saying that TI is appropriate especially in those cases in which a high number of samples need to be analysed, as is the

case with breeding programs given its simplicity, speed and relationship with nutritional effects, as estimated "in vitro" test. However, we are aware of the fact that it needs to be improved and contrasted with other chemical, "in vitro", "in vivo" and even physical-chemical methods such as Near Infrared Reflectance Spectroscopy (NIR),to confirm the results.

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EFFECTS OF RAW AND AUTOCLAVED FABA BEANS (Vicia faba L.) AND FABA BEAN FRACTIONS ON THE INTESTINAL PHYSIOLOGY AND HISTOLOGICAL STRUCTURE IN CHICKS

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Summary

The effects of the inclusion of raw and autoclaved faba beans (RFB and AFB respectively) and faba fractions (cotyledons and hulls) in diets for growing broiler chickens on performance, some digestive parameters and histological structure within gut mucosa were studied. Significantly decreased body weight was observed in those animals fed on diets containing 250 and 500 g/kg of RFB. Birds fed on AFB (500 g/Kg) or autoclaved faba bean cotyledons (AC)(426.4 g/Kg) in the diet showed significantly greater body weight than chicks fed on RFB or raw faba bean cotyledons (RC)(426.4 g/Kg). Dehulling of faba bean seeds produced no significant effect on this parameter, neither did the inclusion of raw faba bean hulls. Significant differences in duodenum, jejunum, ileum and caeca relative lengths, pancreas relative weight, intestinal transit time and plasma zinc concentration of the birds fed on RFB were observed. Birds fed on diets containing AFB or AC showed significantly decreased pancreas relative weight compared with RFB-fed birds. The inclusion of RFB hulls had no effect on these parameters. Dehulling and/or autoclaving of faba beans proved to have no significant effects on duodenum, jejunum, ileum and caeca relative lengths, neither had on plasma zinc concentration. The inclusion of RFB in the diets for growing chickens was shown to produce grave cytopathological changes within pancreas and small intestine mucosa. Birds fed on AFB and AC diets exhibited much more slightly altered intestinal structures as compared with RFB-fed chicks. The results indicate altered intestinal physiology and structure in chicks fed on RFB or RC diets.

Introduction

Faba bean has been one of the most studied specimens of the legume,and considerable interest is being paid to it as a potential indigenous source of plant protein for western Europe. The presence of certain amounts of antinutritional factors (ANF) has been claimed as one of the main reasons for the low nutritional value of these seeds for growing chickens. The aims of the experiments here reported were to study some of the physiological and histological effects observed in chicks fed on RFB as well as the influence of dehulling and/or autoclaving on such parameters.

Material and methods

In Expt. 1, diets contained 0 (control), 250 (C) and 500 (D) g/Kg of RFB. In Expt. 2, diets contained 0 (control), 500 g/Kg of faba beans (diets RFB and AFB), or the amount of cotyledons (426.4 g/Kg) corresponding to the same level of inclusion (diets RC and AC). Diet AC+H contained 426.4 g/Kg of autoclaved cotyledons plus 73.6 g/Kg of raw faba bean hulls. For more details about diets, methods and faba bean ANF analysis see Rubio & Brenes (1988) and Rubio et al. (1988). Intestinal transit ti-

me was measured after a 14 h fast, after this the animals were again offered the food. The period of time lasting from this moment to the first droppings, as detected by visual observation was considered as intestinal transit time.

Results and discussion

Physiology

The significant increases observed in the relative lengths of the intestinal sections of faba bean-fed birds (tables 1 2) can be attributed in part to presence of high amounts of undigested materials into the intestine of the animals.

Table 1. Expt. 1. Growth, relative lengths of intestinal sections, plasma zinc content and intestinal transit time of chicks fed on diets containing 0 (A), 250 (C) and 500 (D) g/Kg of RFB. (0 to 4 weeks)

	A	C	D	SEM
Body weight (g)	705^{a}	637^{b}	580^{c}	18
Relat. lengths (cm/100 g B.W.)				
duodenum	2.9^{a}	3.2^{ab}	3.5^{b}	0.1
jejunum	5.8^{a}	6.7^{ab}	7.3^{b}	0.3
ileum	5.9^{a}	6.8^{ab}	8.1^{b}	0.3
caeca	1.3^{a}	1.5^{ab}	1.8^{c}	0.1
Plasma zinc (ug/dl)	220^{a}	180^{b}	125^{c}	8
Transit time (min)	224^{a}	223^{a}	264^{b}	9

B.W. Body Weight; Means in the same row with different superscript are significantly ($P < 0.01$) different.

The low digestibility of faba bean carbohydrates and proteins has previously been reported (Pritchard et al., 1973; Huyghebaert et al., 1979). In addition, the main faba bean ANF have been reported to exert antienzymatic effects due to phytate and tannins (Griffiths, 1979; Liener, 1986; Thompson, 1986). The increased intestinal transit time (table 1) of those animals fed on faba beans contributes as well to confirm this idea. Heat treatment and the inclusion or absence of the faba bean hulls in the diets have not shown to produce any significant effect on intestinal relative lengths (table 2). This suggests that thermostable cotyledon carbohydrates are the substances mainly responsible for these bowel modifications in those chicks fed on raw or autoclaved faba bean seeds. On the contrary, no significant difference was observed among birds fed on raw (RFB) and heat treated seeds (diets AFB, AC and AC + H)(table 2).

Table 2. Expt. 2. Growth, relative lengths of intestinal sections, plasma zinc content and pancreas relative weight of chicks fed on raw and autoclaved faba bean and faba bean fractions. (0 to 3 weeks)

	O	AC+H	RFB	AFB	AC	RC	SEM
Body weight (g)	474^{a}	355^{bc}	308^{c}	387^{b}	353^{bc}	314^{c}	11
Relative lengths (cm/100 g B.W.)							
duodenum	3.91^{a}	4.61^{b}	4.99^{b}	4.88^{b}	4.67^{b}	5.19^{b}	0.24
jejunum	8.41^{a}	9.66^{ab}	10.09^{b}	9.89^{ab}	9.45^{ab}	11.78^{c}	0.56
ileum	8.32^{a}	9.56^{a}	10.88^{bc}	10.65^{bc}	10.39^{bc}	11.50^{c}	0.53
caeca	1.81^{a}	2.21^{b}	2.46^{b}	2.29^{b}	2.30^{b}	2.48^{b}	0.10
Plasma zinc (ug/dl)	299^{a}	246^{b}	211^{b}	208^{b}	208^{b}	221^{b}	10
Pancreas relat. weight (g/100g B.W)	0.32^{a}	0.34^{a}	0.39^{bc}	0.31^{a}	0.35^{ab}	0.40^{c}	0.01

B.W. Body Weight; Means in the same row with different superscript differ ($P < 0.01$); O (control), AC+H (autoclaved cotyledons plus raw hulls), RFB (raw faba beans), AFB (autoclaved faba beans), AC (autoclaved cotyledons), RC (raw cotyledons).

The significantly decreased plasma zinc concentration in chicks fed on diets containing faba beans (tables 1 2) can not be satisfactorily explained to date. Factors such as food intake, dietary phytate; zinc and calcium x phytate: zinc molar ratios, phytate content of the seeds and tannins appear not to be the most important reasons to explain this effect. Phytic acid is easily hidrolized by autoclaving and we determined no significant differences among treatments with either raw or autoclaved faba beans in the diet. In the other hand, no significant differences were observed among treatments with or without hulls on the plasma content of Zn in the exp. II, thus tannins are not likely to be of great importance in the present circumstances. On the other hand, the marked histological alterations which are very likely to be produced by faba bean lectins (Rubio et al., 1988) and observed in the intestinal mucosa of the animals fed on RFB could greatly contribute to a lower zinc absorption.

Histology

Electron microscopy of the mucosal surface of the jejunum of the birds fed 500 g/Kg of RFB revealed mitochondrial swelling, decreased number of multivesicular bodies, reduction of lisosomes and lipids drops and shortening of microvilli. Nuclei depolarization, hypertrophy of smooth endoplasmic reticulum and dilated Golgi apparatus cisternae were also observed. The pancreas from birds fed 500 g/Kg of RFB showed an irreversible hydropic degeneration in nucleus and cytoplasm, i.e. disorders of intracellular organelles and lack of zymogen granules. The sacules of the rough endoplasmic reticulum were distended, fragmented and filled with a translu-

cent material that can swell the intracellular structures (mitochondrias, Golgi apparatus). The nuclei appeared very translucent and showed few heterochromatic granules. The enterocytes of those chicks fed on diets containing autoclaved faba beans or cotyledons (diets AFB, AC and AC+H) showed a slight mitochondrial swelling. Pronounced strangulations were observed along the microvilli which were similar in length to those of control birds. The presence or absence of faba bean hulls produced no histological changes as detected by electron microscopy. The structural disorders observed in pancreas and gut of RFB-fed chicks are very similar to those reported by others (Pusztai et al., 1981; King et al., 1983) in rats and pigs fed Phaseoulus vulgaris lectins. The atrophy of microvilli and the hidropic degeneration of jejunal enterocytes, which were the major features observed in RFB-fed birds, were not detected in either AFB or AC fed chicks. This gives further evidence to the fact that lectins are the substances responsible for the effects described in RFB-fed birds.

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DETERMINATION OF TANNINS IN FABA BEANS

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Abstract

The tannin content in 15 cultivars of faba beans was determined with four different colorimetric methods, viz. the Folin-Denis method, the Prussian blue procedure, the 'ISO method' and the vanillin assay. With the Prussian blue procedure unsatisfactory results were obtained in that the reproducibility was poor.

The data obtained with the vanillin assay are highly correlated with those obtained with the Folin-Denis method or the ISO method. This is surprising because the nature of the chemical reaction of vanillin with tannins is quite different from the type of reaction that takes place in the Folin-Denis and in the ISO method.

The tannin content in the faba beans was also established with a protein precipitation procedure. No correlation was found between the results of this precipitation procedure and the colorimetric methods mentioned.

Introduction

Tannins are antinutritional substances. The digestion of proteins is interfered by the formation of insoluble complexes between tannins and both feed proteins and proteolytic enzymes.

Moreover, tannins form strong complexes with divalent metal ions such as iron ions resulting in a low biological availability of these metals. Several methods for the determination of tannins have been described in the literature (for a review, see Deshpande et al, 1986). It is important to know which method gives the highest correlation with the antinutritional effect of the tannins found in animal digestibility trials.

Materials and methods

The tannin content in 15 variaties of faba beans and in two pea species was determined with four different colorimetric methods:

- the Folin-Denis method (FD) (Swain and Hillis, 1959)
- the Prussian blue procedure (Price and Butler, 1977)
- the ISO method (ISO, 1987)
- the vanillin-sulphuric acid method (VAN) (Kuhla and Ebmeier, 1981)

The tannin content was also determined by a protein precipitation procedure (Hagerman and Butler, 1978). For this procedure bovine serum albumin (Sigma, St. Louis, MO) was used.

Results and discussion

The tannin contents in the seed coats and in the cotyledons were determined separately. The results are given in Table 1.

The Prussian blue procedure gave unsatisfactory results in our hands

in that the reproducibility was poor.

The results of the FD method, of the ISO method and of the VAN assay were highly correlated. This is surprising because the VAN assay is based on a condensation reaction between vanillin and catechin-like structures, whereas the FD and ISO methods are based on the reduction of metal ions by phenolic groups.

The protein from the cotyledons and the tannins from the seed coats can form insoluble complexes which might affect the tannin determination. The tannin content in the milled whole beans was determined as well. The values found were very similar to the values calculated from the tannin content in the cotyledons and in the seed coats.

No correlation was found between the results of the precipitation procedure and those of the colorimetric methods (Table 2). We assume that the extraction of tannins in the protein precipitation procedure is far from optimal.

This view is supported by the fact that in the whole milled beans lower tannin values were found than were calculated from the contents in the cotyledons and the seed coats.

The results obtained with these analytical methods will be compared with the results of digestibility studies with pigs.

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Table 1. Tannin content in faba beans and peas as determined by different methods

Variety	Cotyledons (%w/w)			Seed coats (%w/w)			Whole bean (%w/w)			Calculated from the cotyledons and the seed coats (%w/w)		
No* Colour of flower	FD	ISO	VAN	FD	ISO	VAN	FD	ISO	VAN	FD	ISO	VAN
1 white	n.d.	0.00	0.06	n.d.	0.05	0.11	0.50	0.00	0.06	n.d.	0.01	0.07
2 coloured	0.57	0.00	0.05	4.70	2.33	4.29	0.95	0.25	0.48	1.11	0.30	0.60
3 white	n.d.	0.01	0.06	n.d.	0.07	0.05	0.54	0.00	0.05	n.d.	0.01	0.06
4 coloured	n.d.	0.03	0.06	7.00	4.64	5.42	1.55	0.78	0.96	1.73	0.85	1.01
5 coloured	0.59	0.02	0.06	7.40	5.08	6.39	1.20	0.85	1.31	1.64	0.80	1.03
6 coloured	0.61	0.03	0.04	5.30	2.48	5.07	0.96	0.33	0.80	1.24	0.36	0.72
7 white	n.d.	0.01	0.05	n.d.	0.11	0.40	0.58	0.02	0.06	n.d.	0.02	0.09
8 white	n.d.	0.00	0.07	n.d.	0.08	0.07	0.49	0.01	0.08	n.d.	0.01	0.07
9 coloured	0.54	0.00	0.04	5.00	3.46	5.80	1.00	0.41	0.72	1.14	0.47	0.82
10 white	n.d.	0.00	0.06	n.d.	0.03	0.07	0.51	0.00	0.06	n.d.	0.00	0.06
11 coloured	0.68	0.04	0.07	4.90	4.12	5.77	1.64	0.78	1.02	1.35	0.69	0.97
12 coloured	0.56	0.00	0.05	6.00	2.26	5.08	0.89	0.31	0.60	1.30	0.31	0.73
13 white	n.d.	0.00	0.06	n.d.	0.02	0.08	0.52	0.00	0.06	n.d.	0.00	0.06
14 coloured	0.59	0.03	0.06	6.20	3.99	5.79	1.53	0.59	1.05	1.56	0.72	1.05
15 coloured	0.52	0.00	0.05	3.60	3.52	5.53	1.28	0.43	0.64	0.95	0.49	0.81
Peas												
16 Brown marrowfat peas	n.d.	0.00	0.07	n.d.	1.77	1.69	0.67	0.20	0.28	n.d.	0.17	0.22
17 Australian peas	n.d.	0.00	0.07	n.d.	2.53	5.92	0.88	0.47	0.72	n.d.	0.24	0.63

n.d. = not determined

FD = Folin-Denis method; ISO = ISO method; VAN = vanillin-sulphuric acid method
* 4 = Alfred, 7 = Blandine, 14 = Herra

Table 2. Tannin content in faba beans and peas as determined by the protein precipitation method.

Variety		Cotyledons	Seed coats	Whole beans	Calculated from the cotyledons and the seed coats
No*	Colour of flower	(%w/w)	(%w/w)	(%w/w)	(%w/w)
1	white	<0.03	0.07	0.05	0.01
2	coloured	<0.03	0.98	0.06	0.13
3	white	<0.03	0.07	0.05	0.01
4	coloured	<0.03	0.69	0.05	0.12
5	coloured	<0.03	1.27	0.09	0.19
6	coloured	<0.03	1.35	0.12	0.18
7	white	<0.03	0.08	0.05	0.01
8	white	<0.03	0.07	0.05	0.01
9	coloured	<0.03	1.50	0.10	0.20
10	white	<0.03	0.07	0.05	0.01
11	coloured	<0.03	1.24	0.09	0.20
12	coloured	<0.03	0.86	0.06	0.12
13	white	<0.03	0.07	0.05	0.01
14	coloured	<0.03	1.58	0.10	0.27
15	coloured	<0.03	1.58	0.10	0.22
Peas					
16	Brown marrowfat peas	<0.03	1.67	0.06	0.16
17	Australian peas	<0.03	0.95	0.05	0.09

* 4 = Alfred, 7 = Blandine, 14 = Herra

DETERMINATION OF TANNIN IN THE SEEDS OF *VICIA FABA* BY NIR

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Summary

A rapid, non destructive method is described for the determination of tannin in whole grains of *Vicia faba*, using near-infrared reflectance (NIR) spectroscopy. A comparison between acidified vanillin (AV) and tannins index (TI) is made. Tannin content could be estimated with high correlation and good accuracy. Calibrations with TI values gave better results than when AV values were used.
Keywords: tannins, near infrared reflectance (NIR), *Vicia faba*.

Introduction

Among the several antinutritional factors present in the seeds of *Vicia faba*, condensed tannins are responsible, significantly, in the reduction of its biological value (Marquardt, 1983). These compounds are polymers of variable molecular weight and its biosynthetic pathway is not fully understood, although it has been proposed that leucocyanidin is a precursor common to cyanidin (anthocyanidin), catechin and condensed tannins (Kristiansen, 1984). This fact explains, in part, the difficulties of reliable analytical procedures. The available methods in some cases do not quantify exclusively tannins; in other cases the degree of polymerization (of high biological significance) is not considered. All of them involve seed grinding followed by such slow, tedious and expensive wet chemical tests that it makes the breeder's work very difficult. A rapid and inexpensive method of analysis with no destruction of seeds is clearly the desire of breeders developing low tannin faba bean cultivars. Near-infrared reflectance (NIR) offers the potential technique for these purposes specially because the tannins are located mainly in the seed coat in faba bean grains.

The objective of the present study was to ascertain the utility of NIR for prediction of tannin content in whole faba bean seeds.

Material and methods

A total of 112 samples of faba beans were used in the study. Seeds for wet analysis were mechanically dehulled for elimination of possible interferences with cotyledons constituents.

The acidified vanillin (AV) procedure (Broadhurst & Jones, 1978) and the tannin index (TI) method (Ford & Hewitt, 1979) were used for wet analysis.

Tannins of faba beans extracted chromatographically were kindly supplied to us by Prof. Tena (E.T.S.I.A. Córdoba). These tannins were used as standard for the transformation of tannin index values into % of tannin. Catechin (+) from Sigma (n. C-1251) and cyanidin chloride from Sarsynthèse (n. 0909) were used.

The near-infrared reflectance spectra of unbreaked whole faba bean samples were measured with a NEOTEC computerized spectrophotometer 6250 model. The region scanned was 1100 to 2500 nm. The number of scans per

sample was 100 and the high fat-high moisture cell was used.

The first and second derivatives of reflectance spectra were used for the linear regression analysis. Several calibrations were developed with the spectral data in order to choose the one that offered the best sample prediction. The reflectance spectra of the pure substances for the wavelength selections were taken into account and those which provided the maximum multiple correlation coefficient and minimum standard error of the regression were chosen.

The different calibrations performed were validated with the percent prediction program, using the 52 population samples not included on the calibration set.

Results and discusion

Laboratory analyses (duplicate determinations) of the seed coat showed the following range: catechin equivalent content 0.01-6.12% ; tannin content 0.01-7.00%. Fig. 1 (a and b) shows the distribution of the samples composition for both parameters.

A set of 60 samples, from the 112 analysed samples, were used for calibration and the rest for validation of the different calibration equations performed. In Fig. 1 the difference between full bar and empty bar are the validation set. It is important to remark that the calibration sample set in each case, AV and TI, was not coincident but was selected in order to have the distributions of analytical values as good as possible.

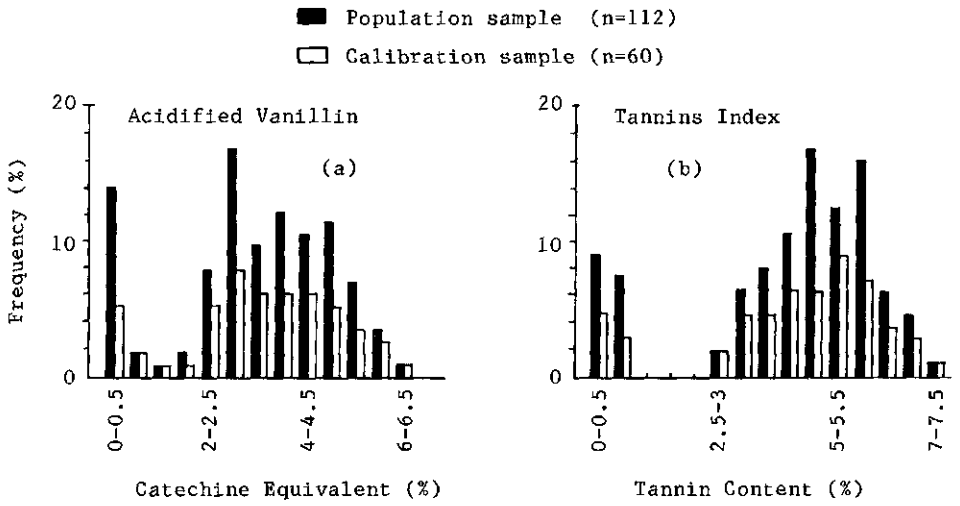

Fig.1. Sample distribution for: a) AV content and b) TI content.

Calibration

In examining the reflectance spectra of catechin, faba bean tannins, and cyanidin (Fig. 2a), several prominent peaks are noted which appeared to be of interest for predicting tannin content. The one around 1700 nm is described as corresponding to the first overtone of C-H links (Osborne & Fearn, 1986) but is our candidate for flavanols because it is common to the three substances (1700 nm for catechin, 1708 nm for faba bean tannins and 1702 nm for cyanidin) and gives the best fit of laboratory and reflectance values. Fig. 2b shows the multiple correlation coefficients

for each wavelength. It is clear that the selected wavelength provides the maximum correlation coefficient and a rounded shape. The absence of this peak on the whole grain is the result of the low concentration of tannin it contains, masked among the background of other constituents of the seed coat, mainly cellulose and related compounds of the cell wall.

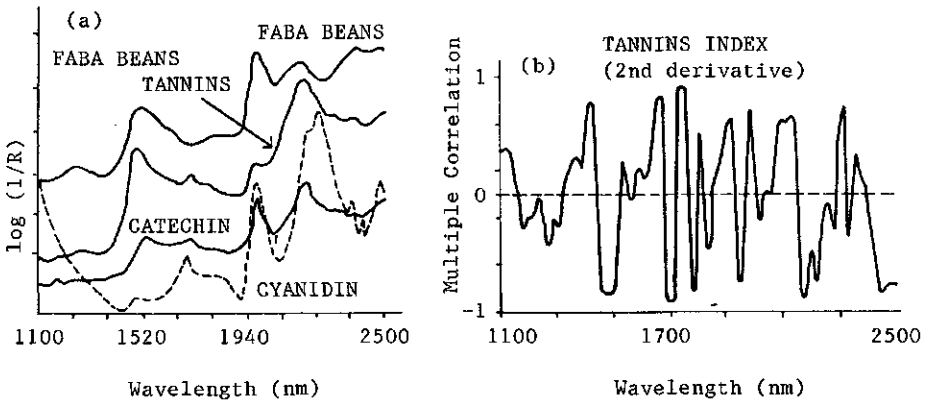

Fig. 2. a) NIR spectra and b) Multiple correlation versus wavelength.

The calibration program was forced to use the primary wavelength near the selected region, obtaining equations for example:
X= K0 + K1 F(L1) + K2 F(L2/L3), where K0, K1 and K2 are constants and X= analytical value of the constituent to calibrate; F(L1) and F(L2/L3) are mathematical funtions (first and second derivative) of the reflectance at the selected wavelength L1, L2 and L3.

The Ks values corresponding to the selected Ls wavelength are obtained with the stepwise regression program of the equipment. The equations with good fitting and with chemical sense for both % of catechin equivalent in seed coat and for % of tannin in seed coat are summarized in Table 1.

Mathematical transformation of the reflectances have to be used given the heterogeneous nature of the samples. Results with second derivative produce better fitting than first derivative in the two cases analysed , % of catechin and % of tannins.

Table 1. Mathematical treatment, wavelength, multiple correlation coefficient and standard error of the estimates.

Parameter Constituent	Math tmt.	L1	L2/L3	R	SEE
% Catechin	1	1724	1414/1684	0.92	0.63
% Catechin	2	1706	1416/2444	0.93	0.59
% Tannin	1	1718	2488/1738	0.97	0.51
% Tannin	2	1700	1488/1694	0.97	0.51

Table 2. Verification data for NIRS tannin analysis equations of Table 1. (52 samples tested).

BIAS	Slope adjustement	Simple correlation	SE prediction
0.258 (S)	0.98 (NS)	0.86	0.84
0.370 (S)	0.94 (NS)	0.88	0.7
0.05 (NS)	0.99 (NS)	0.97	0.50
0.11 (NS)	1.00 (NS)	0.97	0.50

Validation.

Verification of the equations of Table 1 are given in Table 2.

Ranges in concentrations of % of catechin equivalent and % of tannins of the validation sample were similar to those of the samples used for equation development (Fig. 1).

From Table 2 we may conclude that the calibrations using tannins have prediction capacities higher than with catechin. A correction of K0 (bias is significant) is necessary in the catechin cases and again second derivative produced better predictions as we expected for unground samples. Nevertheless, no difference has been found for tannin. This fact could be explained if we admit that the signal in the case of tannin is of such strength that no background noise has to be removed. Calibrations with tannins are of great value for breeding purposes both in first or second derivative. Fig. 3 shows the regression lines of analytical values against the predicted values by NIR. In Fig. 1 and Fig. 3, mainly in the one referred to tannin, a gap corresponding with the difference between zero tannin lines (white flowered plants) and tannin containing lines can be observed. We have done a parallel study eliminating samples from white flowered plants and the results obtained were poorer. We think that the regression coefficients obtained, including the zero tannin grains, reflect the reality in a better way and that the gap observed at the moment will once be filled by breeding work.

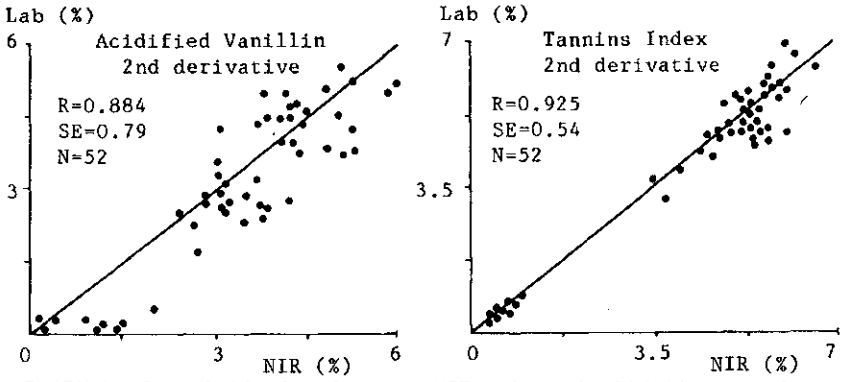

Fig. 3. Plot of analytical values vs NIR values (validation samples set).

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FABA BEANS WITH DIFFERENT TANNIN CONTENTS: ILEAL AND FAECAL DIGESTIBILITY IN PIGLETS AND GROWTH IN CHICKS

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Summary

Results are presented of two experiments to determine the nutritional value in pigs and poultry of different varieties of faba beans with a varying level of tannins.
Significant differences in ileal and faecal digestibility in piglets of different varieties of faba beans were determined, which were likely related to their content of tannins. However, in a growth trial with chicks on diets with 30% faba beans no differences in growth response between the control, high and low tannin faba bean diets were observed.

Introduction

Faba beans (Vicia faba L.) are known to contain different antinutritional factors (ANF). Among them tannins have been often described as being the most important from the nutritional point of view.
Levels of tannins in faba beans are variable, especially between varieties (Cabrera et al., 1986).
Tannins can in some instances reduce feed intake in monogastric animals by their astringent taste. Tannins are known to complex easily with dietary protein thereby reducing its digestibility (Marquardt, 1989). They have also been described as being able to bind to digestive enzymes causing a reduction in their enzymatic function (Griffiths, 1979).

In the present study four varieties of faba beans with different tannin contents and one heat treated variety were used in an experiment with canulated piglets to determine their ileal and faecal digestibilities. The same as well as two additional varieties were tested in a growth experiment with chicks.

Materials and methods

Chemical analysis

The faba bean varieties used in the animal experiments were analysed for tannin content according the following methods: Folin-Denis (FD) (Swain and Hillis, 1959), the vanillin-sulphuric acid method (VAN) (Kuhla and Ebmeier, 1981), the ISO method (ISO, 1987).
Levels of lectins and trypsin inhibitors in beans were determined using, respectively, the haemagglutination assay (Valdebouze et al., 1980) and a modified Kakade method (van Oort et al., 1989).
All faeces and chyme samples were analysed for dry matter, organic matter and protein (N x 6.25).
All faba beans were also analysed for amino acid contents in order to balance chick diets on amino acid contents.

Digestibility trial with piglets

Eighteen barrow piglets of 8-9 kg were surgically fitted with a post valvular T-caecum fistula (PVTC) (van Leeuwen et al., 1988).
The experiment consisted of two test periods and was carried out in a change-over design with six experimental groups. Results were obtained using six animals per treatment.
The basis of diet I (reference diet) consisted of barley, maize, fishmeal and skimmed milk powder. In diets II - VI 30% of the basal diet was replaced by either one of of the four varieties of faba beans (indicated as A, B, C or D) or by 30% heat treated faba beans variety A (diet VI).
The beans for diet VI were heated in an autoclave for 30 minutes at a temperature of 105°C and at a moisture level of 20%.
After 9 days of adaptation to the diets faeces were collected quantitatively for five days, followed by a period of five days in which chyme was collected during 12 hours per day (8.00 a.m. - 20.00 p.m.). Cr_2O_3 was added to the diets as a marker.

Digestibilty of faba beans was calculated by using the digestibility values of the basal diet (diet I) as a reference (difference method).

Growth trial with chicks

One-day-old male broiler chicks were housed in cages and fed a control diet. After five days of adaptation all chicks were weighed and allocated to one of the eleven experimental diets. Each treatment was assigned to six cages with 15 chickens each. The initial mean weight per chick was 123 g. During the experiment chicks were fed ad libitum and weighed weekly. At the same time feed consumption was determined.

Diet I	Control diet with casein/fishmeal as protein sources
II	Control diet with heated soyabean meal as protein source
III - VIII	70% basis as diet I + 30% faba beans variety A, B, C, D, E or F
IX	75.2% basis as diet I + 24.8% cotyledons of faba bean variety D *
X	94.8% basis as diet I + 5.2% hulls of faba bean variety D *
XI	70% basis as diet I + 30% heat treated faba beans variety A

* inclusion levels of cotelydons and hulls equivalent to inclusion of 30% whole faba beans

All diets were balanced with respect to metabolizable energy, protein, lysine, methionine + cystine, threonine, tryptophan and arginine, Ca and P.
The experiment was conducted over a period of three weeks.

Results and discussion

Chemical analysis

Table 1 shows contents of tannins, trypsin inhibitors (TI) and lectins in the beans included in diets in one or both of the two animal trials. The faba bean varieties used had variable levels of tannins ranging from low to high. The levels of TI were moderate but relatively low in variety D and F. Lectin levels were low and were presumed to be of little nutritional significance.

Table 1. Contents of tannins, trypsin inhibitors (TI) and lectins in variety A, B, C, D, E and F, heat treated A and hulls and cotyledons of variety D.

Faba bean	Tannin %			TI*	Lectins**
	FD	VAN	ISO		HA
A	0.57	0.02	0.00	1.33	5
B	1.19	0.40	0.34	1.44	5
C	1.52	0.98	0.86	1.55	5
D	1.55	0.96	0.78	0.70	2
E	0.59	0.00	0.00	1.05	5
F	1.60	0.96	0.88	0.73	5
heated A	0.60	-	-	0.30	-
hulls of D	4.20	5.42	4.64	-	-
cotyledons of D	0.66	0.06	0.03	-	-

* expressed as mg inhibited trypsin per gram product

** haemagglutination units with rabbit red blood cells, 1 HA = 1:1000 dilution step

\- not determined

Digestibility trial

In table 2 digestibility values of the organic matter, protein (N x 6.25) and N-free extract (NFE) of faba beans as determined in young piglets are given.
It can be seen that significant differences exist between the digestibility of organic matter of faba beans with different tannin contents. These differences could be explained by differences in protein digestibility as well as by differences in the digestibility of the NFE fraction.
Cultivar A containing little or no tannins showed the highest digestibility for protein and NFE, and variety D with the highest content of tannins was lowest in digestibility on ileal as well as on faecal level.

Table 2. Ileal and faecal digestiblity of organic matter (OM), protein and N-free extract of faba beans A, B, C, D and heat treated A.

Faba bean	OM		Protein		NFE	
	ileal	faecal	ileal	faecal	ileal	faecal
A	72.0^{a}	88.1^{a}	85.3^{a}	89.3^{a}	72.9^{a}	94.3^{a}
B	70.6^{a}	84.4^{ab}	75.3^{b}	85.2^{ab}	75.4^{a}	93.0^{ab}
C	67.4^{ab}	82.1^{bc}	74.1^{b}	82.4^{bc}	72.7^{a}	90.7^{bc}
D	63.0^{b}	79.0^{c}	68.7^{b}	79.4^{c}	69.1^{a}	89.5^{c}
heated A	69.1^{ab}	86.4^{ab}	82.1^{ab}	88.9^{ab}	69.4^{a}	93.6^{a}

Values with a different superscript within a column differ significantly at $P<0.05$.

A lower digestibility of faba bean protein due to the presence of tannins has also been described by Liebert and Gebhardt (1983) in pigs. The afore mentioned authors found a 2-3.5% lower faecal digestibility of

faba bean protein of beans containing 1.5-1.7% tannins (Folin Denis) compared to the protein of tannin free white flowering cultivars in pigs of 50 kg. In our experiment younger pigs were used which are probably more sensitive to tannins than older pigs.
Heat treating the low tannin variety A destroyed the trypsin inhibitors present but led to a slightly lower digestibility compared to the untreated beans of the same cultivar. This may be due to overheating of the beans.
Further research is needed to explain the relationship between tannin content of faba beans and their digestibility in piglets.

Growth trial

In table 3 results of the growth trial with chicks are presented.

Table 3. Feed intake, weight gain and feed conversion (FC) of chicks fed diets with different varieties of faba beans or faba bean fractions over a period of three weeks.

Diet		Feed intake g	Weight gain g	FC
I	control	1592	1039	1.53
II	control (soya)	1621	1070	1.51
III	bean A	1547	1038	1.49
IV	bean B	1594	1047	1.52
V	bean C	1600	1058	1.51
VI	bean D	1589	1046	1.52
VII	bean E	1561	1031	1.51
VIII	bean F	1607	1042	1.54
IX	cotyledon D	1580	1055	1.50
X	hull D	1600	1049	1.53
XI	heated A	1524	1022	1.49
	LSD (P=0.05)	44	33	0.04

As can be seen from table 3 no significant differences could be observed in weight gain and feed conversion of chicks fed diets with different varieties of faba beans containing variable levels of tannins. Only the diet with heated variety A tended to show a somewhat lower weight gain, due to a lower feed intake or possibly due to some overheating of the beans causing an alteration in the protein structure and therefore possibly its digestibility.
The general level of performance could be described as very high.
No negative effects could be observed from including hulls of variety D, containing nearly all of the analysed tannins (table 1) in the diet in amounts equivalent to a diet containing 30% of whole beans.

In the literature variable results have been presented on chicks fed faba bean diets.
Marquardt and Ward (1979) found a significant influence of the tannin level in faba beans on the growth response of chicks. However, beans were included up to levels of 90% in the experimental diets.
In an accompanying experiment of the same authors water extracts of faba beans containing condensed tannins were added to diets of growing chicks up to levels much higher than obtained with inclusion of 30% faba beans. The correlation coefficient calculated for the parameters tannin intake and weight gain was - 0.90 and for tannin intake and feed conversion was + 0.97. It is questionable whether these values are also relevant for

practical situations in which tannin levels in diets will remain far below the levels as used in the experiment of Marquardt and Ward.

Results from our experiment suggest that feeding faba beans to broilers at a level of 30%, taking into account the amino acid composition of the faba bean protein and the amino acid requirements of chicks, will provide levels of tannins that do not appear to be antinutritional in terms of reducing growth and impairing feed conversion.

Summarizing, we can draw the conclusion that piglets seem more sensitive to tannins than chicks, and it may be presumed that the lower digestibility of the high tannin faba beans as measured in piglets will also lead to a reduced weight gain in growth experiments. This suggestion seems to agree with the results of Huisman et al. (1989) who found that piglets are more sensitive than chicks towards ANF in Phaseolus vulgaris and Pisum sativum.

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ILEAL DIGESTIBILITY OF PROTEIN IN PIGS FED DIETS WITH PEAS OF VARIABLE CONTENT OF PROTEIN AND TANNINS

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Summary

Preliminary results of protein evaluation of different types of peas are presented. Four varieties of white-flowered Pisum sativum and one variety of coloured-flowered P. arvense were analysed for content of protein, amino acids, NDF and tannins, and were used in trials on cannulated pigs to evaluate ileal digestibility of pea nutrients. The true ileal digestibility of different varieties of P. sativum showing protein content (N x 6.25) from 22.4 to 27.7 per cent/DM ranged for protein from 72 to 83, for lysine from 76 to 86, for threonine from 69 to 84, for methionine from 59 to 80 and for tryptophan from 57 to 78. There was a tendency to increased digestibility with increasing protein content in the peas. Coloured-flowered pea showed much lower digestibility of nutrients as compared to the white-flowered peas of similar protein level but of lower content of tannins (7.1 vs 1.6-1.9 mg/g) and NDF (17.2 vs 11.7-13.4%).

Introduction

Pea, in common with other grain legumes, can provide a considerable proportion of dietary protein for pigs. Since the level of protein and of some amino acids in pea is low it is important to have accurate knowledge about all factors which can influence the protein digestibility and amino acid availability in the pig. Additionally to variable protein level, which factor may influence digestibility (Buraczewska et al., 1987), legumes contain various compounds which may result in a reduction in nutritive value as compared to that predicted from amino acid data. Several papers indicate that tannins by their ability to complex with proteins influence nutrient digestibility (Griffiths, 1979; Griffiths and Moseley, 1980) and show harmful effect on animal organs (McLeod, 1974). Considerable differences in nutritive value were found for white and coloured-flowered field beans, containing negligible and high concentration of tannins, respectively (Martin-Tanguy et al., 1977).

This paper is concerned with evaluating ileal digestibility of protein and amino acids in pigs fed diets with white- and coloured-flowered peas showing variable chemical composition.

Materials and methods

Large White x Landrace castrated male pigs ranging in body mass from 40 to 75 kg were fed barley-based rations containing 41.5% of white or coloured-flowered pea of variable chemical composition. The composition of the diets is given in Table 1. Pigs were prepared with a simple T-piece cannula of about 22 mm in diameter, inserted in the terminal ileum. The animals were maintained in metabolism crates, and at 12 h intervals, they were offered an amount of air-dry diet equivalent to 1.9 per cent of their

liveweight.

Table 1. Composition of diets, per cent of air DM.

Ingredients	Diets	
	Control	Experimental[1)]
Barley	51.70	51.70
Maize starch	43.45	2.70
Pea	--	41.50
Min.-vit. mixture	4.40	3.80
Cr_2O_3	0.30	0.30
L-Lys. HCl	0.15	--

[1)]Six diets were prepared with the following varieties of pea: two diets with different crops of "Belinda" and four diets with "Kaliski", "Opal", "Mige" and "Matmal".

Each diet after mixing with water (1:1) was given to 4-6 pigs during two weeks. After seven days of preliminary period faeces (3 days) and digesta (at least 3 x 12 h) were collected. Pooled, freeze dried samples from each animal and the diets were analysed according to methods described by Buraczewska et al. (1987). Tannins were analysed with the method of Jerumanis (1972) modified by Adams and Novellie (1975).

True digestibility of protein and amino acids was calculated by difference method using the digestibility of barley-starch diet as the reference.

Results and discussion

The chemical analysis showed that (Table 2) the white-flowered P. sativum contained from 22.4 to 27.7% of N x 6.25 and not much differentiated quantity of NDF and tannins. The amount of some nutritionally important amino acids expressed in g per 16 g N increased with decreasing level of protein. These results are consistent with observations of Reicher and Mackenzie (1982) on one variety of pea (P. sativum) containing from 14.5 to 28.5 per cent of protein. The authors found that the content of threonine, cystine, glycine, alanine, methionine and lysine were negatively correlated with pea protein content. Gueguen and Barbot (1988) showed large deviations in pea cultivars for proportion of albumins and globulins and of the vicilin/legimin ratio which are related to the nutritional value of the seeds.

Table 2. Chemical composition of pea.

Pea:	Pisum sativum					P. arvense
Variety	Belinda 1	Kaliski	Mige	Belinda 2	Opal	Matmal
Nx6.25, % DM	27.7	26.8	26.1	26.0	22.4	26.6
NDF, % DM	11.8	12.7	11.7	13.4	12.6	17.2
Tannins, mg/g DM	3.0	1.6	1.7	1.9	2.4	7.1
Lys., g/16 g N	7.0	7.2	7.5	7.5	8.0	7.3
Thr., g/16 g N	3.4	3.4	3.8	3.7	3.9	3.8
Trp., g/16 g N	0.8	0.8	0.9	0.9	0.9	1.0
Met+Cys, g/16 g N	2.2	2.3	2.4	2.3	2.4	2.4

As it is shown in Table 3, the true digestibility of nitrogen for the white-flowered peas ranged from 72 to 83. Even more marked differences in digestibility were observed for some amino acids: for methionine from 59 to 80, tryptophan from 57 to 78 and for cystine from 44 to 69. There was a tendency to increased digestibility with increasing protein content in the peas.

Table 3. True ileal digestibility of nitrogen and amino acids of five white- and one coloured-flowered pea.

Pea:	Pisum sativum					P. arvense
Varieties:	Belinda 1	Kaliski	Mige	Belinda 2	Opal	Matmal
N x 6.25	27.7	26.8	26.1	26.0	22.4	26.6
N	83^{a}	81^{a}	76^{ab}	73^{b}	72^{b}	66^{c}
Lys.	85^{a}	86^{a}	79^{b}	76^{b}	76^{b}	72^{c}
Thr.	84^{a}	83^{a}	74^{b}	73^{b}	69^{bc}	65^{c}
Met.	79^{a}	80^{a}	72^{b}	65^{bc}	59^{c}	58^{c}
Cys.	68^{a}	69^{a}	59^{b}	61^{b}	44^{c}	48^{c}
Trp.	77^{a}	78^{a}	68^{b}	67^{b}	57^{c}	58^{c}
Ile	85^{a}	81^{a}	74^{b}	74^{b}	73^{b}	78^{c}

Means for each response criteria not sharing a common superscript letter within a row were significantly different.

In spite of comparatively high protein content in the coloured-flowered pea (26.6), its digestibility was only 66 for protein, 72 for lysine, 65 for threonine, 58 each for tryptophan and methionine and 48 for cystine. In the case of white-flowered pea var. Kaliski of a similar protein content the respective values for digestibility were: 81, 86, 83, 78, 80 and 69. The differences in the ileal digestibility of nutrients between the white and the coloured-flowered cultivars of those peas can be explained by a higher content of tannins (7.1 vs 1.6) and/or fiber (17.2 vs 12.7) in the latter cultivar. However, it is also possible that some additional factors like lectins and trypsin inhibitors, which has not been determined yet in the peas, could affect the digestive processes in the pigs.

More varieties of both types of pea are still under investigation. It may be that an establishment of the relationship between crude protein content in the peas and the ileal digestibility of protein and amino acids will help to predict the amino acid availability on the basis of nitrogen and tannin content.

Since little relation exists between the biological activity of a plant "tannin" extract and its total phenol content (McLeod, 1974), a measure of active tannins must be found to replace the methods of estimating total phenol presently in use.

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DISCUSSION ON TANNINS

Chairman: E.J. van Weerden
Reported by: D.W. Griffith

Tannins

The session opened with a main paper, in which the dietary effects of tannins were comprehensively reviewed. This was followed by three short papers outlining the role of tannins in rumen fluid, the relationship of nutritive value determinations to analytical estimates of tannin content and the effects of raw faba bean on the intestinal physiology of the chick. The resulting discussions, although necessarily brief, concentrated in particular on the possible interactions of tannin with both the intestinal wall and with bacteria inside the rumen. It was considered unlikely that tannin molecules would be able to reach the intestinal wall without reacting with the many potential substrates present in the intestinal fluid. However, although the rumen also contains many potential substrates such as dietary proteins and extracellular enzymes, interactions between bacterial cell wall and/or cytoplasmic membrane proteins and tannins should not be overlooked.

Additionally a cautionary note was introduced to the discussion in warning that not all the observed nutritional improvements in tannin free cultivars of faba bean could be attributed to the absence of tannins and factors such as the digestibility of the constituent carbohydrates may also play an important role. It was also debatable whether the removal of tannins was always desirable particularly in relation to ruminant feeding where their possible role in protecting dietary proteins may be of value although this would obviously be dependent on these proteins being made available further down the digestive tract.

During the discussion of the various posters considerable views were aired over the merits of the various methods available for tannin analysis. The questions ranged over both the specificity and reproducibility of the methods and it appeared very much to depend on whether an accurate assessment of the polyphenolic content of the legume or a measurement relating directly to observed nutritional effects was required. In general methods based on redox reactions were found to be reproducible but it was stressed that many non-tannin compounds could also interfere and thereby elevate artificially the levels found by such methods. Although, in theory, methods based on protein precipitation techniques would appear more appropriate for the determination of biologically active tannins reproducibility with such methods were generally low, possibly due to problems associated with the extraction procedures. It was also of interest to note that although the high tannin faba bean variety Alfred had low levels of trypsin inhibitor and haemagglutinins, whilst the low tannin variety Blandine had high levels of both these ANFs no significant correlation was generally found between tannin content and the proteinacious anti-nutritive factors.

In the case of the posters relating to the significance of tannins in vivo the discussions centred on the relative merits of introducing tannins into experimental diets at levels higher than would normally be included in what might be considered more practical levels of feeding or to increase the number of animals under test thus increasing the accuracy of the results obtained in vivo and enabling smaller anti-nutritional effects to be seen more clearly. Both approaches appeared to have various merits perhaps depending on whether the interest lay more in the mode of action of the tannin or in the practical consideration of diet formulation advice for 'on farm' feeding.

Session alkaloids

METABOLISM, TOXICITY AND NUTRITIONAL IMPLICATIONS OF QUINOLIZIDINE (LUPIN) ALKALOIDS

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Summary

The general properties of alkaloids are reviewed. The major emphasis is on the quinolizidine alkaloids (QA) of Lupinus spp. The QA have diverse biological effects, including feeding deterrancy, neurological effects and teratogenicity. The QA are based on a bicyclic quinolizidine ring. The most common QA in lupins include bicyclic (e.g. lupinine), tricyclic (e.g. angustifoline, cystisine) and tetracyclic (e.g. lupanine, sparteine, anagyrine). The metabolism of QA in animals is relatively simple. They are oxidized by cytochrome P-450 mediated reactions in the liver, producing reduced metabolites (e.g. dehydrosparteine) via oxidation of the tertiary nitrogen. Metabolic effects of QA and metabolites are primarily neural inhibition, producing acute toxicity signs of convulsions and respiratory paralysis. Anti-palatability effects of lupin QA might be mediated in part through neurological effects.

The principal Lupinus spp. grown as grain legumes are the white lupins, L. albus, and the narrow leaved spp., e.g. L. angustifolius. The main QA in both species is lupanine.

Swine and poultry differ in their response to dietary lupin seed. Swine are very sensitive; growth rate is reduced at all levels of inclusion of lupin. Feed refusal and vomiting are noted. Adverse effects are directly correlated with dietary QA level. Heat treatment of lupin seed does not modify its adverse effect on swine. With poultry, no depression in performance is noted with lupin levels at least as high as 20% of the diet. Rabbits can tolerate very high dietary levels (50% or more) of dietary lupin.

Another deleterious factor in L. albus is manganese (Mn). L. albus may accumulate Mn levels as high as 6000 ppm, and lead to toxic Mn levels when L. albus seed is incorporated into diets. Limited evidence indicates amelioration of Mn toxicity with iron supplementation.

Numerous perennial Lupinus spp. indigenous to U.S.A. rangelands contain anagyrine, a tetracyclic QA which causes skeletal deformities in the bovine fetus. There is no evidence that grain lupins contain anagyrine or have adverse effects on reproduction.

Key Words: Lupins, quinolizidine alkaloids, manganese toxicity, teratogenicity, swine, poultry, rabbits.

Introduction

Alkaloids are very widely distributed in the plant kingdom, although their occurrence in crop plants is quite limited. Chemically, they are a

diverse group of compounds, sharing only the features of alkaline properties and having nitrogen in a heterocyclic ring. The principal role of alkaloids and other secondary compounds in plant tissues appears to be one of chemical defense against herbivory. Alkaloids are bitter compounds, acting as anti-palatability factors to mammalian herbivores. Although there are a number of forages which contain alkaloids, they are found in only a few crop plants. These include potatoes which contain steroid alkaloids, grains which may be infected with ergot alkaloids, and lupins which contain quinolizidine alkaloids.

Lupins are grown as crop plants for grain, as green manure crops, and as garden ornamentals. Many wild species of lupin are used as forage by grazing animals. In the USA, many of the wild lupins contain toxic levels of alkaloids, causing poisoning of cattle and sheep. Low alkaloid sweet lupins (in contrast to high alkaloid bitter lupins) have been developed. These are widely grown in Australia, where lupins have become a major crop. This is because they thrive on the sandy, semi-arid soils that characterize much of Australia, and when grown in rotation with wheat, improve wheat yields because of their soil enriching effects. The main lupin species grown for grain in Australia, Europe and North America are Lupinus albus (white lupin), L. angustifolius and L. mutabilis. High alkaloid bitter lupins (L. utabilis) have been grown for human food for many centuries in the Andes of South America. They are debittered by being boiled in water for 30 minutes followed by steeping in running water for 3 days. While this removes the alkaloids, it also removes a significant portion of water-soluble carbohydrates and other nutrients.

Over 100 species of wild lupins grow in North America. Some of the most important toxic species are L. leucophyllus, L. leucopsis, L. argenteus and L. sericeus. These are particularly significant because they cause acute toxicity in sheep and teratogenic effects (crooked calf disease) in cattle.

Structure of Lupin Alkaloids

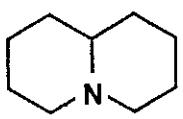
The lupin alkaloids are based on the bicyclic quinolizidine ring, and include bicyclic, tricyclic and tetracyclic structures (Fig. 1). Some of the more important quinolizidine alkaloids (QA) will be mentioned.

The most simple QA in chemical structure are the bicyclic alkaloids, of which lupinine is representative. It occurs in L. luteus, one of the sweet lupins. Examples of tricyclic QA are angustifoline, albine and cytisine. These are found in sweet lupin species such as L. angustifolius (angustifoline) and L. albus (cytisine). Most of the QA in Lupinus sp. are tetracyclic. These include sparteine, lupanine and anagyrine. Numerous optical isomers exist for each of the principal QA, so the potential number of QA in individual Lupinus sp. is large. The major QA in grain lupins is lupanine. For example, over the period 1982-85 in western Australia, the alkaloid profile for QA in L. angustifolius in commercial channels was 42-59% lupanine, 24-45% 13-hydroxylupanine, 7-15% angustifoline and 1-1.5% other QA (Petterson et al., 1987). In L. albus, lupanine is the major QA (Ruiz et al., 1977). The main QA in L. mutabilis are lupanine, 13-hydroxylupanine, 4-hydroxylupanine and sparteine (Hatzold et al., 1983).

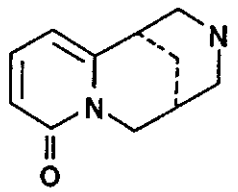
Anagyrine is of particular importance because it is teratogenic in some species. In the USA, numerous range lupin species contain anagyrine,

which is teratogenic in cattle, causing skeletal deformities referred to as crooked calf disease. Fortunately, anagyrine does not occur in grain lupins (Keeler & Gross, 1980). One unfortunate example of teratogenesis in humans has been linked to anagyrine. Severe skeletal deformity in a child in California has been presumptively linked with milk-transferred anagyrine (Meeker & Kilgore, 1987). The child's mother consumed milk from goats grazing L. latifolius. Anagyrine constituted 86% of the total QA and 1.14% of the plant dry matter, and was isolated from goat's milk.

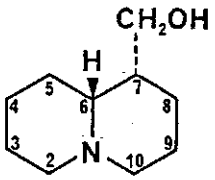
Fig. 1. Structures of representative quinolizidine alkaloids (QA)



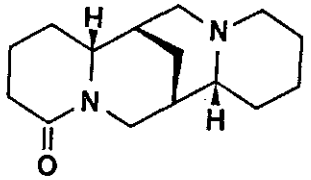
Quinolizidine nucleus



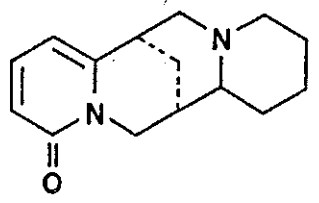
Tricyclic QA:
e.g. cytisine



Bicyclic QA:
e.g. lupinine



Tetracyclic QA:
e.g. lupanine



anagyrine

The major site of QA synthesis in lupins is in the actively growing leaves (Wink & Witte, 1984). The QA are translocated via phloem to other parts of the plant, especially to the seeds. Wink and Witte (1984) speculated that QA may play a role in nitrogen transport, since there is a very rapid turnover of QA in plant tissue and marked diurnal variations in concentration. QA are stored in vacuoles in plant tissue (Wink, 1987). A major role of QA in plants is in chemical defense; they deter mammalian and insect herbivores (Wink, 1987).

Metabolism of QA

Many plant toxins are metabolized in animals by undergoing biotransformation in the liver, usually mediated by the cytochrome P-450 system (Cheeke & Shull, 1985). Relatively little information has been published on the metabolism of QA. Ohnhaus et al. (1985) studied metabolism of sparteine in rat and rabbit liver microsomes. The major metabolite was 2-dehydrosparteine. In humans, sparteine metabolism exhibits genetic polymorphism, with two phenotypes: the extensive and poor metabolizers. About 5% of the European people are poor QA metabolizers, and excrete the alkaloids unchanged in the urine (Ohnhaus et al., 1985). In the other group, sparteine is metabolized to 2-

dehydrosparteine. Lupanine is excreted unchanged in horse (Haywood, 1978) and rat (Wittenburg & Nehring, 1965) urine; rats also excrete hydroxylupanine.

Some pharmacological data is available on QA. In terms of acute toxicity, the following results have been reported. The oral LD_{50} of sparteine and lupanine in mice is 220 and 410 mg/kg (Yovo et al., 1984). Petterson et al. (1987) reported that the oral LD_{50} of a mixture of L. angustifolius QA was 2279 mg/kg, and for lupanine was 1464 mg/kg. From these data it appears that QA have a low acute toxicity.

The principal signs of QA toxicity are neurological effects. In laboratory animals, tremors, convulsions and pulmonary arrest are noted. Livestock such as sheep show similar symptoms, with depression, labored breathing, trembling, convulsions and respiratory paralysis (Kingsbury, 1964). The signs are those of respiratory paralysis and are not specific for QA. The QA act at the ganglionic level, inhibiting the ganglionic impulse transmission of the sympathetic nervous system (Yovo et al., 1984). Acute poisoning of livestock occurs primarily with sheep on western rangelands of the USA. Sheep avidly consume the seed pods, and can readily consume a toxic dose.

There is little evidence of chronic toxicity of QA. Ballester et al. (1984) fed L. albus flour (0.025% lupanine) to rats over two generations, and did not observe any deleterious effects. Rats which survived acute toxicity trials showed no further clinical signs after recovery, and subsequently gained as well as untreated animals of the same age (Petterson et al., 1987).

Effects of QA in Grain Lupins on Livestock

The low alkaloid or sweet lupins are used quite extensively for livestock feeding, particularly in Australia where lupins are widely grown. With nonruminant species, animal performance often shows some degree of impairment. Although there may be a number of deleterious factors involved, the lupin alkaloids are particularly important.

Swine

Swine appear to be more sensitive than other livestock species to lupin alkaloids. In general, feeding trials with sweet lupins of various species have given growth depression in pigs. Common observations include feed refusal, low feed intake, and vomiting. Typical results of feeding trials with swine are shown in Table 1.

Godfrey et al. (1985) showed quite conclusively that growth depression of swine fed lupin seed was largely a reflection of alkaloid content. These workers used different combinations of low alkaloid seed (L. albus cv. Ultra) and a high alkaloid type (L. angustifolius cv. Unicrop) to produce a series of diets varying in QA content. The results (Table 2) show that as QA content increases above 0.20% of the diet, growth rate is reduced, largely as a result of reduced feed intake.

The mechanisms of action of lupin QA in reducing swine performance are not totally clear. The most obvious possibility is that because the QA are bitter compounds, they may directly inhibit feed intake via taste responses. Alternatively, the neurological effects of QA might play a

role in the feed intake and vomition responses. No conclusive data are available to assess the validity of these proposed actions. Young pigs are more sensitive to inhibitory effects of lupins than are older animals that have been exposed to dietary lupin throughout the grower phase.

Table 1. Performance of growing-finishing pigs fed various levels of L. albus cv. Ultra seed (Kelly & Cheeke, 1988).

Item	% Dietary lupin			
	0	10	20	30
Grower phase (30-57 kg):				
Avg. daily gain (g)	863	817	681	636
Avg. daily feed (g)	2361	2270	1952	1998
Feed/gain	2.74	2.78	2.87	3.14
Finisher phase (58-100 kg):				
Avg. daily gain (g)	953	908	908	772
Avg. daily feed (g)	3314	3133	3087	2860
Feed/gain	3.47	3.45	3.40	3.70

Table 2. Effect of QA concentration on performance of growing pigs (Godfrey et al., 1985).

Item	Treatment					
	1	2	3	4	5	6
% Diet QA	0.12	0.20	0.28	0.36	0.44	0.52
% Lupin in diet	29.7	28.7	27.7	26.6	25.7	24.7
Avg. daily gain (g)	627	624	576	563	501	440
Feed intake (g/day)	1.78	1.81	1.69	1.62	1.45	1.34
Feed/gain	2.83	2.90	2.95	2.86	2.92	3.03

Assuming that low palatability is a major cause of the growth-depressing effects of lupin seed in pigs, the addition of feed flavors or other attractants might be expected to have positive effects. However, Kelly and Cheeke (1988) found no improvement in growth of pigs when a 30% lupin diet was supplemented with molasses (Table 3).

Batterham et al. (1986a) observed that heat treatment of lupin seed did not alter growth rate of pigs fed the seed, and concluded that there were no heat-labile deleterious factors in lupin seed. However, they observed

a low availability of lysine in lupin, and concluded that the low bioavailability of lysine in lupin seed is a major factor explaining the growth depressing effects. Subsequent work (Batterham et al., 1986) gave lysine bioavailability estimates of 44-57% for lupin and 80% for soybean meal, indicating a low availability of lupin lysine in the pig. Heat treatment did not improve lysine utilization. The low lysine availability does not seem to be related to QA content.

Table 3. Effect of lysine, molasses and iron supplementation on performance of growing pigs fed 30% lupin (cv. Ultra) seed (Kelly & Cheeke, 1988).

Item	Treatment				
	control	30% lupin	30% lupin + 0.25% lysine	30% lupin + 5% molasses	30% lupin + 135 ppm Fe
Avg. daily gain (g)	863	681	699	613	590
Avg. daily feed (g)	2270	1952	1771	1725	1634
Feed/gain	2.63	2.87	2.53	2.81	2.77
Hematocrit (%)	39	41	--	--	41

Another possibility to explain the adverse effects of lupins in swine diets is high manganese concentrations. L. albus accumulates Mn in the seed, particularly when grown on acid soils which have a high available Mn content. L. albus accumulates much higher seed Mn levels than other Lupinus spp. Levels as high as 6900 ppm Mn have been reported (Oram et al., 1979). L. albus may have 20 times the Mn concentration of L. angustifolius when both species are grown in the same soil (Hung et al., 1987). It is not apparent if the Mn content is related to QA, although Oram et al. (1979) noted that high QA lines of L. albus had lower Mn concentrations than low QA lines. In L. albus, about 80% of the Mn is in the seed endosperm, while in L. angustifolius only 40% of total Mn is in the endosperm.

The toxic level of Mn for pigs is not well established, but may be about 1000 ppm (Batterham, 1979). This level could be approached when high Mn L. albus seed is incorporated into swine diets. Peters et al. (1986) supplemented lupin diets with 400 ppm iron and noted lupin-Fe interactions for gain and feed efficiency. Iron may have a role in reversing the adverse effects of excess Mn; excess Mn results in inhibition of hemoglobin formation through inhibition of iron absorption (Hurley & Keen, 1987). Kelly and Cheeke (1988) found a significant depression in average daily gain when 0.05% ferric sulphate was added to a diet containing 30% L. albus vs. Ultra seed (Table 3).

No reports of reproductive problems in swine fed lupin seed have been published. The inadvertent substitution of lupin seed for soybean meal in a commercial swine diet in the US Midwest apparently caused reproductive

failure, as well as the well-recognized problems of poor growth and vomiting (Cheeke, unpublished observations).

Poultry

Poultry are much more tolerant of dietary lupin than are swine. Karunajeewa and Bartlett (1985) found that dietary levels of L. albus cv. Hamburg of at least 22% in starter diets did not depress performance of chicks. Growth rate was depressed when 30% lupin, which replaced 100% of the soybean meal, was used. The lupin seed contained 2320 ppm Mn; the authors concluded that the apparent appetite depression at 30% lupin may have been due to a combined effect of QA, excess Mn and a marginal level of tryptophan. Watkins and Mirosh (1987) evaluated L. albus cv. Ultra as a protein source for layers, at dietary levels of 10, 15, 20, 25 and 30%. Except for a slight depression of egg production at the 30% level, there were no adverse effects noted (Table 4). Halverson et al. (1983) fed levels of Ultra lupin seed at levels of 15, 30, 45 and 60% to young turkeys. Growth rates were depressed to 94, 89 and 85% of control values with 30, 45 and 60% dietary lupin, respectively. The growth depression observed in poultry with high dietary lupin levels appears to be an appetite-depressing action attributable to the QA (Guillaume et al., 1979). Poultry are sensitive to the taste of bitter substances such as quinine sulfate and high saponin alfalfa (Cheeke et al., 1983).

Table 4. Performance of layers fed L. albus cv. Ultra (Watkins & Mirosh 1987).

Item	% Dietary lupin					
	0	10	15	20	25	30
Trial 1						
Feed intake (g/day)	109	108	108	109	109	--
Egg production	91	89	89	91	89	--
Egg weight (g)	57	57	57	57	57	--
Trial 2						
Feed intake (g/day)	109	110	112	113	116	115
Egg production*	87	88	86	87	86	84
Yolk color (I-15 Rochefan)	8.05	8.65	8.57	9.02	9.55	9.25

* % hen day basis.

Limited data suggests that heat treatment does not improve the utilization of lupin by poultry. Watkins and Mirosh (1987), for example, compared raw, autoclaved and extruded lupin seed for poultry and observed no beneficial effects of heat treatment. However, Boldaji et al. (1986)

found that autoclaving lupin meal increased the TME by about 10% for poultry (13.3 MJ/kg for cooked, 12.1 MJ/kg for raw).

Rabbits

Rabbits seem to be quite tolerant of lupin alkaloids. Kelly and Cheeke compared two cultivars (Ultra & Kiev) of L. albus in diets for weanling rabbits. The results (Table 5) show no growth depression until the level of dietary lupin reached 62.5%, at which level lupin provided 100% of the supplementary protein. The reduced gains at this level may at least partially reflect amino acid balance rather than a QA effect. With the same batch of Ultra lupin, growth of weanling pigs was significantly reduced at 20% dietary lupin. Thus rabbits are much more tolerant of lupin than swine. Rabbits are quite tolerant of bitter substances such as quinine sulfate and saponins (Cheeke, 1987), so it is likely that the lack of growth inhibition was because the lupin seed was not unpalatable to this species.

Table 5. Effect of dietary lupin seed meal on performance of weanling rabbits (Kelly & Cheeke, 1988).

Item	% Dietary lupin					
	0	10	20	30	50	62.5
Trial 1						
cv. Ultra						
ADG*, g	42.7	36.1	42.7	42.4	37.7	35.1
ADFI*, g	148	139	157	160	140	123
F/G	3.5	3.9	3.6	3.8	3.7	3.6
cv. Kiev						
ADG, g	42.7	42.7	43.1	41.1	38.7	30.4**
ADFI, g	148	155	148	141	137	106**
F/G	3.5	3.6	3.4	3.4	3.5	3.5

* ADG = average daily gain.
ADFI = average daily feed intake.

** Different from other means in same row ($p < 0.05$).

Teratogenic Effects

In the western USA, various species of rangeland lupins are involved in a syndrome called crooked calf disease. In an extensive series of studies, Keeler and associates demonstrated that teratogenicity was associated with the presence of anagyrine in toxic lupins. Skeletal

deformity occurred in fetuses when pregnant cows consumed toxic lupins between day 40 and day 70 of pregnancy (Keeler, 1976). Anagyrine does not occur in sweet lupins used as food and feed (Keeler & Gross, 1980). The only known case of teratogenesis in humans linked to QA is that reported by Meeker and Kilgore (1987) in which anagyrine was consumed by a pregnant woman who drank milk from goats grazing L. latifolius.

Lupinosis

Lupinosis is considered to be the most economically significant plant-related toxicosis in Australia (Edgar & Culvenor, 1985). Lupinosis has been observed in most countries where lupins are extensively grown, including Germany, Poland, South Africa, New Zealand and Australia. The disease is caused by a mycotoxin produced by fungi infecting the lupin plant. In Australia, lupinosis has mainly been a problem in sheep and cattle grazing lupin stubble after harvest. Signs are typical of liver damage, and include poor growth, jaundice and massive liver necrosis. Fatty infiltration of the liver occurs, causing it to be greatly enlarged, bright yellow or orange in color, and very greasy when cut. Photosensitivity is commonly seen. Australian researchers have isolated and chemically identified the toxins involved, named phomopsin A and B. The phomopsins are cyclic hexapeptides and are extremely toxic, with an LD_{50} in sheep of about 10 µg/kg body weight. Phomopsins are elaborated by the fungus Phomopsis leptostromiformis which infects the lupin plant.

Elevations in liver copper concentrations are noted in lupinosis (Allen et al., 1979), as well as increases in selenium and decreases in zinc. Similar effects are noted with other hepatotoxins such as the pyrrolizidine alkaloids (Swick et al., 1982a,b).

Although most reports of lupinosis have involved grazing animals, the lupin seed can be contaminated with phomopsin. An awareness of the potential for lupinosis in swine and poultry is important.

Other Factors Affecting Lupin Utilization

Lupin seed contains poorly digested carbohydrates such as galactans (Aguilera et al., 1985). These may accumulate in the hindgut, and stimulate bacterial fermentation. The hindgut of lupin-fed pigs can be grossly enlarged (Cheeke, unpublished observations). Batterham et al. (1986a) similarly noted that "Lupin-seed meal accumulates in the hindgut of pigs and it is essential that results be assessed on a carcass rather than a live-weight basis". This valid concern unfortunately has not generally been taken into account. In essence, it means that the growth inhibition generally seen when lupins are fed to pigs is actually more severe than usually reported, if gains in tissue weight rather than total body weight are used.

Because of the poorly-digested carbohydrate in lupin seed, evaluation of the effects of commercial enzyme preparations on the digestibility of lupin would appear warranted.

Lupin seed has a high fraction of its protein present as globulin storage proteins (Batterham et al., 1986a). The globulin proteins are deficient in sulfur amino acids. Adequate methionine supplementation when using lupins in swine and poultry feeding is important.

Improvements Through Plant Breeding

Most "new" crops have a number of deleterious factors which can be modified through plant breeding to improve their utilization by animals. One of the best examples is rapeseed, for which improved cultivars have been developed with low glucosinolate, low crucic acid, low tannin, herbicide resistance and yellow hull (to resemble soybean meal). Obviously, lupins can be improved. The selection of low alkaloid sweet lupin has been a major advance. However, in general, the QA content is still excessively high, particularly for pigs. It appears (Cheeke, unpublished observations) that reversion to higher alkaloid content is a continual threat. There still seem to be major problems with lupin QA.

Selection of L. albus for lower manganese content is feasible (Oram et al., 1979). It is particularly important that both Mn and QA be monitored when such selection is made; low Mn lines of lupin tend to have higher QA levels. Although L. angustifolius has lower Mn levels than L. albus, the latter species is more desirable in having higher crude protein, lysine and oil and less fiber than L. angustifolius (Batterham, 1986).

In the case of lupinosis, there are cultivar differences in susceptibility to infection with the fungus (Wood & Allen, 1980). Thus with the application of modern plant breeding techniques, it should be possible to modify the lupin plant to produce a product more suitable for animal feeding. Sight must not be lost of why plants contain toxins - i.e. as chemical defenses. Selection for lower toxin levels is basically selection against "nature's pesticides", and thus decreased vigor and yield are likely (Cheeke & Shull, 1985). A balance must be struck between agronomic needs and the quality of the end product. From a plant breeder's point of view, the ideal lupin would be one having a high yield and resistance to pests. It would probably be very high in alkaloid and useless as a feed. Conversely, the ideal lupin from an animal scientist's point of view would be alkaloid-free, very palatable and digestible. Unfortunately, it would probably be virtually impossible to grow it because of susceptibility to pests and diseases. Thus these two conflicting points of view must reach a compromise, so that the crop has adequate yield and pest resistance, while being reasonably palatable and causing minimum negative effects on animal performance.

Priorities for Research

1. Plant Breeding for Improved Feeding Value

The alkaloid problem in lupins remains unsolved. The so-called "sweet" lupins often give unsatisfactory animal performance, particularly with pigs. Selection for reduced QA levels is needed. With L. albus, selection to reduce seed Mn levels in conjunction with reduced QA concentrations is required.

A major constraint in utilizing lupins as feedstuffs is the variability in composition. The crude protein contents reported in the literature vary from less than 30 to over 40% (Table 6). Presumably, selection for a uniform, preferably high protein content is possible.

Table 6. Literature values for composition of lupin seed.

Species	Reference	% Crude protein	% Meth & cyst	% Lys	Mn (ppm)	% Crude fiber	Total QA
L. albus cv. Buttercup	Kemm et al., 1987	30.3	1.39	0.91	—	16.5	< 0.01
L. albus cv. Hamburg	Karunajeeva and Bartlett, 1985	37.0	1.67	0.69	2320	11.0	—
L. albus cv. Hamburg	Batterham et al., 1986b	36.3	1.50	0.90	2400	11.1	0.14
L. albus cv. Hamburg	Kemm et al., 1987	30.7	1.38	0.89	—	15.9	0.13
L. albus cv. Kiev	Kemm et al., 1987	35.0	1.47	0.88	—	17.1	0.01
L. albus cv. Multolupa	Aguilera et al., 1985	41.3	1.69	0.56	1580	13.0	< 0.01
L. albus cv. Ultra	Batterham et al., 1986b	35.5	1.60	0.70	1800	12.1	0.08
L. albus cv. Ultra	Kemm et al., 1987	36.1	1.41	0.94	—	12.4	0.01
L. albus cv. Ultra	Watkins and Mirosh, 1987	32.0	1.72	0.85	—	10.6	0.008
L. albus cv. Ultra	Halvorsen et al., 1983	32.0	1.59	0.86	—	11.6	—
L. albus cv. Ultra	Peters et al., 1986	34.7	1.99	0.55	1157	16.1	0.002
L. albus cv. Ultra	Batterham, 1979	31.5	1.80	0.73	3750	14.6	< 0.01
L. angustifolius cv. Unicrop	Batterham, 1979	28.7	1.55	0.52	42	14.5	< 0.01
L. angustifolius cv. Unicrop	Batterham et al., 1986a	34.7	1.60	0.80	—	17.2	< 0.11
L. angustifolius cv. Uniharvest	Batterham et al., 1986a	33.3	1.60	1.00	—	17.9	< 0.11

2. Mode of Action of QA

The mechanisms of metabolic action of QA in reducing animal performance are not fully identified. Is the appetite-suppression in pigs strictly a palatability effect, or a post-absorptive neural involvement? If a palatability effect, perhaps feed flavors could be useful in overcoming the effects. Further studies are necessary to determine the specific reasons why pigs are more sensitive to dietary lupin than other species. Presumably, their sensitivity is related to the effects of lupin QA, but involvement of other factors cannot be totally ruled out. Data on QA metabolism is quite limited. Further studies on QA metabolism in various animal species are needed in order to fully assess the biological effects of QA.

3. Improvement of Digestibility

The carbohydrate fraction of lupin seed contains galactans which are poorly digested. The use of commercial yeast culture and other sources of enzymes to improve utilization of lupin seed should be investigated.

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THE EFFECT OF LUPIN ALKALOIDS ON GROWTH PERFORMANCE OF RATS AND CHICKEN

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Summary

Feed intake and body weight gain were measured in rats and chicken fed on diets containing white lupin seed with different alkaloid (A) contents. A content in the diets varied from 0.12 to 5.10 and from 0.12 to 3.64 mg/g for rats and chicken, respectively. Feed intake and body weight gain in both species were negatively affected by increasing amounts of dietary lupin alkaloids but chicken were more tolerant to lower and more sensitive to higher levels than rats. The effect of A on rats was less pronounced when dietary proteins were increased from 10 to 14 per cent. Similar response was observed in chicken when diets were enriched in tryptophan and fed in pelleted form. The enlargement of liver weight with increasing dietary A content was greater in chicken than in rats.

Keywords: lupin alkaloids, rats, chicken, feed intake, weight gain.

Introduction

According to analytical survey by Matyka et al. (1985) the content of alkaloids (A) in fodder lupin seeds grown in Poland ranges from 0.5 to 8.3 mg/g which means that the animals fed on diets containing lupins as the main protein supplement may be exposed to dietary levels of A as high as 2 mg/g. Little is known about the response of different species, including rat as the laboratory animal, to the increasing subtoxic levels of A, especially in relation to different dietary conditions. In some experiments the effect of A on feed intake and body weight gain could not be separated from the influence of poor amino acid balance in lupin seeds (e.g. Tannous et al., 1968; Muindi & Rundgren, 1981). In the present study we have observed the response in feed intake and body weight gain of rats to increasing percentages of lupin A in diets containing 10 or 14% of lupin protein supplemented with Met. and Trp. The same study was performed on chicken fed with diets supplemented with either Lys. and Met. or Lys., Met. and Trp.

Material and methods

Animals. Groups consisting of five 21 days old male rats of Ifz:JAZ strain and of six 7 days old male Cornish x White Rock chicken kept in individual cages with free access to water were fed ad libitum with different diets. Feed intake and body weight gain were recorded weekly and, after about three weeks of feeding, animals were killed. The liver weights of the animals on diets with the lowest and the highest A content were recorded.

Diets. Composition of reference diets based on sweet lupin Kalina only, containing 10% (LP) or 14% (HP) of protein for rats and with (+Trp) or without (0Trp) Trp supplementation for chicken is presented in the footnote. Experimental diets were prepared as reference diets but obtained following amounts of A: 1.00, 1.88, 2.75, 3.64 and 6.10 mg/g (HP diet only); this was achieved by progressive replacement of lupin Kalina by half-bitter cv. PRH-182. Alkaloid content was determined according to method described by Wiewiórowski & Skolik (1959).
Statistics. Evaluation of data obtained from animals kept on reference diets was performed on original data using Student's t-test. The effect of A was tested on values expressed as percent of respective reference values using the nonparametric, Kruskal-Wallis test and computer program described by Theodorsson-Norheim (1986).

Results and discussion

Mean values of animal performance obtained for groups kept on reference diets are given in Table 1.

Table 1. Animal performance on reference diets during 20 or 21 days for rat or chicken respectively. Mean (SD).

Diets	Feed intake (g)	Body gain (g)	Feed/gain (g/g)	Liver weight (g/100g bm)
rat LP	264 (22)	83.5 (8)[a]	3.17 (0.08)[a]	4.43 (0.35)
rat HP	258 (14)	104 (13)[b]	2.50 (0.22)[b]	4.71 (0.17)
chicken 0Trp.	1043 (130)	489 (82)	2.14 (0.14)[a]	2.30 (0.34)
chicken +Trp	1118 (142)	589 (138)	1.92 (0.26)[b]	2.27 (0.17)

a and b differ significantly ($P < 0.1$) within species.

Those data were used as a basis for percentual expression of animal responses to different amounts of dietary alkaloids. The relationship between changes of A level and feed intake, body gain, feed to gain ratio is presented in Fig. 1, and liver weight in Fig. 2. Statistical evaluation of group differences resulted in not significant ($P < 0.05$) effects on rat performance, nevertheless the linear decline in feed intake and body gain with increased A content was observed. In case of chicken there was an increase in feed intake at 1 mg A level ($P < 0.05$) only without Trp supplementation. Declining tendency in feed intake as well as in body gain can be observed only at highest dose of A (3.64 mg/g) being significant for body gain in 0Trp group ($P < 0.01$). In this group the feed to gain ratio was increased at A level of 2 and 3 mg/g ($P < 0.05$ and 0.001, resp.) as opposed to +Trp group. The enlargement of liver weight, commonly used as an indication of the presence of dietary toxins, was observed in rat as well as in chicken even at low A level and was more pronounced in the latter species (Fig. 2).

Footnote: Composition of lupin Kalina based reference diets supplemented with amino acids, g/kg.

Animal	Diet	Lupin Kalina	Sucrose	Casein	Sova oil	Min.& vit.	Wheat	Starch	Met	Trp	Lys	Alkaloids
rats	LP	271	120	-	20	50	-	536.6	2.0	0.4	-	0.12
rats	HP	380	120	-	20	50	-	428.6	2.8	0.6	-	0.17
chicken	0Trp	271	-	30	-	41	657.0	-	0.5	-	0.5	0.12
chicken	+Trp	271	-	30	-	41	656.5	-	0.5	0.4	0.5	0.12

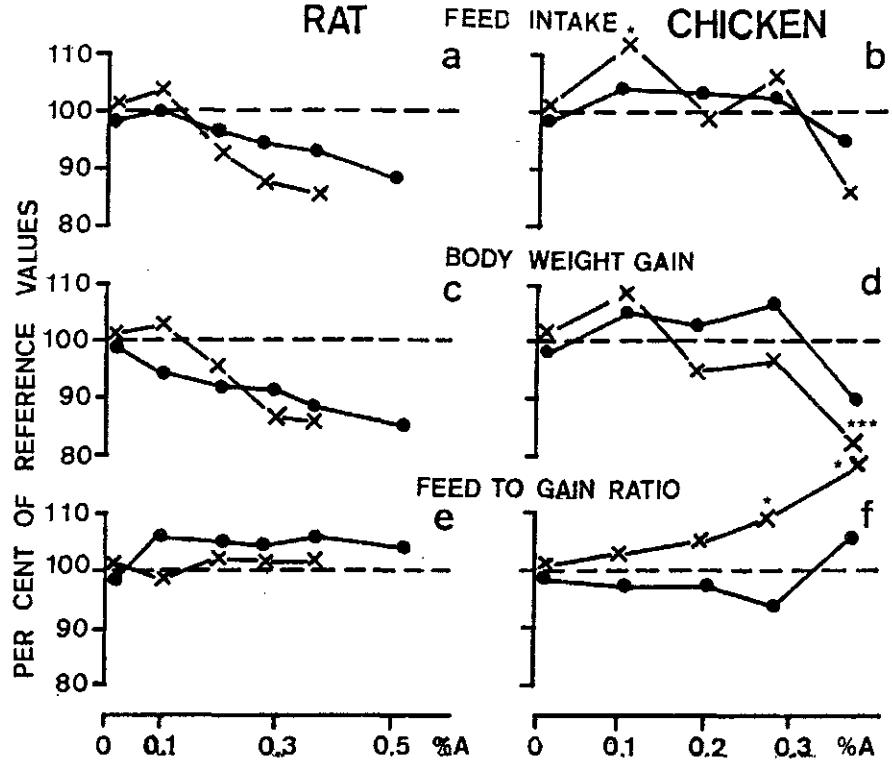

Figure 1. The effect of alkaloids on feed intake (a, b), body weight (c, d) and feed to gain ratio (e, f). Rats fed with 10% (x-x) and 14% (•-•) protein in the diet. Chicken fed without (x-x) and with (•-•) tryptophane supplementation. The data are expressed as percentage of values obtained for animals fed with reference diet containing lupin v. Kalina only.

($P < 0.05$ - *; $P < 0.01$ - **; $P < 0.001$ - *** vs reference).

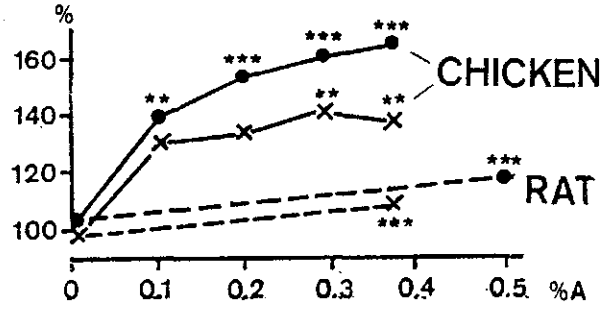

Figure 2. The effect of alkaloids on liver weight in rats and chicken. For reference see text to Figure 1.

The general effect of A on all studied parameters was negative, irrespectively of the type of diet. Nevertheless, the indicated changes in response of chicken to A follwed a different pattern than that of rats. Feed intake and body gain in chicken remained approximately normal up to the highest level of A and only then dropped to values lower than in rats while in the chickenwas systematically depressed indicating possible metabolic disturbances. This was confirmed by greater increase of liver weight in chicken than in rats. The magnitude of rat response to A level as high as 5.1 mg/g was only 10% depression of feed intake and 14% of body gain which confirms the observation of Ruiz et al. (1977) on relatively great tolerance of rats to A up to 1.5 mg/g in the diet. However, our results do not support their explanation that this tolerance is due to nibbling feeding behaviour of rats since the response to A of the two studied species having similar feeding behaviour was different.

It should be emphasized that the tolerance to alkaloids is greater when animals are fed with better diet.

Acknowledgement

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GASTROINTESTINAL EFFECTS OF SOME MEMBRANOLYTIC PLANT CONSTITUENTS

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Summary

Many vegetable foods, medicinal plants and animal feedstuffs contain membranolytic constituents such as saponins and glycoalkaloids. The influence of such compounds on gastrointestinal permeability was assessed *in vitro* by measuring the depolarisation of isolated rat-intestine. Everted segments of jejunum were incubated with α-solanine, α-chaconine, Quillaja saponin, alfalfa saponin, clover saponin or guar saponin at concentrations of 0.5 and 4 mM. There was considerable variation in activity between the compounds; alfalfa saponin produced a steep depolarisation at both concentrations, whereas the clover saponin had very little effect even at 4 mM. The physiological effects of membranolytic saponins *in vivo* were studied by feeding a diet containing *Gypsophylla* saponins (ca. 1.5%) to rats for 7 days. There was no apparent lesion of the intestinal mucosa, but there were morphological and cyto-kinetic changes consistent with an increased rate of crypt-cell mitosis.

Introduction

Saponins occur in over one hundred plant families including many which are used as feedstuffs and human foods. The great majority of saponins possess surface active properties, owing to the presence of a lipophilic aglycone moiety linked to hydrophilic mono- or oligosaccharides (Price, Johnson & Fenwick, 1987). Many saponins interact avidly with membrane sterols to form stable micelle-like structures containing central fluid filled pores (Seeman, 1974). This leads to a rapid loss of membrane integrity and accounts for the characteristic haemolytic activity of many saponins. Recently we have decribed the permeabilisation of isolated gastrointestinal tissue in the presence of some isolated saponins (Johnson *et al*, 1986). *Gypsophylla* saponin and the glycoalkaloid, α-tomatine, gave rise to a rapid de-polarisation of the glucose-dependent transmural potential difference (PD), coupled in the case of *Gypsophylla* saponins with a rise in the permeability to passively absorbed sugars and a loss of active transport activity. In contrast, the monodesmosidic saponins of soy beans possessed only weak activity. The present report describes further studies on a range of saponins and glycoalkaloids using the *in vitro* system, and a feeding study designed to assess the physiological effect of highly membranolytic plant constituents in the diet.

Methods

Materials

The glycoalkaloids α-solanine and α-chaconine were extracted from potato sprouts (*Solanum tuberosum*) by the method of Coxon *et al* (1979). The isolation and purification of *Gysophylla*, alfalfa and clover saponins has

been described by Southon et al (1988), and Jurzysta et al (1988). Guar saponin was isolated by the method of Curl et al (1986). Quillaja saponin was kindly supplied by Dr DG Oakenfull.

Transmural PD

Male Wistar rats (200-250g) were fed a commercial pelleted feed prior to sacrifice under barbiturate anaesthesia. The entire small intestine of each rat was removed and three 5 cm everted sacs were prepared from the proximal jejunum, attached to glass cannulae and ligatured at the free end. Sacs were then filled with Krebs bicarbonate Ringer and suspended in a similar medium containing glucose (28mM) at 37°. The steady-state transmural glucose potential was monitored with silicone rubber KCl-agar-bridges, connected via calomel half-cells to a Keighley 177 digital voltmeter (Keighley Electronics, Reading, UK). The time-course for changes in PD after transfer of the sacs into identical control media, or media containing membranolytic compounds at 0.5 mM or 4 mM, was measured with a chart recorder. After allowance for changes in control PD, the mean change in PD at each time point was calculated for 5 rats and plotted against time.

Saponin feeding study

Twenty male Wistar rats were divided at random into groups of 10 and fed either a saponin free semi-synthetic diet or an identical diet containing unpurified Gypsophylla saponin (ca. 1.5%). The animals were housed singly in wire-bottomed cages and fed the diets ad libitum for 7 days. Food and water intake, faecal production and growth were assessed daily. On the final day of the feeding period all rats were given an intra-peritoneal injection of vincristine sulphate (Sigma, Poole, UK) and sacrificed individually at 9 minute intervals. Samples of mucosa from the proximal jejunum and distal ileum were collected and placed in fixative. A further length of jejunum was isolated and used for transmural PD measurements in saponin-free saline. The crypt cell production rate (CCPR) and the morphology of jejunal and ileal crypts and villi were determined by vincristine blockade and light microscope histology.

Results

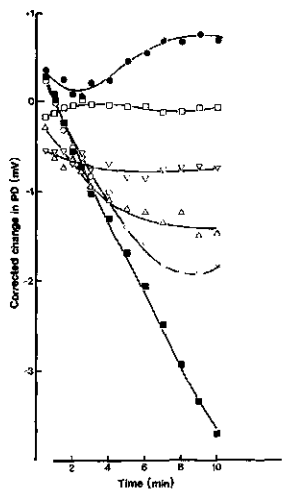

Fig. 1. Changes in transmural PD in the presence of 0.5 mM: α-solanine (□), Quillaja saponin (●), α-chaconine (○), clover saponin (▽), guar saponin (△), alfalfa saponin (■).

Changes in transmural PD in isolated rat jejunum exposed to a variety of saponins and glycoalkaloids at 0.5 mM are shown in Figure 1. There was a considerable variation in response, even amongst structurally related compounds such as α-solanine and α-chaconine, which differ only in their trisaccharide moieties. At the higher concentration all the compounds except the clover saponin led to a steeper depolarisation, and there was little difference in the effects of the two glycoalkaloids.

In the feeding study, the rats showed a marked aversion to the diet containing Gypsophylla saponins, and their rate of weight gain was significantly reduced. There was no macroscopic evidence of inflammation in the jejunum in these animals, and no significant difference in glucose-dependent transmural PD of saponin fed rats (6.45 mV) compared to the controls (5.79 mV).

Both groups of rats had typical leaf-shaped villi, but the saponin-treated group showed a significant enlargement of the villi and crypts, especially in the jejunum (Table 1). The increased crypt length would be consistent with a faster rate of crypt-cell proliferation, and this interpretation was supported by the evidence of a higher CCPR. There was marked variation about the regression lines in the saponin-fed group however, and the difference in CCPR's was not statistically significant.

Table 1. Mucosal morphology and cell proliferation in jejunal mucosa of control and saponin treated rats.

Feature	Control Group	Saponin Group
Villus height (mm)	0.50 ± 0.01*	0.56 ± 0.02
Villus width (mm)	0.35 ± 0.03	0.39 ± 0.01
Crypt length (mm)	0.16 ± 0.05**	0.22 ± 0.07
CCPR (cells/crypt/h)	19.7 ± 1.5	25.5 ± 6.9

Values are means ± SEM
Control and test means differed significantly: *$p<0.05$; **$p<0.001$

Discussion

The present study demonstrates that a wide range of saponins and glycoalkaloids are capable of depolarising the transmural potential difference, which is characteristic of the functionally intact small intestinal mucosa. In earlier studies it was shown that this reduction in PD is accompanied by increased permeability to sugars and polyethyleneglycol 4000 (Johnson et al, 1986). It is probable therefore that the effect depends upon the formation of permeable lesions in the mucosal brush-border membranes. The basic glycoalkaloids in damaged potato tubers, and the complex bisdesmosides from Gypsophylla, Quillaja and alfalfa were the most potent compounds in the in vitro system. Soyasaponins, fortunately perhaps, are the predominant saponin source in

the UK diet, but intakes are high in groups from particular ethnic communities, who also consume significant quantities of more active forms such as guar saponins (Ridout et al 1988).

The permeabilisation of mucosal cells can be expected to increase their rate of exfoliation. The feeding study produced evidence of morphological changes consistent with an enhanced rate of cell turnover in the proximal intestine of saponin-fed rats, but there was no evidence of the damage to the mucosa which occurred in vitro. It is probable that the ability of the mucosa to respond to damage by increased cell production serves as a protective mechanism against membranolytic food constituents. However the consequences of prolonged intake of such compounds which may be encountered in, for example, new food legumes or health foods such as alfalfa supplements, remains to be studied. It has recently been shown that saponins increase the uptake of rabies vaccine in mice, and hence act as an oral adjuvant for the allergic response (Maharaj et al 1986). The possibility that a similar process could lead to sensitisation to food allergens in man requires investigation.

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DISCUSSION ON ALKALOIDS

Chairman: E.J. van Weerden
Reported by: D.W. Griffith

Alkaloids

This session commenced with a main paper reviewing the anti-nutritional properties of alkaloids and in particular the quinoline alkaloids present in lupins together with an examination of the possible importance of manganese toxicity in certain types of lupins. The short paper reported results of the feeding of lupin alkaloids to rats and chicks. The resulting discussions were again necessarily brief and although the importance of alkaloids was accepted as a major anti-nutritional factor present in lupins it was emphasised that particularly for pig feeding other factors such as α-galactosides and the amount of the limiting amino acids were also of great importance when considering the nutritive value of lupins, thus serving as a reminder that not all the observed reductions in nutritive value can be totally attributable to anti-nutritive factors and of the possible dangers of direct extrapolation of the effects and importance of these compounds from one animal species to another.

Session technology

EFFECTS OF PROCESSING ON ANTINUTRITIONAL FACTORS (ANF) AND NUTRITIONAL VALUE OF LEGUME SEEDS FOR NON-RUMINANT FEEDING.

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Abstract

The role of legume seeds in non-ruminant feeding could be more important if the negative effects of several limiting (a.o. ANF) factors can be adequately eliminated. Nutritional evaluation of both processed and untreated legume seeds have to set processing standards for optimum nutritional value. On that base, legume seeds can be traded on quality characteristics (nutritional value; ANF). Also strict control criteria have to be defined. This is already done for processed soya and is reflected in its price.

Priorities in studying the effects of processing of available varieties of legume species are optimization of in vivo protein nutritional value i.e. ileal protein (amino acid) digestibility and amino acid availability, rather than the reduction in the levels of ANF. Furthermore, for some legume species optimization of energy digestibility has to be studied more intensively in relation with digestion of carbohydrates in the hind-gut.
Optimizing energy utilization seems neccesary for both Ph.Vulgaris, Lupins and soyabeans. Primary and secundary processing efficacy have to be established and also quality control criteria.

Keywords: Processing, thermal treatment, dehulling, Phaseolus, pea, fababeans, soyabeans, lectins, trypsin inhibitor, tannins, protein utilization, non-ruminants.

Introduction

The feeding value of some particular feedstuffs is lower than is expected on the basis of their chemical composition, because of physical and chemical properties. These reduce the biological availability and the digestibility of one of more nutrients. Therefore, processing techniques have to be involved before these feedstuffs are applied as animal feed. In order to establish optimal utilization of nutrients from feed ingredients, under intensive animal production, both raw materials (primary processing) and complete diets (secondary processing) should be subjected to one or more commonly used techniques.
In Europe an obvious group of legumes to grow and use in animal feed are fababeans (V.faba L.), peas (P.sativum L.), common beans (Ph.vulgaris L.), soybeans (G. max), lupins (Lupinus spp.), lentils (Lens culinaris) and chick peas (Cicer arietinum). These leguminous seeds contain an ample variety of antinutritional factors (ANF).

These include a wide range of digestive inhibitors, toxins and other substances. These factors may interfere with appetite, absorption and/or metabolism in the animal (Chubb, 1983; Liener, 1980).
Many of the ANF found in raw legumes are inactivated by adequate heat treatment. These can be employed in primary processing for livestock feeding. The nutritional significance of residual ANF-activity and enhanced protein and energy digestion after processing, however, is not fully understood and has to be properly assessed in view of the efficiency of the various processing methods.
In order to make nutrients and energy more available to the animal, it is important to establish those processing conditions which are necessary for a more optimal protein and energy utilization. The following aspects will be discussed:
- research into legume composition and utilization by animals
- processing for reduction of ANF levels
- general processing effects in different legume species and in some particular crops as fababean, common bean, peas and lupins.

Legume composition and utilization by animals

Before an optimal use of legume seeds by the animal can be made one needs to know to what extent various processing techniques improve nutritional value.
In this respect, problems arise from the fact that the different legumes have different levels and/or activities of several ANF (for review, see Liener, 1989). Even within cultivars of the same legume, various activities of ANF are found. For this reason the contribution of each ANF to the magnitude of negative effects in production traits of several animal species has not yet been fully elucidated.
Therefore, research into the potential utilisation of legume seeds is an important feature. This field of research can be arranged within certain topics as shown in Table I.

Table I: Research into legume seed utilization by animals.

1. Chemical identification and characterisation of ANF in legume seeds.
2. Variation in composition of legume seeds (including those of genetic origin). Distribution of ANF in different fractions and establishing the negative effects of ANF in various animal species.
3. Technology: processes designed to inactivate ANF and to rupture plant cell walls; thus making nutrients and energy more available to the animal.
4. Effects of legume seeds, raw or processed, on animal performance (animal species;different age/sex;digestibility/growth).
5. Economic feasibility and practicality of the various processes.

A considerable amount of fundamental research has been focussed on biochemical characterization without direct assessment for the utilization of legumes for livestock feeding. This work has included studies on (1) isolation, identification, characterization and analytical methodology of individual toxic constituents. Furthermore, (2) location in seeds and distribution after fractionation, and (3) heat-sensitivity of proteinaceous ANF have been thoroughly studied but studies are too noumerous to be cited here.
This paper will relate some physical and chemical factors in seeds with biological effects, after applying to animals.

It seems critical to establish, firstly, the negative effects of antinutritional factors in the different feed legumes.
From other papers of this ANF Workshop (Liener, 1989; Marquardt, 1989; Pusztai, 1989) it is obvious that the overall effects of different ANFs are not the same for all feed legumes, their varieties nor the effect in different animal species.

The consequences of the occurence of different ANF in legume seeds can be seen to some extent in data of the proximate analyses and in vivo apparent digestibility data.
The potential improvement in the efficiency of utilization of legume seeds by processing or plant breeding may be derived from Table II. This Table has been compiled from several recent studies (CVB, 1988; Huisman & van Weerden, 1987; Van der Poel & Huisman, 1988; Van der Poel et al., 1989)

Table II: Proximate analyses (main nutrients; % dry weight) and pig apparent digestibility for several feed legumes. a

	Proximate composition (%)				Pig apparent digestibility (%)			
	CP	Cfat	Cfib	NFE	Protein		NFE	
					ADTI	AFD	ADTI	AFD
Ph. vulgaris (Steam heated)	21.8	1.6	4.3	55.3	neg-40	43-79	42	91-95
Glycine max. (Steam heated)	36.5	19.0	5.5	23.4	80-83	90-93	24-43	86-93
P. Sativum (untreated)	22.8	1.2	6.3	51.8	74-76	88-89	71-86	100
V. Faba	24.6	1.7	7.4	47.5				
(untreated,HTV)					72	78	65	90
(" ,LTV)					77	85	74	96

a See list of abbreviations

Results given in Table II show that digestibility of protein at the end of the ileum in Phaseolus is very low. The data on Phaseolus shows that a lot of potential improvement is possible. This potential improvement is less in peas and fababeans. For energy digestibility, however, data in Table II show the need for research in Phaseolus and in soyabeans, in particular, and for peas and fababeans to a lesser extent.

In evaluating processing techniques by means of in vivo experiments, ANF have to be taken into account in the evaluation of feed. These factors affect the utilisation of nutrients negatively in the diet as a whole. The protein and the carbohydrate fractions, however, are the components of primary interest.

The greatest need in the animal feed industry for protein evaluation methods are methods which allow the nutritive values of individual ingredients to be evaluated. These methods should give separate values, which need to be summated to predict the nutritive value of the whole diet (Fuller, 1988).

Three components of this protein evaluation are:

a. the amino acid content of the feed	: a.acid content
b. the absorbed fraction of a.	: digestibility
c. fraction of it in an utilizable form	: availability

The value of feed is consequently calculated as a x b x c.

It can be expected that protein digested in the ileum will be a better predictor of potential use because absorbed nitrogen from the hindgut is not in the form of amino acids (Pond, 1987; van Es, 1982). Indeed, the amount of digestible crude protein and amino acids, measured at the terminal ileum, has a higher correlation to the protein deposited in pigs (Low, 1982; Just et al., 1985 and Moughan & Smith, 1985).

Energy value of feed is derived from the addition of the contribution of each component to the energy value. In pigs, digestible energy can be calculated from various amounts of digestible components (ARC, 1981). This method is used in various countries. In other countries the net energy system is used, and in The Netherlands we use the Rostock formula (Schiemann et al., 1971). In this method net energy is calculated as the addition of the contribution of each apparently digestible component to the net energy. In this formula, faecal digestibilities are used. This is, because especially carbohydrates digested in the large intestine can also contribute to the energy value of the feed.

Processing

To improve the nutritional quality and to provide effective utilization of legume seeds to a level that it is maximal in diets for non-ruminants, it is essential that ANF-activity be removed and that a higher protein and energy digestibility be obtained.

Preparation of separate feedstuffs as well as complete feeds have been drastically modified. Also, the degree of processing has increased considerable in the last years.
Feed processing can be applied by physical, chemical, thermal and/or bacterial means. Any other treatment or alteration of a feed or feed ingredient, before it is fed to the animal, is also termed feed processing.
Feeds are processed for several reasons, but it is clear that the modifications brought about by processing may directly alter the feeding value of the products treated.
In the following we will focus on in vivo protein digestibility.

At least four factors in general do influence the in vivo protein digestibility of legume seeds, each of which is influenced by processing to a certain extent (Krogdahl, 1986; Rackis et al., 1986; Van der Poel, 1989). These factors are:
1. Proteinaceous antinutritional factors; antigenic factors
   (Trypsin inhibitors and lectins; sensitive to denaturation)
2. Protein resistance to proteolysis
   (Protein constituents - albumins, globulins - can react differently upon denaturation)
3. Cell wall constituents and other fibers
   (Flow alteration of digesta; coating nutrients; compound binding)
4. Antinutritional factors, other than proteinaceous in nature
   (I.e. phenolic compounds, phytates)

In processing, both primary processes such as steam-treatment (toasting) and secondary processing (like pelleting) can be distinguished.
Approaches from various disciplines can be used to eliminate ANF from legume seeds (Table III). However, some of these have side-effects related to correlated primary effects.

Table III: Possibilities for reduction or elimination of ANF in feed legumes for livestock from various disciplines.

Technology
1. Breeding and genetic manipulation
2. Feed formulation
- selection of ingredients
- supplementation with amino acids
3. Processing
3.1 Primary processing
-chemical treatments
-enzymatic treatments
-physical treatments
-fractionation
-heat treatments
3.2 Secondary processing
-conditioning/(double) pelleting

Breeding and genetic manipulations are considered long term efforts in establishing the removal or reduction of ANF. These efforts are being made with regards to lectins, TI and tannins (Osborn & Bliss, 1985; Hymowitz, 1986; Bond & Smith, 1989).

Physical treatments, based on separation of seed fractions, is a further possibility for removal of at least part of the ANF. The distribution of several ANF in fractions has been studied after dehulling and pin-milling/subsequent air-classification. These procedures are sometimes used but are efficaceous only in the case they produce a higher (protein and/or energy) nutritional value in relation to the levels of ANF in the material produced.

Extensive efforts have been made to define processing conditions in inactivating activity of ANF in legume seeds, based largely on thermal treatment. Especially heat processing has been proven to be an effective method for decreasing activity of protease inhibitors and lectins. This effect is based on heat denaturation of these proteinaceous inhibitors.

Thermal treatments

The nutritive value of vegetable protein is improved by heat treatment. It has been suggested that the mechanism through which legume proteins are rendered nutritionally available by thermal treatment is the result of an increased accesability of protein by enzymatic attack and inactivation of proteinaceous ANF, primarily lectins and trypsin inhibitors. These inhibitors require their structural integrity in order to inactivate proteolytic enzymes by complex formation (Rackis et al., 1986).
On the other hand, overheating caused by a too high temperature and/or to long exposure, may adversely affect some of the desirable nutrients, such as lysine, methionine and cystine, in intact bean proteins (Rios-Iriarte & Barnes, 1966; Skrede & Krogdahl, 1985). Therefore, accurate control of the heating proces is critical to the processing of bean protein with maximum nutritional value. The determining of available lysine values (ALV) after heating of legume proteins has to be established more frequently.

The effectiviness of heat treatment on the nutritional value of legume seeds depends on a combination of process temperature, heating time, particle size, initial moisture content and eventually the amount of water added during the heat process.
Several investigators have studied TIA and HA as a function of time and temperature. Most of protein inhibitor activity present in the original products is readily destroyed during normal cooking procedures (Rackis et al., 1986).

When soaking or boiling with water, ANF are expected to be extracted from the product. However, with water, also water soluble compounds (tannins; phytate) are being removed. Furthermore, other compounds may be desintegrated with water and/or cooking.
Moreover, studies on soaking and subsequent boiling do not simulate actual procedures used for animal feed processing.

Processing of legume seeds for livestock consumption involves the use of treatments which are feasible. Toasting (atmospheric and pressurized steaming), autoclaving, extrusion (wet and dry), jet-sploding and roasting have been studied as ways to improve the nutritive value of legume seeds by industrial and experimental heat processing. For each of the mentioned processes there is variation in moisture content. Also, various combinations of temperature/time , intensity of pressure etc. at appropriate stages are being used.

The degree of denaturation of the protein is dependent on the combined effects of temperature, time and moisture. Protein solubility decreases during the processing steps with an increase of any of these variables.

Much less research efforts have been undertaken to evaluate the processing of beans especially for livestock feeding.

Processing effect on different ANF

For extended overviews on the effect of heat on several ANF, the reader is referred to the detailed information on the effect of thermal treatments on tannins (Marquardt, 1989), trypsin inhibitors (Rackis et al., 1986; Burns, 1987) and lectins (Van der Poel, 1989).

Combined data of processing effects on ANF destruction or inactivation in legumes are difficult to evaluate due to extensive studies involving different treatment procedures and the use of several legume species and varieties.

Also, these data give no definite explanation for the mechanisms through which legume proteins and carbohydrates are rendered nutritionally available. On the other side it is clear that heat treament permits the nutritional potential of legume proteins to be realized. A overview of the overall effect of heat on ANF is given in Table IV.

Table IV: ANF as affected by heat.

Factor	Active principle	Effect of heat
lectins	protein	+
trypsin inhibitor	protein	+
α-amylase	protein	+
tannins	polyphenols	-/+ ?
metal-binding	phytate/protein	-/+ ?
alkaloids		-
protein digestion		*
starch digestion		*
flatulence		-

+ = pos; - = neg; * = pos, unless overheating

These general effects on ANF, however, cannot be considered as generally valid. Differences have been observed for ANF in various feed legumes. For example, assayable contents of fababean tannins (Marquardt, 1989) and Phaseolus tannins (Van der Poel et al., unpublished) are partly reduced by heat, whereas sorghum tannins are not (Marquardt, 1989). Furthermore, the reductions in the level of ANF after thermal treatment are only of value if a clear relationship is present with biological effects. For example, lectins and TI's from Phaseolus vulgaris are clearly inactivated by heat (Table V) although protein nutritional value in vivo of these heat processed beans, is still very low (see Table II).

Table V: Reduction in TIA and lectin activity, expressed as % of original activity (range) by thermal treatments of (whole or broken) Phaseolus beans.

treatment	inactivation of	
	TIA	HA
steam treatment (100°C; >15 min)	65- 97	90-100
autoclaving (121°C; >15 min)	85-100	99-100
dry roasting (various temp./time)	54- 82	85- 99
extrusion (145°C; 16 sec)	70- 98	93- 98

Data derived from Van der Poel, 1989.

Data in Table V show that a great reduction in TIA and HA can be achieved by heat treatments. TIA levels are reduced to a somewhat lower extent. With dry roasting lower effects in TIA are sometimes noted.

Processing effects in different legume species

Animal nutritionists are still hesitant to incorporate legume species other than processed soya beans routinely in compound diets for non-ruminants.
The reasons, in general, for this are:
- there is a considerable variability in nutrient content within cultivars of the same legume species. The price, however, is not always adjusted according to nutritive value.
- the level of antinutritional factors in certain cultivars is, sometimes, so high that ANF can reduce the nutritional value.
- processing efficacy becomes questionable in the case that the content of ANF is variable.
- reduction in levels of specific ANF differs between various legume species.
- variability in supply of the legumes between years and in trade criteria.

In Table VI. an overview is shown of processing applications for different legume species and criteria for in vitro evaluation. In this Table. reference has been made to the ANF in the different legume species. apart from possible overall effects.

Table VI. Principle reasons for processing (a). applied processes and quality parameters (b) for different legume species.

Feed legume	Processing Yes	No	Reason for processing	Applied processes	Criteria for evaluation
Ph.vulgaris all var.	*		lectins protein (TI ?)	heat treatment	HA:ELISA:FcLIA DBV: TIA: ALV
V. Faba					
HT-var.	*	*	tannins fibre	dehulling reconstitution heat treatment	FD;Vn11n;DBV: PP-A
LT-var.		*			
P.Sativum					
HTI-var	??		TI (?) tannins (?) lectins (?)	heat treatment	TIA: (FD) (HA)
LTI-var		*			
Glycine Max all var.	*		Protein TI	heat treatment	TIA:Urease: PDI:DBV:ALV
Lupinus spp. coloured var.	??		Alkaloids	??	ALV

a primary processing for inactivation of ANF-effects
b see list of abbreviations

From Table VI it can be concluded that primary processing is commonly applied for G. max and Ph. vulgaris. and is applied only incidentally for HT-Fababeans.
For soya (G. max) much research efforts have led to the availability of processed soya(products) on the market. These products are based on strict quality control criteria which reflect nutritional value and price.
Other legume species are not always processed to within known quality standards. since the role of specific ANF is not yet clear. Consequently. ANF treshold levels and subsequent recommended inclusion levels for legumes in diets for non-ruminant animals are not very well known.
As mentioned before. both the variability in the level of ANF and the variability in activity between legumes seeds. emphasize the need for evaluating processing effects for the various legumes rather than ANF.

Effects of processing for each crop

For fababeans, peas, common beans and lupins, data are selected below on recommended levels found in literature for feed formulation (% maximum inclusion) for the inclusion of a specific legume specie in compound diets for non-ruminants. Combining several legume species in one particular diet reveals further retrictions for combinations of species and is common practice in Dutch standards. For these recommended levels, the range of maximum inclusion levels found in literature are given, instead of recommended inclusion levels.

(1) Fababean

Table VII. Range for maximum inclusion levels (%) a

pigs		poultry	
piglets	5-10	broilers	
pigs		0-3 wks	5
-growing	10-15	3-6 wks	7.5
-finishing	10-15		
sows	0	laying hens	7.5-10

a range: data by Tychon & Vanbelle, 1986 and Dutch standards.

The main antinutritional factor present in faba beans in coloured flowered varieties has been shown to be the condensed tannins (Marquardt et al., 1977; Ward et al., 1977). Furthermore, in relation with non-ruminants the TIA (possibly tannin related) and the level of fibre have to be considered.
Several approaches have been considered in neutralisation of the negative effects of fababean tannins (tannin-binding agents; reconstitution; ammonia solutions). The applied processing techniques for animal feeding , however, are largely based on dry fractionation (primary processing) and thermal treatments (primary and secondary processing).
Dehulling will increase the level of protein and decrease the contents of tannins and fibre (or NDF/ADF) in the remaining product, and will result in an increased nutrient digestibility (Henry & Bourdon, 1973; Pastuszewska et al., 1974; Marquardt et al., 1978; Edwards & Duthie, 1973).
Thermal treatments are known to reduce the assayable content of fababean tannins to a certain level. Treatment effects of processing are known to improve the nutritional value of fababean for both pigs and poultry (for review: see Marquardt, 1989). However, these in vivo effects may not be the same for all varieties, for low-tannin varieties have a higher protein but lower starch digestibility in poultry (Laccassagne et al., 1988) Benefits from processing are, furthermore, believed to be greater in young stock. Threshold levels for pigs and poultry, therefore, should include specific values for both Fababean varieties and processed (dehulled; heat-processed) products, based on both protein and energy digestion data.

(2) Phaseolus vulgaris
(Steam-treated)

Table VIII. Range for maximum inclusion levels (%) a

pigs		poultry	
piglets	0	broilers	
pigs		0-3 wks	5
-growing	5	3-6 wks	7.5
-finishing	7.5		
sows	7.5	laying hens	7.5

a data: Dutch standards.

Factors which influence the utilization of Phaseolus bean protein are mainly associated with the resistance of inherent protein to proteolyses and with lectins and protease inhibitors. Especially heat processing has been proven to be an effective method for decreasing activity of PI and lectins, based on heat denaturation of these proteinaceous inhibitors and all varieties are suggested to be heat treated prior to use in diets for nonruminants (Grant et al., 1983).
The in vitro lectin-activity as well as TIA is greatly reduced upon thermal treatment (Van der Poel, 1989). However, it is known that processed beans still have no optimal protein value for pigs (Myer & Froseth, 1983; Rodriquez & Bayley, 1987; Van der Poel & Huisman, 1988). Therefore, reduction of lectin by processing (based on Haemagglutination Assay) and for TI (based on TIA-activity) cannot be sole criteria in the processing of Phaseolus beans.
The importance of tannins and associated polyphenolic compounds has to be recognized, particularly in coloured seeded strains (Ma & Bliss, 1978) although levels may be too low for actual antinutritive action. The qualitative effects of tannins on the nutritive value of Phaseolus spp. are not exactly known.
Attention must be given to processing characteristics (amino acid availability) and possible other reasons for the observed low protein digestion. The mode of action of residual ANF or unidentified factors may be related to the animal digestion (endogenous protein).
On that basis, future processing of Phaseolus beans needs to be optimized and criteria for quality control can subsequently be set.

(3) peas

Table IX. Range for maximum inclusion levels (%) a

pigs			poultry		
	LTI	HTI		LTI	HTI
piglets	0-15	0-15	broilers		
pigs			0-3 wks	5-30	10-20
-growing	7.5-25	7.5-15	3-6 wks	10-30	10-20
-finishing	10-50	10-20	laying		
sows	0	0	hens	10-15	10-15

a range: data by Tychon & Vanbelle (1986), Grosjean & Gatel (1989) and Dutch standards.

Pea tannin levels are related to breeding varieties and are low for spring varieteis grown for yielding pea seeds. Peas of the latter varieties contain appreciable amounts of lectins (Huisman & Van der Poel, 1988) and of TI, especially in winter type peas (Grosjean, 1985).
The nutritive influence of lectins seems to be very low (Bertrand, 1984) compared with Phaseolus spp. The requirements of processing, therefore, is especially related to TI in winter-type peas, to inherent protein denaturation, as well as to the overall carbohydrate structure. Indeed, both primary and secondary processing of pea will improve nutrient digestibility in maize and wheat based diets (Carre et al., 1987; Longstaff and McNab, 1987).
The amount of TIA is reduced by extrusion in both winter pea (Grosjean & Gatel, 1989) and spring pea (Van Zuilichem & Van der Poel, 1989), and, for pelleting, data are controversial (Stappers et al., unpublished; Grosjean & Gatel, 1989).
The fact that in The Netherlands, peas are evaluated differently in either meal or pelleted diets, respectively, requires further elucidation of the possible consequences of pea processing.

(4) Lupins
(sweet white)

Table X. Range for maximum inclusion levels (%) a

pigs		poultry	
piglets	0	broilers	
pigs		0-3 wks	5 -30
-growing	5	3-6 wks	7.5-30
-finishing	5-10	laying	
sows	0-10	hens	7.5-10

a range: data by Leuillet (1984) and Dutch standards.

Sweet lupin seeds approaches equivalence to soya as a protein supplement for nonruminants. The main disadvantages of low alkaloid varietes appear to be the S-containing amino acids (more marked than in most legume seeds) and the fibrous testa (Hill, 1977). Protein digestibility of lupin seed meal is likely to be high (Taverner et al., 1983) and lysine availability is increased by heat treatment (Batterham et al., 1986a,b) compared with raw samples. However, lysine availability decreases with exposure to steam treatment for 20 minutes or longer (Ceron & Gutierrez, 1981). Pig performance was not improved by extruding the lupin (Hale & Miller, 1985).
Approximately 60% of the gross energy from lupins was not digested by gut enzymes (Taverner et al., 1983) which indicate the need for further investigations on energy digestibility of lupin varieties.

Conclusions

The quality of legume species after processing has been the subject of many investigations. However, there have only been few systematic studies on legume quality after processing dealing with the optimization of processing based on in vivo data.

It is clear, that the reduction on the level of antinutritional factors, lectins and protease inhibitors in particular, can be accomplished by thermal treatments. Residual activities alone, however, cannot always be used to asses protein quality. Due to the inherent structure of proteins and carbohydrates, present in legumes, other factors have to be studied also, including lysine and S-amino acid availability and overall protein digestibility (Burns, 1987; Van der Poel, 1989).
It is obvious that changes, induced by thermal processing need to be studied biochemically in both protein and carbohydrates, since amino acid availability and digestion of protein and energy, respectively, may change to a different extent after processing. Further studies on ANF-loss after processing, together with loss of essential nutrients and optimization of in vivo digestion, are needed for the legume seeds.
For a correct assessment of ANF and removal by technological means, assays are needed to measure functional ANF and the effects of ANF on animal characteristics before and after processing.

These studies will contribute a more complete understanding of processing consequences for nutritional value of legume seeds for non-ruminant feeding and will contribute to the optimization of processing conditions.
It will also give valuable information, needed to evaluate the possible ANF treshold levels in seeds in relation to future breeding objectives.

List of abbreviations

ADTI : Apparent digestibility terminal ileum
AFD : Apparent faecal digestibility
ANF : Antinutritional factor(s)
AVL : Available lysine value
CP : Crude protein
Cfat : Crude fat
Cfib : Crude fiber
DBV : Dye binding value
ELISA : Enzyme linked immuno-sorbent assay
FgLIA : Functional globulin lectin immunoassay
FD : Folin Denis
HA : Haemagglutination activity
HT : High tannin
HTI : High trypsin inhibitor
LT : Low tannin
LTI : Low trypsin inhibitor
NFE : Nitrogen free extract
PDI : Protein dispersibility index
PI : Protease inhibitors
PP-A : Protein precipitation assay
TI(A) : Trypsin inhibitor (activity)
Vnlln : Vanillin-HCl/-H2SO4

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EFFECT OF MOISTURE AND PROCESSING TEMPERATURE ON ACTIVITIES OF TRYPSIN INHIBITOR AND UREASE IN SOYBEANS FED TO SWINE

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Summary

Growth performance of 72 crossbred pigs was used as a criterion to evaluate the influence of percentage moisture and elevated temperature on activities of trypsin inhibitor and urease in whole soybeans (Glycine max var. Bragg). Percentage moisture of soybeans used in the comparison was adjusted to 10.3 or 20.5 prior to processing. Soybeans were then heated to 110 C or 125 C in a gas-fired roaster, ground through a hammermill and included with ground corn to be fed as a meal. The respective activities of trypsin inhibitor and urease were lowered by 25.9 and 7.6 percent when soybeans containing 10.3 percent moisture were heated to 110 C. A 57.0 percent reduction in trypsin inhibitor resulted when soybeans containing 10.3 percent moisture were heated to 125 C or when soybeans containing 20.5 percent moisture were heated to 110 C. The respective activities of trypsin inhibitor and urease were lowered by 83.0 and 87.3 percent when soybeans containing 20.5 percent moisture were heated to 125 C. Weight gain and feed consumption of pigs were measured bi-weekly. Pigs fed diets containing soybeans with 10.3 percent moisture heated to 125 C consumed more feed ($P<.05$) had higher ($P<.05$) weight gain and were more efficient ($P<.10$) in utilization of feed than those fed diets containing the same percent moisture heated to 110 C. Further, pigs fed diets containing soybeans with 20.5 percent moisture heated to 110 C or 125 C displayed improved ($P<.05$) feed intake and weight gain when compared to those fed diets heated to either temperature at 10.3 percent moisture. These data suggest that elevating moisture concentration effectively lowered the requirement of heat processing to inactivate trypsin inhibitor and urease in soybeans fed to young swine.
Keywords: moisture, temperature, trypsin inhibitor, urease activity, soybeans.

Introduction

The earliest written accounts of the soybean (Glycine max (L) Merrill) revealed that it was domesticated by Chinese farmers approximately 3,000 years ago (Hapgood, 1988). The primitive predecessor of the domesticated soybean was a wild recumbent vine-like legume that produced very small, hard seeds considered to be indigestible by humans and nonruminant livestock. Selections for more easily cultivated and higher yielding varieties of soybeans were made also in China and by the year 1100 BC, free-standing varieties had been identified.

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Today, there are more than 7,000 varieties of soybeans which confirm a massive potential for expression of genotypic and phenotypic traits. Among these, the seed of the soybean contains natural antinutritional factors that are known to vary in concentration from one variety to another. A partial listing of antinutritional factors intrinsic to soybean seeds are lectins, saponins, antithyriod agents and trypsin inhibitors. The importance of these chemical compounds to the soybean seed is not completely understood, however, some are thought to provide a margin of protection against pests that threaten to devour the embryo along with its nutrient capsule.

Antinutritional factors also interfere with the physiological processes of digestion in higher mammals. In nonruminant species such as swine, trypsin inhibitors ingested in soybean seeds interfere with the normal function of pancreatic proteolytic enzymes. Fortunately, trypsin inhibitors are heat-labile and can be partially or completely denatured when the soybean is exposed to elevated temperature during commercial processing.

Commercial processing of whole soybeans to extract the oil component, inactivate trypsin inhibitors and improve nutrient availability of the resulting meal is also heavily dependent upon fossil fuels. Within the past decade there has been a global increase in the cost of fossil fuels and this trend is expected to continue into the forseeable future. Therefore, new and more innovative methods for processing soybeans used in swine feeds are being actively researched.

Attempts to lower energy expended in processing soybeans by increasing moisture content prior to the application of heat is one method currently under investigation. In this report we present the results of a nutrition study which evaluated growth performance of swine fed whole soybeans containing 10.3 or 20.5 percent moisture that were subsequently heated to 110 C or 125 C. We believe that these data could be of benefit in designing new strategies for more efficient commercial processing of soybeans, yet, the resulting product would have acceptable nutrient quality when fed to swine.

Methods and materials

Seventy-two Yorkshire-Hampshire x Duroc crossbred pigs with an average initial bodyweight (BW) of 5.0 kg were allotted by litter origin, BW and sex to receive one of four dietary treatments. Each treatment was replicated three times into pen units which contained six pigs each. The four dietary treatments were formulated to contain corn as the primary grain source. The following protein supplements were then added directly to the grain component: (1) soybeans at 10.3 percent moisture roasted to a mean temperature of 110 C or (2) 125 C and (3) soybeans at 20.5 percent moisture roasted to a mean temperature of 110 C or (4) 125 C. The moisture content was adjusted by simple additions of distilled water to increments of whole, full fat soybeans of the Bragg variety. When moisture content of 10.3 or 20.5 percent had reached equilibrium within each increment of soybeans, they were passed through a conventional gas-fired roaster (Roast-A-Tron, Mix-Mill, Inc., Bluffton, Indiana, USA). The transit time (30 to 90 seconds depending upon treatment) through the roasting unit was carefully controlled so that the desired core temperature (110 C or 125 C) was reached as the soybeans emerged from the process. The roasted soybeans were then ground into a meal form prior to inclusion into their respective diets. Each diet was formulated to be isonitrogenous and isocaloric with respect to others fed and all contained recommended levels of

supplemental vitamins and minerals to support optimal growth of swine (NRC, 1979). The composition of diets is given in table 1.

Table 1. Composition of diets.

Ingredient	International feed number	kg/100 kg
Ground corn, yellow dent, grain	4-02-935	56.90
Soybeans, full fat, roasted	5-04-597	39.90
Dicalcium phosphate	6-28-335	1.70
Calcium carbonate	6-01-069	.80
Sodium chloride	6-04-152	.25
Mineral premix, commercial		.10
Vitamin premix, commercial		.10
Antibiotic, ASP-250		.25
		100.00
Protein, chemical analysis (%)		18.00
Metabolizable energy (Kcal/kg)		3304

Trypsin inhibitor activity of soybeans, before and after processing, was determined by the method of Hammerstrand et al (1981); urease activity by the method of Caskey and Knapp (1944).

During the 35-day feeding trial, pigs were housed in an enclosed nursery in elevated pens over expanded metal floors. Feed and water were offered ad libitum. All pigs were weighed at the beginning of the feeding trial and at 14-day intervals thereafter. Feed consumption was also recorded for each weight period.

Data were subjected to a computerized analysis of variance (SAS, 1979) for the randomized complete-block design, where blocks represented replications. These data were further subjected to a 2x2 factorial analysis to assess the main effects of moisture content and roasting temperature on growth performance of pigs. Duncan's multiple range test (Steel and Torrie, 1980) was applied to interpret significant differences among means.

Results and discussion

Table 2 presents the values of trypsin inhibitor and urease activities in soybeans before and after heating. Trypsin inhibitor activity in soybeans containing 10.3 percent moisture and heated to 110 C was lowered 25.9 percent. Soybeans containing 10.3 percent moisture but heated to 125 C had approximately 57.0 percent lower activity of trypsin inhibitor which represented about the same level of activity measured in soybeans containing 20.5 percent moisture and heated to 110 C. Urease activities followed a similar trend as trypsin inhibitor when moisture and temperature were increased. The most effective treatment was the application of 125 C to soybeans containing 20.5 percent moisture. This combination of higher temperature and moisture lowered the activity of trypsin inhibitor by 83.0 percent and that of urease by 87.3 percent.

Table 2. Relative activities of trypsin inhibitor and urease in soybeans following heat treatments.[a,b]

Exit temp. deg.C	Moisture %	Trypsin inhibitor mg/g	Urease pH
Unheated[c]	10.3	53.95	1.97
110	10.3	40.00	1.82
110	20.5	24.00	1.30
125	10.3	22.88	0.55
125	20.5	9.18	0.25

[a]Roast-A-Tron, Mix Mill, Inc., Bluffton, Indiana, USA.

[b]Process time was 30-90 seconds depending on exit temperature required.

[c]Included to establish a baseline of reference.

The growth performance of pigs fed the four dietary treatments is given in table 3. Average daily gain was highest for pigs fed the diet containing soybeans at 20.5 percent moisture heated at 125 C and lowest for those fed soybeans at 10.3 percent moisture heated to 110 C and differences were significant. Pigs fed the diets where soybeans contained 10.3 percent moisture heated to 125 C or those fed soybeans containing 20.5 percent moisture heated to 110 C displayed similar average daily gains. Average daily feed consumption was highest for pigs fed the diet containing soybeans at 20.5 percent moisture heated at 125 C when compared with those in other treatments and means also were different (P<.05). Feed efficiency was improved for pigs fed diets containing soybeans adjusted to 20.5 percent moisture and heated at 110 C or 125 C when compared with groups fed soybeans heat processed (either temperature) at 10.3 percent moisture.

Table 3. Performance[a] of pigs fed diets with soybeans initially containing 10.3 or 20.5 percent moisture and heated to 110 C or 125 C.

	Soybeans, var. Bragg				
Process temperature, C	110	110	125	125	
Moisture, %	10.3	20.5	10.3	20.5	SE[b]
Initial wt, kg	5.02	5.03	5.03	5.03	-
Final wt, kg	7.65	9.75	11.03	13.71	-
Daily gain, kg	.06[e]	.13[d]	.17[d]	.25[c]	.02
Daily intake, kg	.32[d]	.41[d]	.39[d]	.55[c]	.02
Feed-to-gain	5.23[c]	3.09[d]	2.33[d]	2.21[d]	.41

[a]Least squares means.

[b]Standard error.

[c,d,e]Means in rows with different superscripts differ (P<.05).

The factorial analysis for main effects of moisture content and heat treatment are presented in table 4. The main effects of increasing roasting temperature or moisture content were to significantly improve daily gain and daily intake of feed. In addition to improved average daily gain and feed intake, the factorial analysis revealed that the efficiency of conversion of feed to BW gain was improved ($P<.05$) when the processing temperature was raised from 110 C to 125 C.

Table 4. Factorial analysis for main effects of soybean moisture content and process temperature on growth performance of pigs.

	Temperature		Moisture		
	110 C	125 C	10.3%	20.5%	SE[a]
Initial wt, kg	5.02	5.02	5.02	5.02	-
Final wt, kg	8.69	12.41	9.35	11.78	-
Daily gain, kg	.10[b]	.21[c]	.12[b]	.19[c]	.04
Daily intake, kg	.37[b]	.47[c]	.35[b]	.48[c]	.03
Feed-to-gain	4.16[d]	2.27[e]	3.78	2.65	.79

[a]Standard error.

[b,c,d,e]Means in rows by effect (temperature or moisture) with different superscripts differ ($P<.05$).

These data collectively show that increasing moisture content of soybeans prior to heat processing lowers the temperature required to inactivate trypsin inhibitor and urease. Increasing moisture content of whole, full fat soybeans prior to heat processing also improved their nutritional value and may offer an alternative method of processing requiring lower energy expenditures.

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METHODS OF REMOVING TANNIN FROM Vicia faba L.

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Summary

Ground or whole seeds of a commercially available faba bean of Spanish origin (INIA-06) were subjected to the following treatments for the removal of tannins: soaking in 4% NaOH solution, autoclaving at 121ºC, addition of adsorbents (polyvinylpyrrolidone -PVP- and polyethylene glycol -PEG 4000-) and husking. The effect of the treatments on tannin content, acid detergent fiber, acid detergent lignin, soluble N and "in vitro" digestibility of dry matter(IVDDM) and of crude protein(IVDP) was studied. The effects were compared to those resulting from genetic improvement. Treatment with NaOH and autoclaving did not improve the nutritive value of the faba beans in spite of reducing tannin content by 97% and 57%, respectively. Husking and treatment with adsorbents increased IVDDM and IVDP values to levels similar to those found in white flower lines obtained through genetic selection.
Keywords: Vicia faba L., tannin, methods of removing, autoclaved, alkali, husking, adsorbent, genetic breeding.

Introduction

The decrease in nutritive value caused by the presence of tannins in different products has led to the study of different methods of removal: alkali treatment, cooking, addition of adsorbent like PVP, PEG, etc. and the search for tannin free cultivars through genetic breeding. Besides the direct effect of the treatments on tannin removal, dependent on the conditions that are employed (temperature, time, concentration, etc.), and on the type of product treated, these may also affect other of its components (protein, starch, etc.) as well as their overall nutritive value. This last aspect has been recently reviewed by Garrido (1987).

The quantification of these effects still warrant further study, especially in V. faba. Thus, the purpose of the present paper is to compare different methods of removing tannin: husking, alkali treatment, autoclaving, the use of adsorbents and genetic breeding by quantifying the decrease in tannin levels and its influence on the nutritive value of the faba bean seeds.

Material and Methods

Alkali treatment, autoclaving and the use of adsorbents were carried out on a commercial cultivar of faba beans (INIA-06); husking on 12 commercial cultivars of Spanish origin and also were compared, 8 lines of white flower (WF), 8 of normal flower (NF) and 8 of diffused flower (DF) obtained from a genetic breeding programme designed to produce lines with low tannin content.

The analytical methods for neutral detergent fiber (NDF),acid detergent

fiber (ADF) and acid detergent lignin (ADL) were those proposed by Robertson & Van Soest (1977). NDF was previously treated with B.subtilis α-amylase (McQueen & Nicholson, 1979); "in vitro" digestibility of the protein (IVDP),HCl-pepsin (A.O.A.C.,1975); soluble nitrogen, SN (Verité & Demarquilly, 1978); "in vitro" digestibility of dry matter, IVDDM (Tilley & Terry, 1963) and for tannin the Tannin Index, TI (Ford & Hewitt, 1979).

Alkali treatment was carried out with 4% (weight/volume) NaOH solution (0.005 M and 0.05 M) at 100°C for 5 and 20 minutes and at 30°C for 1 hr. and 24 hrs. Autoclaving was done at 121°C for 15, 30 and 60 minutes following the recommendations by Marquardt & Ward (1979). Two synthetic polimeres, polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG 4000), were used.

The SAS software programme (SAS User's Guide Statistics, 1982) was used to describe the variables, groups and subgroups, variance analysis, comparision of means and for the analysis of the principal components.

Results and discussion

Table 1 summarizes the results of the treatments carried out. Only the average values of the most effective treatments in the reduction of tannin content are shown.

Table 1. Comparison of different tannin removal treatments

Treatment	TI A_{535nm}	NDF DM%	ADF DM%	ADL DM%	SN TN%	IVDP %	IVDDM %
Control (1)	0.86	12.1	12,2	0.4	69.8	92.9	89.2
Husking (2)	0.02	-	2.2	0.0	-	97.4	92.8
Alkali (3)	0.03	-	14.0	1.2	38.7	92.3	90.7
Autoclaving (4)	0.37	14.0	12.4	2.1	13.9	92.8	90.7
Adsorbents (5)	0.04	-	-	-	-	97.0	92.6
Genetic selection (6)	0.02	-	10.5	0.21	76.3	97.2	93.9

(1):INIA-06 cultivar without treat.; 2:Mean 12 commercial cultivars;
(3):NaOH, 0.05M, 100ºC, 20'; (4):121ºC, 60'; (5):160 mg PVP/mg tannin.

Husking drastically reduced tannin content and structural components. This is in accord with results found by Newton & Hill (1983) and Marquardt & Bell (1986).

Alkali treatment and autoclaving, despite producing important reductions in the TI (97% and 57%, respectively), did not improve IVDP and IVDDM. A general comment for both could be that the two produce "heat damage" responsible for such low values of SN (38.6% and 13.8%, respectively). The possible liberation of phenolic compounds and their polymerization with the formation of "artifact lignin" would be responsible for the increase in ADL values. The nutritional importance and analytical interference of these liberated compounds has been pointed out by Van Soest (1982) and together with the protein alteration, could be the cause of the low increases in the IVDDM value, especially when compared to the other treatments studied.

With the autoclave tannin removal (TI) is not complete. This could be

explained by a possible thermoresistence of tannins, as has been reported for other legumes (Aw & Swanson, 1985). The effects produced by the heat on this and other anti-nutritive factors as well as on other components of the product are extremely interesting, for example, the damage it causes to starch (Van Soest, 1982). Increases in NDF with important difficulties in filtration, probably produced by an insufficient disolution of the starch in the neutral detergent solution (Asp & Johanson, 1984) were detected.

In treatment with adsorbents quantity is a decisive factor. In our case, 160 mg PVP/mg tannin were needed to obtain maximum precipitation.

The results obtained for the three groups of colour flower show differences ($p<0.05$) for all the characteristics studied. The high variability detected in tannin contents and in IVDDM for the NF group complete current data (Cabrera & Martin, 1986) as to the possibility of selecting low tannin content lines from the NF group.

An analysis of the principal components with 68 samples and all the variables studied has also been carried out. Two factors explain 80.4% of the variance; 58% corresponds to the first and is associated to "nutritive quality" and 22% to the second, associated to a higher protein content and tannin level. The projection on the two factors (Fig. 1) clearly shows the discrimination between groups.

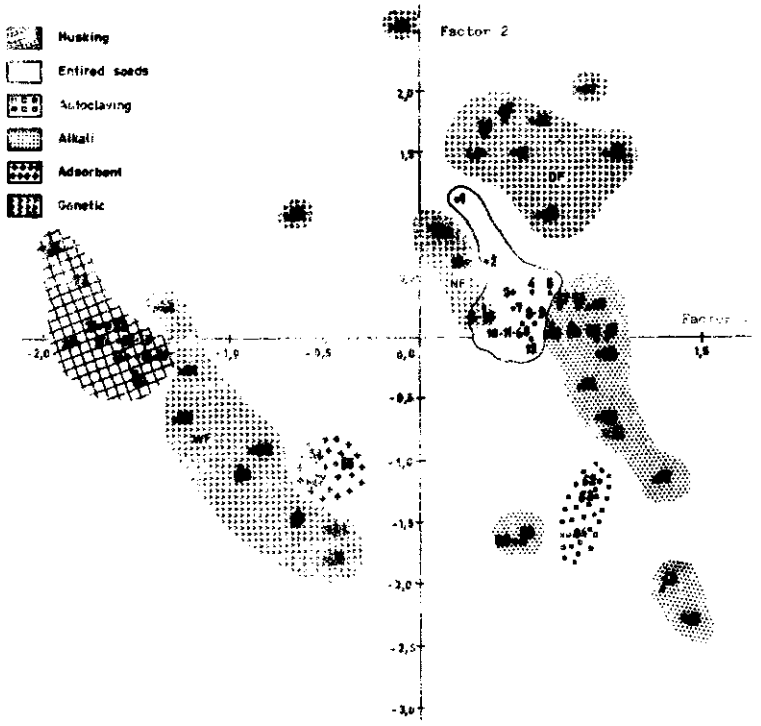

Fig. 1. An analysis of the main components

All the treatments produced a decrease in tannin content, although their effect on nutritive quality varied. The high variability in the results of the alkali and autoclave treatments is due to differences in treatment conditions. However, in all cases the "nutritive quality" is far behind the WF lines. Moreover, husked grains and those treated with adsorbents have somewhat similar values. The high variability within the WF group induces us to consider the possibility of intragroup genetic breeding. This variability could be attributed to differences in the monomeric phenolic compounds given their known presence, although with limited effects on the use of nutrients (Marquardt et al., 1977). This last aspect warrants further studied.

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FEEDING VALUE OF PISUM SATIVUM FOR PIGS :

- INFLUENCE OF TECHNOLOGY
- INFLUENCE OF GENOTYPE (TRYPSIN INHIBITOR ACTIVITY)

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Summary

Like other pulses, pea (Pisum sativum) contains antinutritional factors. In the pea varieties which are grown in France, the main antinutritional factors are trypsin inhibitors. However, some varieties have low trypsin inhibitor activity and can be used raw up to 45 % in diets for growing finishing pigs without any consequence on growth and carcass performance. High trypsin inhibitor activity varieties lead to lower performance between 25 and 60 kg. Trypsin inhibitor activity is not affected by storing the pea during one year, is little affected by pelleting a diet containing 30 % pea, and is greatly reduced by extrusion.
Keywords : Pea, trypsin inhibitor activity, storage, pelleting, extrusion, pigs.

Introduction

Pea has been grown more and more in Europe for the past ten years, especially in France, since in 1988 the production is estimated at 3,525,000 tons in the EEC, of which 2,350,000 tons in France. This production is mainly consumed by pigs, especially between 25 and 100 kg live weight.

Like other pulses, pea (Pisum sativum) is known to contain antinutritional factors (Grosjean, 1985) mainly trypsin inhibitors, hemagglutinins, tannins and α-galactosides. The effects of α-galactosides have been little studied. Tannins are not found in the pea varieties which are grown in France for monogastrics because they are Pisum sativum hortense, with white flowers. Tannins are only found in Pisum sativum arvense which is grown for forage and not for seed since the seed yield is lower than with the hortense type. The hemagglutinins seem to have little effect in the French pea (Bertrand et al., 1988). So, among the antinutritional factors found in French pea, only trypsin inhibitors may cause lower performance in pig feeding. Trypsin inhibitors are proteins and their activity can be reduced by heat. Moreover, trypsin inhibitor activity is quite variable. High activity is found in winter varieties (from 8.2 to 15.9 trypsin inhibition units / mg dry matter - T.I.U./mg D.M.) while low activity is found in spring varieties (from 2.3 to 4.5 T.I.U./mg D.M.) (Valdebouze et al., 1980; Valdebouze & Gaborit, 1985). Recent winter varieties can have 6-8 T.I.U./mg D.M.

Effect of storage

A one year storage did not change the trypsin inhibitor activity in a winter pea batch (cv. Frimas) (9.8 T.I.U./mg D.M. after harvest and 10.2 one year later). The one year stored batch was compared to a batch of the same variety grown a year later (12.9 T.U.I./mg D.M.) in a feeding experiment with growing-finishing pigs. The two batches led to identical growth and carcass performance when incorporated at 36% in maize soyabean meal diets (Grosjean & Castaing, 1983).

Effect of extrusion

Extruding winter pea (cv. Frimas) with an Instapro apparatus (Dievet technic) reduced its trypsin inhibitor activity (from 10.5 to 0.3 T.I.U./mg D.M.). A feeding experiment with maize-based diets containing the raw and the extruded pea batches had been carried out with pigs. Relatively to the control diet (maize, soyabean meal with minerals and vitamins), the diet containing 30 % raw winter pea and 60% maize led to a higher feed/gain ratio (25 %) between 25 and 60 kg live weight. The diet containing 30 % extruded pea and 60% maize led to a feed/gain ratio only 7 % higher ($p<0.01$) than the control one. This 7% difference may be explained by the insufficient sulphur amino acid (and tryptophan?) level of the diet. Between 60 and 100 kg, the three diets allowed the same performance (Grosjean & Castaing, 1983).

Effect of pelleting

The effect of pelleting an amino acid balanced diet containing 30 % pea was studied simultaneously with the effect of pea genotype, in a recent digestibility trial and in two growth experiments carried out with Institut National de la Recherche Agronomique (INRA) and feed manufacturer SANDERS (Grosjean et al., 1989). Pelleting was made at 80°C with steam, and pea varieties were winter pea Frisson (9.8 T.I.U./mg D.M.) and spring pea Finale (3.9 T.I.U./mg D.M.). The trypsin inhibitor activity of diets containing pea is little reduced by pelleting (2.7 vs 3.2 T.I.U./mg for the diets containing winter pea and 1.2 vs 1.3 T.I.U./mg for the diets containing spring pea).

The faecal digestibility of energy reached 86.0 - 87.1 - 84.2 and 89.0 % respectively for winter pea in meal, winter pea in pellet, spring pea in meal and spring pea in pellet. The digestibility of pea nitrogen reached respectively 86.9 - 88.3 - 84.8 and 87.5 %. So pelleting has little effect on digestibility of energy and nitrogen of the pea.

In the two growth trials, there was no interaction between pelleting and pea genotype effects on pig performance. In the two trials, pelleting improved feed/gain ratio :12.3 % ($p<0.05$) between 25 and 60 kg and 8.4 % ($p<0.05$) between 60 and 100 kg in the first trial, and 4.5 % ($p<0.05$) and 1.0 % (non significant) in the second trial.

The lack of interaction between pelleting and trypsin inhibitor activity of the diet had already been found in a previous work comparing maize soyabean meal diets containing 0

or 30 % winter pea and given in meal or in pellet. Only the positive effect of pelleting and the negative effect of winter pea had been found (Castaing & Leuillet, 1981).

Effect of pea genotype

A comparison of the feeding value of high and low trypsin inhibitor activity peas was studied in five experiments.

In only one trial, there was no difference between diets containing winter or spring pea, regardeless of the live weight of the animals and the diet form - meal or pellets - (Grosjean et al., 1989).

In an other trial, Grosjean et al. (1989) compared wheat based diets in meal and containing 0 - 30% high trypsin inhibitor activity pea (cv. Frisson) and 30% low trypsin inhibitor activity pea (cv. Finale). The control diet led to better feed/gain ratio than the diets with high and low trypsin inhibitor activity pea between 25 and 60 kg live weight (5.3 and 1.2 % respectively - $p<0.05$) but no difference was observed at 100 kg live weight.

In the other trials, high trypsin inhibitor activity pea was less efficient than low trypsin inhibitor activity pea, especially in the begining of the trials.

In one of the two trials testing the interaction between pelleting and pea genotype (Grosjean et al., 1989), the two diets containing winter pea (cv. Frisson) led to lower performance than the control diets without pea or the diets containing spring pea (cv. Finale) between 25 and 60 kg live weight (feed/gain ratio increased by 9.5 and 7.5 % - $p<0.05$). These differences lessened in the finishing period (4.1 and 0.2 % between 60 and 100 kg live weight - $p>0.10$).

This result, obtained with wheat based diets, is in agreement with our previous works comparing raw pea varieties with low, medium or high trypsin inhibitor activity in maize soyabean meal diets (Castaing & Leuillet, 1981 ; Grosjean et al., 1986). The diets with 30% high trypsin inhibitor activity pea led to lower growth performance than the diets with 30% low trypsin inhibitor activity pea: 5.9 % ($p<0.01$) between 25 and 60 kg, and 3.4 % ($p=0.10$) between 60 and 100 kg. The diets containing low trypsin inhibitor activity pea led to the same performance as the control diets. The diet with 30% medium trypsin inhibitor activity pea led to intermediate results.

The high feeding value of low trypsin inhibitor activity pea was recently confirmed in three experiments with amino acid balanced diets containing 0 or 40-45 % pea. Diets containing pea were used by pigs as efficiently as the control diets (Gatel et al., 1989).

Conclusion

In conclusion, these works reveal that the pelleting effect is not important in comparison with the extrusion one. It can be supposed that during pelleting, trypsin inhibitors are not heated enough to be inactivated. During extrusion, feed temperature is higher than during pelleting and trypsin inhibitors are inactivated. However improvement of the feeding value of high trypsin inhibitor activity pea cannot be only ex-

plained by the decrease of trypsin inhibitor activity because temperature modifies also starch (Champ & Delort Laval, 1987). In practice, extrusion is an expensive technic and is scarcely used for growing finishing pig feeds.

With low trypsin inhibitor activity peas (which represent 95 % of the French production), technology is not necessary. These pea varieties can be incorporated without limit (i.e. up to 40-45 %) in amino acid balanced diets for growing finishing pigs.

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EFFECT OF COOKING AND AMINO ACID SUPPLEMENTATION ON THE NUTRITIVE VALUE OF LENTILS (Lens culinaris M.)

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Summary

Four cultivars of lentils (Lens culinaris M.) were analysed for their proximate contents. Each raw or cooked lentil was combined into semi-purified diets and fed to male weanling rats. The ground lentil provided the sole source of protein in each diet. Overall, the True Digestibility of the cooked lentil was higher than the raw lentil. The Biological Value was also improved by cooking. The brown seeded lentil, Olympic, had a higher Biological Value for both the raw and cooked seed when compared with the other three lentils. In all cases addition of methionine increased the overall Biological Value of the diet but the response was different for raw and cooked lentils.
Keywords: lentils, proximate analysis, cooking, rats, protein quality, True Digestibility, Biological Value, Net Protein Utilization, methionine supplementation, trypsin inhibitor.

Introduction

Lentils (Lens culinaris Medikus) were among the earliest food crops cultivated by Neolithic man. They are now very important sources of protein in the diet of millions of people in the Mediterranean area, Africa, the Middle East, Southern Asia and South America. The nutritional evaluation of this crop has been summarised by Aykroyd & Doughty (1964), Abu-Shakra & Tannous (1981) and by Savage (1988). Lentils are noted for their protein content. They are good sources of lysine and threonine which are deficient in cereals, but are poor sources of methionine and tryptophan (Venkat Rao et al., 1964; McCurdy et al., 1978). Many studies for instance, Agarwal & Bhattacharya (1983) have published values for the proximate analysis of improved varieties of lentils but few studies have included determinations of protein quality of both raw and cooked lentils (Savage 1988).

The nutritive value of lentils is limited by antinutritive factors present in the raw seed. Most of these, for instance, trypsin inhibitors and haemagglutinins can be inactivated by heat treatment (Savage, 1988) and/or leaching with water (Venkat Rao et al., 1964). Saponins, on the other hand, appear to have a positive nutritional effect by lowering blood cholesterol levels in humans. They are heat stable but they are extracted in hot water (Fenwick & Oakenfull, 1983).

The purpose of this investigation was to study the nutritive value of four different cultivars of lentils grown in comparable conditions in New Zealand and to determine the effect of cooking on the nutritional quality of the seed. In addition the optimum level of methionine supplementation on the overall protein quality of raw or cooked seed was also investigated.

Animal trials

Titore, a small red seeded cultivar and 3 brown seeded lentils, Big yellow, Laird and Olympic provided the sole source of protein in a semi-purified diet (Johnston & Savage, 1987). Nine g of each diet was fed to 5 male Wistar albino rats housed individually in metabolism cages (Thompson, 1970). Water was provided ad lib. The rats were given a 5 day pre-test period followed by a 7 day balance trial during which quantitative collections of urine and faeces were made. The True Digestibility (TD), Biological Value (BV) and Net Protein Utilization (NPU) were calculated using the Thomas-Mitchell method (Mitchell, 1924). Johnston & Savage (1987) give details of this procedure and the method of calculation of metabolic faecal nitrogen (MFN) and endogenous urinary nitrogen (EUN).

In a following experiment the basal diets formulated from either raw or cooked lentils were then supplemented with DL-methionine increments ranging from 0.05 to 0.7% of the diet.

The data were subjected to analysis of variance (Snedecor, 1965). Duncan's multiple range test was used to test for significant differences between means (Duncan, 1955).

Results

The proximate analysis of the raw and cooked lentil seeds is shown in table 1. The results are in general agreement with the data reviewed by Savage (1988) and with similar varieties grown in New Zealand (Savage et al., 1986).

Table 1. Proximate analysis of raw and cooked lentil cultivars (g/100 g DM)

	Titore		Big yellow		Laird		Olympic	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Dry matter	86.5	92.6	87.8	93.0	86.4	95.4	87.1	96.2
Total ash	2.2	1.4	2.2	1.4	2.4	1.7	2.2	1.5
Ether extract	1.4	1.5	1.2	1.4	1.4	1.6	1.1	1.4
Crude protein	21.8	24.8	21.5	24.6	22.9	27.3	20.9	24.4
Crude Fibre	3.3	3.1	2.9	2.7	3.6	3.4	3.0	2.7
N-free extract	57.8	61.8	60.0	62.9	56.1	61.4	59.9	66.2
Energy MJ/kg DM	19.0	17.7	19.0	17.9	18.9	17.3	18.9	17.0

The True Digestibility of raw lentil protein ranged from 81 to 84% (Table 2). Cooking improved the True Digestibility of all varieties except Olympic. The Biological Value of raw Olympic was significantly ($P< 0.05$) higher than the other three varieties. Savage et al., (1986) also observed that the numbered variety 299184 grown under the same

conditions as Olympic also gave a significantly higher BV when compared to other varieties. In each case cooking resulted in significant ($P< 0.05$) increases in biological value when compared to raw seed. The NPU of Olympic was significantly ($P< 0.05$) higher than the other three varieties while the NPU of cooked Olympic was only marginally higher than the other three varieties.

Table 2 Nutritive value of the raw and cooked lentil cultivars

Cultivar	True Digestibility		Biological Value*+		Net Protein Utilization	
	Raw	Cooked	Raw	Cooked	Raw	Cooked
Titore	80.7^{a}	83.6^{ab}	34.1^{b}	44.1^{b}	26.5^{b}	36.8^{a}
Big yellow	82.0^{a}	83.4^{ab}	34.3^{b}	43.7^{b}	28.2^{b}	36.5^{a}
Laird	80.7^{a}	85.1^{a}	34.8^{b}	43.3^{b}	28.1^{b}	36.8^{a}
Olympic	84.2^{a}	81.7^{b}	41.5^{a}	48.7^{a}	35.0^{a}	39.8^{a}
Standard Error of Mean	1.38	0.71	2.31	1.29	2.00	1.24

Mean values with different superscripts in each column are significantly different ($P< 0.05$)

* MFN 1.005 mg N g DM consumed
\+ EUN 949.5 mg N/(kg bodyweight)$^{0.75}$

The effect of addition of methionine to diets containing raw or cooked lentil cultivars is shown in Figs. 1 to 4. The maximum BV (65 - 68) for raw lentil protein was reached on addition of between 0.5 and 0.6% methionine to the diet. Addition of 0.7% methionine in the case of raw Big yellow and Titore produced a markedly reduced response which was also shown to a limited extent by raw Olympic and Laird. The maximum BV reached for the cooked lentils was 84 - 86% with addition of between 0.3 and 0.35% methionine.

Discussion

On the whole, cooking lentils improves the True Digestibility of nitrogen. It is interesting to note that Olympic which had the highest TD for the raw protein showed a small fall on cooking and was marginally lower than the other three cooked lentils. The improvement in BV on cooking is probally due, at least in part, to the destruction or elimination of some antinutritive factors in the lentils. Savage et al (1986) showed that cooking reduced the trypsin inhibitor activity to very low levels. The trypsin inhibitor activity of these New Zealand-grown whole raw lentils ranged from 0 U/g (Olympic) to 122 U/g (Laird).

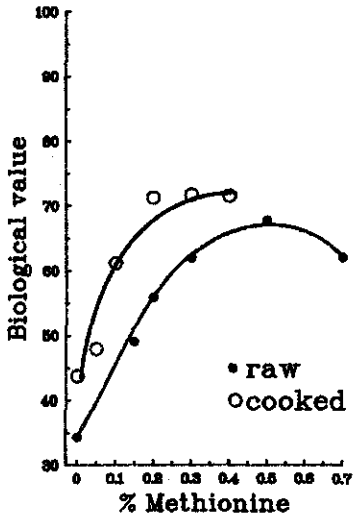

Fig. 1. Big yellow

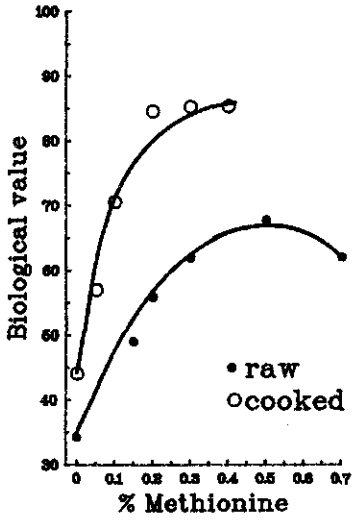

Fig. 3. Titore

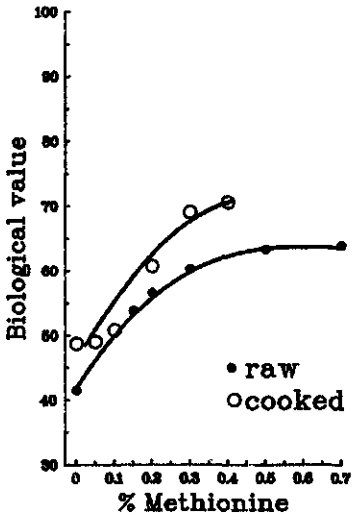

Fig. 2. Olympic

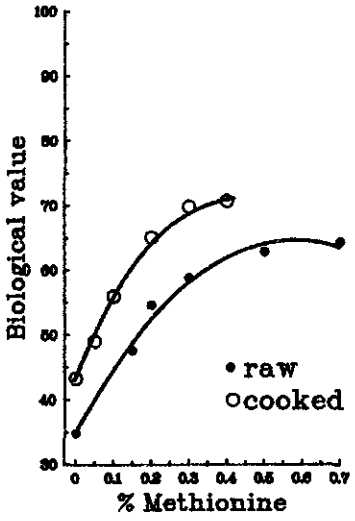

Fig. 4. Laird

The differential response to methionine in raw or cooked lentil based diets is more interesting. Addition of methionine to a protein source known to be deficient in the essential amino acid methionine would be expected to give an increased response until it was no longer the first limiting amino acid. Addition of further amounts of methionine would then have a negative effect as the animal would have to degrade the excess. In this experiment cooking would have largely eliminated the trypsin inhibitor which would have prevented dietary protein from being digested completely in the raw lentil. In addition the pancreas may function abnormally when trypsin inhibitors are present so dietary amino acids are diverted from synthesis of body tissue to the synthesis of extra pancreatic enzymes. As pancreatic proteolytic enzymes are rich in sulphur containing amino acids, increased pancreatic activity would divert cystine and methionine from synthesis of body tissue to pancreatic enzyme synthesis. This would accentuate the deficiency of sulphur amino acids in lentils. This explains why supplementation with methionine does improve the Biological Value of raw lentils. The improvement in the BV on addition of methionine to cooked lentil diets confirms that lentil protein is deficient in methionine. The fact that the maximum BV attained in the raw lentil diets with methionine supplementation is still below that reached for cooked lentils suggests that the presence of trypsin inhibitors in raw lentils does not provide the whole explanation for the lower BV of raw lentils.

Lentils also contain tannins, a diverse group of polyphenolic compounds which can depress the apparent digestibility of protein. Phenolic compounds are generally not toxic but if they are absorbed by mammals their detoxification involves methylation which would put further stress on the limited methionine content of lentils. The generally small increase in True Digestibility of the cooked lentils in this experiment (Table 2) may suggest that some tannins were eliminated during cooking but this does not provide a complete answer to the differential response of raw and cooked lentils on addition of methionine.

Conclusions

The difference in response to graded additions of methionine in the raw or cooked lentils in this experiment could be an indication of the overall metabolic effect of the trypsin inhibitors which were largely eliminated by cooking. Since lentils are widely used in the diets of millions of people and many of these people are at or below subsistence level, improvement in the quality of lentils by reduction in the trypsin inhibitor and tannin contents would have a considerable beneficial effect. In addition research priority should be given to screening lentil lines for high amino acid content, particularly methionine. The large range in methionine contents observed by Savage (1988) would suggest that selection for higher methionine content from existing lines would be quite feasible.

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ACTIVITY AND THERMAL INACTIVATION OF PROTEASE INHIBITORS IN GRAIN LEGUMES

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Summary

Trypsin and chymotrypsin inhibitor activity in a range of leguminous seeds was investigated. Soybeans contained the highest levels of both trypsin and chymotrypsin inhibitors, the lowest levels being recorded in lupins. There was a wide variation in both trypsin and chymotrypsin inhibitor activity between the varieties of each legume and this was more pronounced among the soybean cultivars. The activity of chymotrypsin inhibitor was higher than the trypsin inhibitor in some legumes and not in others. Both trypsin and chymotrypsin inhibitors of soybeans and mungbeans were resistant to dry heat and retained more than 50% activity after heating at 105°C for 15 minutes. Autoclaving for 5 minutes at 121°C virtually destroyed trypsin and chymotrypsin inhibitor activities in both legumes. The autoclaving process appears to be more effective in destroying protease inhibitors compared to dry heat under the conditions employed.
Keywords: protease inhibitors, legumes, inactivation, thermal.

Introduction

The low molecular weight protein with a unique ability to inhibit the proteolytic activity of certain enzymes (Ryan, 1973) are known to be present throughout the plant kingdom, especially the legumes (Wagner & Riehm, 1967; de Lumen & Salamat, 1980; Al-bakir, 1982; Tan et al. 1984). In fact, all lugumes that have been looked at to date have been found to contain protease inhibitors (trypsin and chymotrypsin) to varying degrees. These inhibitors have attracted the attention of food chemists and nutritionists because of their possible effect on the nutritive value of food proteins (Erickson et al., 1979; Tan & Wong, 1982). Several of these inhibitors are double headed and are capable of inhibiting both trypsin and chymotrypsin (Haynes & Feeney, 1967; Richardson, 1977).

The extent to which the protease inhibitors in legumes are destroyed by heat is a function of the temperature, duration of heating, type of heat and moisture content. Comparatively, there are only a few detailed studies which have dealt with the effect of various types of heat treatments on the trypsin and chymotrypsin inhibitor contents of grain legumes. In this paper, the trypsin and chymotrypsin inhibiting activities of several legumes are investigated and the effect of various heat treatments on their stability is reported.

Results and discussion

In the present study the trypsin and chymotrypsin were extracted in tris-buffer (pH 8.0, 0.1 M containing 10 mM $CaCl_2$) and a complete extraction was achieved at two hours. Whole dry seeds were ground in a Udy cyclone mill to pass through a 100 mesh sieve before the preparation

of extracts. When this procedure was compared with the sample preparation method, extraction medium and assay procedure (AOCS official method) developed by Kakade et al. (1969), a close agreement between the trypsin inhibitor activities was achieved. Synthetic substrates N-benzoyl-L-arginine-p-nitroanilide (BAPNA) and N-benzoyl-L-tyrosine-p-nitroanilide (BTPNA) were used for assaying trypsin and chymotrypsin inhibitor activities respectively. Chymotrypsin inhibitor activity was determined by using a method modified from Hirado et al. (1981).

The range of mean results for duplicate analysis of various legumes representing a number of varieties are given in Table 1.

The highest concentration of both trypsin and chymotrypsin inhibitors were found in soybeans with a mean average of 15.77 and 9.96 mg inhibitor/g sample respectively. Adzuki beans showed quite a high level of trypsin inhibitor and this value was greater than a number of soybean cultivars examined. The chymotrypsin inhibitor level was below the average value recorded for soybeans but was higher than the values obtained for other legumes. The lowest concentrations were recorded in lupins and some cultivars of lupins were devoid of chymotrypsin inhibitor activity. The levels of trypsin and chymotrypsin inhibitors in chickpeas were higher than those recorded for field peas, mungbeans, pigeon peas and cowpeas. Desi-type chickpeas had higher concentrations of both trypsin and chymotrypsin compared to kabuli-types. Also, the concentration of chymotrypsin inhibitor was greater than the trypsin inhibitor in chickpeas and a similar trend was observed for field peas and lupins. The other legumes, e.g. mungbeans, pigeon peas, adzuki beans, cowpeas and soybeans indicated higher levels of trypsin than chymotrypsin inhibitor.

There was a wide variation in the levels of both trypsin and chymotrypsin inhibitors between the varieties of all legume species. The range was much wider between the soybean cultivars and quite narrow in the case of chickpeas. A similar trend was obvious for chymotrypsin inhibitor activity. The majority of the mungbean cultivars were devoid of chymotrypsin inhibitor activity.

These results clearly show that there are variations in the activity of both trypsin and chymotrypsin among the varieties of these legumes. Such varietal differences are previously reported for limabeans (Ologhobo Fetuga, 1983) and lentils (Weder, J.K, personal communication). Whether these inhibitors from various varieties of these legumes will differ in their physiological effects on animals remains to be tested.

Samples of soybeans and mungbeans were subjected to various heat-treatments. Duplicate lots of mungbeans and soybeans were placed to a depth of approximately 10 mm in metal petri-dishes and heated in a forced-draught oven to a temperature of 90, 105, 120, 135 and 150°C for 15 minutes. The oven was allowed to reach the desired temperature before heating the seeds. After cooling, the seeds were fine ground. The patterns of trypsin and chymotrypsin inhibitors depletion were slightly different when the seeds were subjected to dry or wet heat treatments.

Table 1. Trypsin and chymotrypsin inhibiting ability of grain legumes.

Grain	No. of samples	Trypsin Inhibitor[a]		Chymotrypsin inhibitor[b]	
		range	mean	range	mean
Pisum sativum					
Field peas	9	0.56 - 1.90	1.08	0.88 - 2.64	1.6
Phaseolus mungo					
Mungbeans	6	1.84 - 3.25	2.37	1.12 - 1.26	1.19
Cajanus cajan					
Pigeon peas	7	2.33 - 5.20	3.79	1.65 - 2.75	2.34
Phaseolus angularis					
Adzuki beans	1	-	12.40	-	5.70
Vigna sinensis					
Cowpeas	1	-	3.49	-	1.10
Cicer arietinum					
Chickpeas					
desi	11	3.73 - 5.04	4.12	4.00 - 5.20	4.78
kabuli	3	2.10 - 3.33	2.63	3.35 - 4.50	3.97
Lupinus spp.					
Lupins	8	0.16 - 0.29	0.20	0.00 - 0.70	0.64
Glycine max					
Soybeans	13	11.47 - 21.06	15.77	8.00 - 12.20	9.96

[a], activity expressed as mg inhibitor/g sample, on the basis of 62% centration of active trypsin in the commercial sample used as standard.

[b], activity expressed as mg inhibitor/g sample, assuming the concentration of active chymotrypsin in the commercial sample as 100%.

There was a gradual decrease in the activity of trypsin inhibitor with only 30% decrease after heating at 105°C for 15 minutes (Table 2). There was a sharp decline up to 120°C and almost complete elimination occurred after heating at 135°C for 15 minutes. A similar pattern was observed in chymotrypsin inhibitor activity.

Duplicate lots of soybeans and mungbeans were also subjected to autoclaving at 121°C for 5, 15, 25, 35 and 45 minutes (Table 3). More than 80% of trypsin inhibitor activity was removed after 5 minutes autoclaving and there was a slow depletion at subsequent times. More than 95% chymotrypsin inhibitor was destroyed after 5 minutes autoclaving. Mungbeans used in this study were devoid of chymotrypsin inhibitor activity.

Table 2. Trypsin and chymotrypsin inhibiting abilities of soybeans and mungbeans heated to various temperatures for 15 minutes.

Temperature °C	Trypsin inhibitor activity		Chymotrypsin inhibitor activity	
	soybeans	mungbeans	soybeans	mungbeans
0	13.5	2.2	11.7	0.0
90	10.4	2.2	10.1	0.0
105	9.5	1.4	9.2	0.0
120	2.3	0.3	3.5	0.0
135	0.7	0.2	1.7	0.0
150	0.5	0.2	1.4	0.0

Table 3. Effect of autoclave treatments on trypsin and chymotrypsin inhibitor activity of soybeans and mungbeans.

Autoclaving time, min	Trypsin inhibitor activity		Chymotrypsin inhibitor activity	
	soybeans	mungbeans	soybeans	mungbeans
0	13.5	2.2	11.7	0.0
5	1.7	0.4	0.3	0.0
15	0.3	0.2	0.0	0.0
25	0.2	0.1	0.0	0.0
35	0.1	0.0	0.0	0.0
45	0.1	0.0	0.0	0.0

It appears that these inhibitors are extremely resistant to dry heat. Autoclaving on the other hand is quite effective in eliminating both trypsin and chymotrypsin inhibitors from these grains. Similar differences in the thermal stability of trypsin and chymotrypsin inhibitors in winged beans have been reported (Tan & Wong, 1982; Tan et al., 1984). It is therefore important that, if dry heat treatment is applied, high temperatures above 120°C be selected.

It is now well established that the increased intake of raw beans, high in trypsin and chymotrypsin inhibitor activity stimulates pancreatic juice secretion and causes pancreatic hypertrophy and growth inhibition. Nevertheless,the exact nutritional significance of these inhibitors is not clear, because there does not appear to be any clear-cut correlation between the protease inhibitor content of various legumes and the beneficial effect which heat has on their nutritional value (Borchers & Ackerson, 1950). The presence of other anti-nutritional factors in these legumes no doubt tends to obscure whatever detrimental effect the trypsin inhibitors per se may have on animal health and growth.

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EXTRUDED MIXTURES OF BEANS (PHASEOLUS VULGARIS) AND SOYBEANS AS PROTEIN SOURCES IN SWINE AND CHICK DIETS

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Summary

Experiments involving swine and young chicks indicated that simple dry extrusion of beans (Phaseolus vulgaris) and soybeans together may offer a practical and easy method for heat processing beans, and perhaps other low lipid legume seeds, for use in non-ruminant animal diets. This procedure would be partially applicable in the utilization of cull beans and other legume seeds not suited for or usually used for human consumption.
Keywords: Extrusion, Beans (Phaseolus vulgaris), Soybeans, Swine, Chicks

Introduction

Heat processing, such as autoclaving, has been shown to decrease the detrimental effects of many antinutritional factors in dry beans (Phaseolus vulgaris) (Liener 1976). The laboratory autoclave, however, is not a practical processing method for beans, or other legume seeds requiring heat processing, for use in livestock feeding. The extrusion process, which has been reviewed by Harper (1978), offers a practical heat processing method. Simple extrusion cooking has been successfully used to heat process whole soybeans for use in diets of non-ruminant animals (Pond and Maner 1984). Feeding trials conducted with chicks and young pigs have shown the extrusion process to be as effective as autoclaving for heat processing of cull red beans (Myer et al., 1982; Myer and Froseth, 1983a). However, the extrusion of dry beans is difficult because of the beans' low lipid content. Water or steam must be added to dry beans if they are to be extruded alone. Conversely, extrusion of whole soybeans is relatively easy because of the soybeans' high content of oil, which acts as a lubricant during the extrusion process. Thus, extruding a mixture of beans and soybeans could be a practical method for heat processing raw cull beans, those beans not suited for human consumption, for use in non-ruminant animal diets.

Experimental

Two experiments with pigs (one with young, starting pigs and the other with older, growing-finishing swine) and one experiment with chicks were conducted to evaluate extruded mixtures of beans (small red, recleaned culls) and soybeans as dietary protein supplements (Myer et al., 1982;

Myer and Froseth, 1983b). Simple dry extrusion[1] process was utilized in the extrusion of the bean and soybean mixtures. In the chick trial, diets containing one-half of the dietary protein from the various extruded products or soybean meal were evaluated. The 20 treatments included 10 different test protein sources either with or without supplemental DL-methionine (0.2% of diet). In both swine trials, the various extruded products or soybean meal provided the sole supplemental protein source in barley-based diets. In all the feeding trials, the diets were formulated following USA National Research Council (NRC) recommendations and were offered ad libitum in the meal form.

Results and discussion

The cull small red beans used contained, on a dry matter basis, an average of 27.4% crude protein (N x 6.25) and 1.4% ether extract. The beans also averaged 7.4, 5.1 and 2.5 g/100 g protein of lysine, threonine and the sulfur amino acids, respectively. In comparison to the soybeans used, as a percentage of their crude protein content, the beans contained 16% more lysine and 9% more threonine, but only 69% as much of the S-amino acids.

Extrusion reduced trypsin inhibitor and lectin activities of the bean-soybean mixtures used in the chick and swine starter trials by at least 90%, but the mixtures used in the growing-finishing trial still contained 10 to 20% residual activities after extrusion. Moisture pretreatment by injecting water (2 to 8% w/w) in the first extrusion chamber during the extrusion process was found to be more effective in reducing trypsin inhibitor and lectin activities in the mixtures used in the chick and swine starter trials than dry extrusion, and agrees with that observed with soybeans (Campbell et al., 1986). The small red beans used were found to contain a significant amount of tannin (6 to 9 mg/g catechin equivalent). Tannin concentration of the various mixtures reflected the proportion of red beans in the various mixtures.

The chick and swine starter trials utilized the same extruded products. Results are summarized in Tables 1 and 2.

In the chick trial, day-old chicks fed diets containing one-half of the total dietary protein from extruded beans had lower ($P<.01$) 2-week gains and gain-to-feed ratios than chicks fed soybean meal. Chicks fed diets containing extruded mixtures of one-third beans plus two-thirds soybeans with supplemental methionine had similar 2-week gains and higher ($P<.05$) gain-to-feed ratios compared to chicks fed the soybean meal diet with supplemental methionine. Chicks fed diets containing extruded mixtures of two-thirds beans plus one-third soybeans with supplemental methionine had decreased ($P<.05$) 2-week gains but similar ($P>.10$) gain-to-feed ratios. Supplementation of diets with methionine, in general, resulted in greater increases in weight gains and gain-to-feed ratios for chicks fed the bean or the 2/3 - 1/3 bean-soybean mixture containing diets compared to chicks fed the soybean diets.

1. Insta-Pro Model 2000 Extruder, Insta Pro Division, Triple F Feeds, Inc., Des Moines, IA (USA)

Table 1. Performance of chicks fed diets containing extruded small red beans, extruded soybeans, or extruded mixtures of beans and soybeans with and without supplemental methionine[1].

Test protein source[2]	Avg. 2-week gain, g		Gain/feed	
	- methionine	+ methionine	- methionine	+ methionine
Soybean meal control	218^{fgh}	226^{hij}	$.73^{de}$	$.75^{ef}$
Dry extruded (DE) soybeans (S)	228^{hij}	241^{j}	$.77^{gh}$	$.80^{hi}$
Wet extruded (WE) soybeans	236^{ij}	230^{hij}	$.79^{hi}$	$.80^{hi}$
WE beans (B)	153^{a}	178^{b}	$.61^{a}$	$.68^{b}$
1/3 WE B, 2/3 DE S	210^{efg}	231^{hij}	$.71^{cd}$	$.78^{ghi}$
2/3 WE B, 1/3 DE S	207^{efg}	200^{cde}	$.68^{b}$	$.73^{de}$
1/3 B, 2/3 S, WE together	210^{efg}	221^{ghi}	$.74^{ef}$	$.78^{ghi}$
2/3 B, 1/3 S, WE together	186^{bc}	228^{hij}	$.68^{b}$	$.75^{ef}$
1/3 B, 2/3 S, DE together	202^{def}	229^{hij}	$.74^{ef}$	$.76^{fg}$
2/3 B, 1/3 S, DE together	190^{bcd}	217^{fgh}	$.69^{bc}$	$.74^{ef}$

[1]Each mean represents three replicates (pens) of ten chicks each.
[2]One-half of the total dietary protein from these sources; the other half was from the corn-soybean meal-fish meal-basel.
[a,b,c,d,e,f,g,h,i,j]Means within each measurement with a common superscript are not significantly different ($P<.05$).

In the starting pig trial, the substitution of extruded mixtures of beans and soybeans as the supplemental protein for soybean meal in the diet for young, starting pigs (average initial weight of 7 kg) decreased average daily gains slightly; however, feed efficiency was not detrimentally affected ($P>.05$). The proportion of beans and soybeans in the mixtures (1/3 - 2/3 vs. 2/3 - 1/3) did not affect performance ($P>.05$) of starting pigs. In both the chick and starting pig trials, the method of extrusion (separate or together) of the various bean-soybean mixtures did not affect animal performance ($P>.05$). Also, in spite of its beneficial effect on reducing toxic factors in the extruded mixtures, moisture pretreatment during the extrusion process had no effect on chick or young pig performance ($P>.05$).

Table 2. Performance of starter pigs fed barley-based diets containing extruded soybeans, extruded beans or mixtures of extruded soybeans and beans as supplemental protein sources[1].

Supplemental protein source[2]	Daily weight gain, g	Feed to gain ratio	Daily feed, kg
Soybean meal	425^{ab}	1.75^{ab}	$.74^{a}$
Dry extruded soybeans	470^{a}	1.61^{c}	$.75^{a}$
Wet extruded soybeans	434^{ab}	1.62^{c}	$.72^{abc}$
Wet extruded beans	346^{cd}	1.80^{a}	$.62^{bcd}$
2/3 DES-1/3 WEB	344^{cd}	1.68^{abc}	$.58^{cd}$
1/3 DES-2/3 WEB	370^{bcd}	1.70^{abc}	$.63^{abcd}$
2/3 S-1/3 B, WE	379^{bcd}	1.72^{abc}	$.65^{abcd}$
1/3 S-2/3 B, WE	325^{d}	1.67^{bc}	$.54^{d}$
2/3 S-1/3 B, DE	408^{abc}	1.64^{bc}	$.67^{abc}$
1/3 S-2/3 B, DE	376^{bcd}	1.62^{c}	$.62^{bcd}$

[1]Four replicates of four pigs each per treatment; average initial body weight, 7.2 kg; 5 wk trial.
[2]2/3 DES-1/3 WEB = 2/3 dry-extruded soybeans, 1/3 wet-extruded beans; 1/3 DES-2/3 WEB = 1/3 dry-extruded soybeans, 2/3 wet-extruded beans; 2/3 S-1/3 B, WE = mixtures of 2/3 soybeans, 1/3 beans, wet-extruded together; 1/3 S-2/3 B, WE = mixture of 1/3 soybeans, 2/3 beans, wet-extruded together; 2/3 S-1/3 B, DE = mixture of 2/3 soybeans, 1/3 beans, dry-extruded together; 1/3 S-2/3 B, DE = mixture of 1/3 soybeans, 2/3 beans, dry-extruded together. Diets were formulated to a constant ME to crude protein ratio of 178 kcal ME/% protein. Animal fat (2.0% of diet) was added to the SBM and WEB diets and DL methionine (0.1% of diet) was added to all diets.
^{a,b,c,d}Means in the same column without a common superscript differ ($P<.05$).

Results of the growing-finishing swine trial are summarized in Table 3. In growing pigs (from 22 to 54 kg), rate of gain was similar to or greater ($P<.05$) for pigs fed various dry extruded bean-soybean mixtures ranging from 1/8 to 3/4 beans than that of pigs given soybean meal as the protein supplement; feed efficiency was not different ($P>.05$). There were no differences in performance ($P>.05$) of finishing pigs (from 54 to 91 kg) fed any of the dry extruded bean-soybean mixtures or soybean meal.

Conclusion

The results of the above three studies indicates that concurrent simple dry extrusion of beans and soybeans may offer a practical and easy method for heat processing cull beans for use in non-ruminant diets, especially for growing-finishing swine. Of the bean-soybean mixtures used, those containing up to 50% beans were dry extruded with no difficulty.

Table 3. Performance of growing-finishing swine fed barley-based diets containing dry-extruded soybeans or dry-extruded mixtures of beans and soybeans as supplemental protein sources[1].

Supplemental protein source[2]	Daily weight gain, g	Feed to gain ratio	Daily feed, kg
Soybean meal	683[b]	3.01	2.06[a]
Dry extruded soybeans	710[ab]	3.28	2.33[ab]
7/8 S-1/8 B[3]	760[a]	3.13	2.37[b]
3/4 S-1/4 B	740[a]	3.12	2.30[ab]
5/8 S-3/8 B	721[ab]	3.18	2.30[ab]
1/2 S-1/2 B	708[ab]	3.16	2.20[ab]
3/8 S-5/8 B	743[a]	3.12	2.32[ab]
1/4 S-3/4 B	708[ab]	3.09	2.18[ab]

[1]Each mean represents six replicates of two pigs each; on experiment from an average weight of 22 to an average weight of 91 kg.
[2]Grower (22 to 54 kg) and finisher (54 to 91 kg) diets were formulated according to NRC (USA) guidelines and contained 4,300 and 5,100 kcal ME/% lysine, respectively. Animal fat (2.0% of grower and 1.2% of finisher diets) was added to the SBM diets and DL-methionine (0.1% of diet) was added to all grower diets.
[3]Proportion of soybeans (S) and beans (B) in the dry-extruded mixtures.
[a,b]Means in the same column without a common superscript differ ($P<.05$).

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EFFECT OF PROCESSING ON TRYPSIN INHIBITORS IN PEAS (PISUM SATIVUM) AND INCIDENCE ON RAT GROWTH

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Summary

Raw, steam-flaked, micronised and extruded peas (PISUM SATIVUM) were analysed. The trypsin inhibitor capacity was reduced to a minimum level in all of the processed seeds, without alteration of the available lysine and the pepsin soluble protein. On the other hand, pea's starch was gelatinized only by extrusion.
A digestibility trial was conducted with 18 male rats and 108 growing rats to determine the growth rate and the conversion efficiency of diets which contained 60% of raw, steam-flaked or extruded peas. It appeared that processing improved N retention and the dry matter conversion efficiency of the diet, probably in relation to the decrease in ANF levels.

Introduction

In recent years, the culture of peas has been encouraged in Western countries by the EEC Common Agricultural Policy. So PISUM SATIVUM is utilised more and more for feeding monogastric livestock. However, like other leguminous seeds, the pea contains anti-nutritional factors (trypsin inhibitors, lectins, tannins) which must be inactivated by heating, to improve the nutritional value of the seed (Van der Poel & Daris, 1988).
The effects of steam-flaking, micronisation and extrusion on starch gelatinization, on the protein quality and on the trypsin inhibitor activity of peas have been compared. Utilization of steam-flaked and extruded peas by growing rats has been determined.

Material and methods

Various kinds of peas (unknown varieties) were analysed:
- 6 raw peas (5 from Belgium, 1 from England);
- 6 samples of peas steam-flaked at atmospheric pressure in a Bülher apparatus by the INTERAGRI company-Belgium;
- 4 samples micronised by the Micronising Company (U.K.) Limited;
- 4 samples extruded by a Clextral apparatus (3 at the Agronomical Faculty of Gembloux-Belgium, and 1 by the Royal-Canin Company-France).

Some parameters of nutritional significance were determined:
- the starch degradability by porcine pancreatin (Osman & al., 1970);
- the pepsin N solubility (Goering & Van Soest, 1970);

- the available lysine (Williams, 1967);
- the trypsin inhibitor capacity (Hamerstrand & al., 1981).

Raw, steam-flaked and extruded peas from the same origin have been used in an investigation of the effects of processing on the utilization of peas by growing rats.
In a first experiment, 18 Wistar x Brown-Norway male rats of approximately 150 g liveweight were distributed among the 3 tests diets. All animals were housed in individual metabolism cages fitted with urine and faeces separators. After a preliminary period of 14 d, total outputs of faeces and urine were measured over a 11 d collection period.
The composition of the experimental diets was per kg: 600 g peas + 350 g manioc + 25 g soja oil + 20 g minerals and vitamins + 5 g methionine. Chemical composition on a dry matter basis was 16.5 % crude protein and 6.2 % crude fiber. Dry matter intake, organic matter and N apparent digestibility coefficients, and N retention were calculated.

A second experiment was conducted to determine the effects of processing on the pea's diets conversion efficiency. 108 Wistar x Brown-Norway rats (1 month old, approximately 75 g liveweight) were distributed among the 3 diets described for trial 1, such that each diet had 36 replicates. Animals were housed by twos in plastic cages, on sawdust. They were fed ad libitum for a 28 d period and weighted on days 0, 14 and 28. Dry matter intake, growth rate and dry matter conversion efficiency were calculated.

Results of both rat experiments were submitted to analysis of variance.

Results and discussion

Nutritional parameters of processed peas (Table 1)

Table 1. Effects of processing on some parameters of nutritional significance for peas.

Peas	Raw	Steam-flaked	Micro-nised	Extruded
Pancreatin starch degrad. (mg/gDM/30 min)	33	60	69	258
Pepsin soluble N, % N	97	96	97	96
Available lysine, % lys.	94	95	97	97
Trypsin Inhibitor Capacity (mg trypsin inhib./gDM)	8.0	0.8	0.8	0.3

The results obtained show that the trypsin inhibitor capacity of peas may be reduced to a minimum level in any cases by steam-flaking, micronising or extrusion, without loss in availability of lysine or pepsin N solubility. These results agree with those of Van der Poel & Daris (1988) for extruded peas, and of Van der Poel & Van Vuure (1988) for

infrared exposed peas. However, another aim of processing is usually a better starch utilization. As pancreatin starch degradability is a good indicator, it appears that a real starch gelatinization of peas only occurs with extrusion.

Experiment 1 (Table 2)

Table 2. Effects of processing on the utilization of peas (PISUM SATIVUM) by rats.

Peas	Raw	Steam-flaked	Extruded
DM Intake (g/day/rat)	16.6	16.8	16.4
Apparent digest. coefficient			
- organic matter	0.86	0.86	0.86
- N	0.74	0.75	0.74
N retention, % N ingested	25.6[a]	27.5[ab]	28.5[b]

a: different superscripts denote significant differences ($P < 0.05$)

Processing doesn't influence dry matter intake of the peas based diets nor their apparent digestibility coefficients. On the other hand, N retention was significantly increased by extrusion ($P < 0.05$). N retention for steam-flaked peas diet was intermediate between raw and extruded peas. These results are consistent with the decrease of ANF level in processed seeds. Processing improves the N utilization of peas.

Experiment 2 (Table 3)

The better N utilization of processed peas shown in experiment 1 failed to induce a faster growth rate in the young rats, but is accompanied by a significantly better DM conversion efficiency ($P < 0.05$). The fact that this improvement is equal for steam-flaked and extruded peas is indicative of a probable decrease in ANF levels, and further suggests that the gelatinization of pea starch is of little nutritional interest!

Table 3. Effects of processing of peas on the growth rate and the dry matter conversion efficiencies by growing rats.

Peas	Raw	Steam-flaked	Extruded
DM Intake (g/day/rat)	13.5	12.8	13.0
Growth rate (g/day)	4.3	4.4	4.4
DM conversion efficiency (g DM/g growth)	3.1[a]	2.9[b]	2.9[b]

a: different superscripts denote significant differences ($P < 0.05$)

Conclusion

The pea's antinutritional factors are reduced to a minimum level by steam-flaking, micronizing or extrusion. Consequently, the N utilization of peas by growing rats is significantly improved by processing involving steam-flaking and extrusion. However, this improvement remains quite limited. For this reason their use in animal feeding necessitates an economic study to compare the costs of these process with their nutritional benefits.

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EFFECT OF HTST TREATMENT OF PISUM SATIVUM ON THE INACTIVATION OF ANTI-NUTRITIONAL FACTORS

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1. Introduction

Earlier studies with soya showed that HTST treatments like extrusion and infrared radiation were favourable in the inactivation of ANF's in the crop. Since there is a trend in Europe to promote the use of peas (Pisum sativum) for livestock feeding purposes it is logic to investigate the effect of both treatments on Pisum sativum varieties. The activities of ANF's like trypsine inhibitors, lectines and tannines, are investigated. In order to describe the severeness of the heat treatment at the same time the decrease of the available lysine, total lysine and the protein dispersibility index were determined.

2. Materials and methods

Two varieties of Pisum sativum were investigated, being Pisum sativum convar sativum (Final; round-seeded pea) and Pisum sativum spp arvense (C 306; wrinckled-seeded pea). These varieties are further referred to as seed pea and feed pea, respectively. Both were taken from the Cebeco Company in Rotterdam. For the extrusion cooking trials a CM 45 counter-rotating twin screw extruder, made by Cincinnati Milacron was used (Kouzeh Kanani et al., 1981). The infrared equipment, made by Almex-Kösters is a Dutch machine and is designed as an endless belt above which 8 burners fixed with Dutch natural gas are mounted (Van Zuilichem et al., 1984).

The moisture content of the peas for the I.R. trials was set at equilibrium conditions (14%) and at 20% by soaking in water. The residence times in the belt were chosen as 30, 45, 55 and 65 s. at which roughly 3 levels of products temperature were attempted to reach respectively 105 °C, 116 °C and 124 °C. In order to investigate the effect of a holding time the peas were kept in an insulated container for 0, 10, 20 and 30 minutes respectively. For the extrusion-cooking trials 4 levels of moisture content were applied (15%, 20%, 25% and 30%), while also 4 temperature levels in the extruder were used (105 °C, 115 °C, 125 °C and 135 °C). Standard analytic measurements were selected for the determination of the ANF's, the PDI and lysine numbers. The PDI was measured following the AOCS method 46-24. The trypsine inhibitor was measured according to a modified procedure of Kakade et al. (1974). The determination of lectines was done using a haemagglutination test, at which the ultimate dilution of lectins extract is determined at which agglutination is caused in red blood-cells of rabbits after 16 hrs of contact. The determination of the tannin content (polyphenol) is done with the Folin-Denis reactant, resulting in molecular bindings with tannins, which can be recognised by spectrofotometing at 760 mm. Finally the available lysine content is given by the method of Hall et al. (1973).

3. Results

3.1 Extrusion cooking trials

3.1.1 The trypsin inhibitor (TIA), originally 1,75 mg/g in the untreated seed peas Finale, was totally inactivated after each extruder run. Undependent of process conditions values were found to be lower than 0.1 mg/g. The TIA level in the feed pea C 306 could be inactivated at higher temperatures compared with the seed pea Finale. Extruding at 105 °C gave a reduction whilst at temperatures > 125 °C good values of a 0.5 mg/g could be realised, (see Fig. 1).

3.1.2 The lectins activity was found to be dependent of temperature and moisture content, (see Fig. 2). The lectin content could well be correlated with the PDI-number, (corr. coefficient > 92%) for the feed pea (originally HA 16). In contrast with this, this could not be found for the seed pea, originally HA 10, but showed a strong dependency with the temperature (see Fig. 4). A similar effect like this TIA is found for the temperature effect on the lectin content. Mild conditions (105 °C, 25% moisture) result for seed peas in a residual lectine content of 12,5%, whilst feed pea do not react in those mild conditions.

The tannin content, generally referred to as heat stable, could not be fully removed by extrusion cooking (see Fig. 3). After the process the tannin content decreased with 30% maximum in the seed peas. For feed peas a maximum decrease of 40% could be realised. Finally the data of available lysine decreased with (8-30)% and (16-20)% for seed peas and feed peas respectively, (see Fig. 4).

3.2 Infrared treatment

In Figures 5 - 8 the results are given for the 4 groups of products. From Figures 5 and 6 can be concluded that ANF reduction can be realised for each of the parameters focussed on seed peas, whereas Figures 7 and 8 give the same parameter values for feed peas. As to the TIA values for seed peas a zero value is reached for temperatures above 120 °C at 14% moisture and for feed peas above 130 °C. There is a small dependency on the water content. Here the favourable effect is found that the available lysine data are practically not influenced by the heat treatment and remain at the original levels. The PDI levels decrease for all the products but still values of about 30 - 35 could be found after the residence times in the belt. For all the products is measured that the inactivations rate for lectine is faster compared with the TIA-inactivation. Also here tannins are inactivated but only for a small percentage (± 25%). In the meantime it is not clear if tannins are destroyed or could not be detected properly due to possible polymerisation effects. Also here the lectin content correlates properly with the PDI data. Comparing Figures 5 and 6 with 7 and 8 learns that soaking with water promotes the ANF reduction, in this way that soaking accelerates the heat penetration.

Residual TI vs temperature

for extruded products

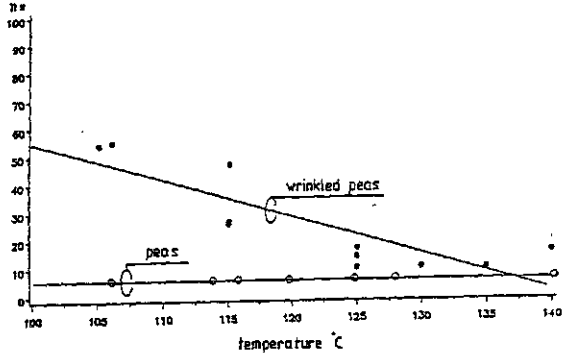


fig. 1

Residual lectines vs temperature

for extruded products

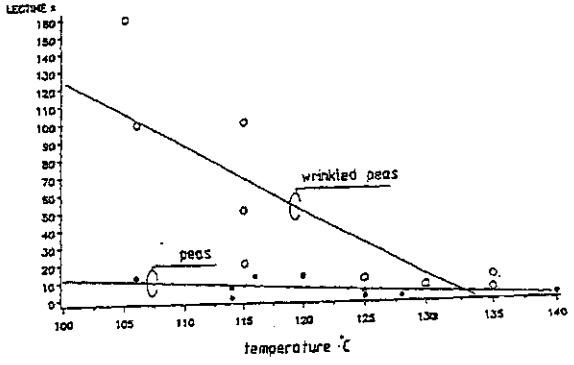


fig. 2

Residual tannins vs temperature

for extruded products

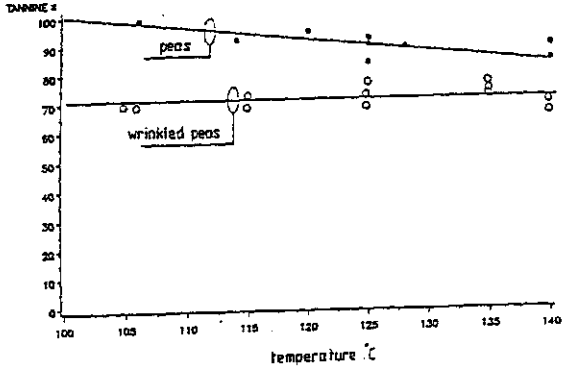


fig. 3

AV lysine vs temperature

for extruded products

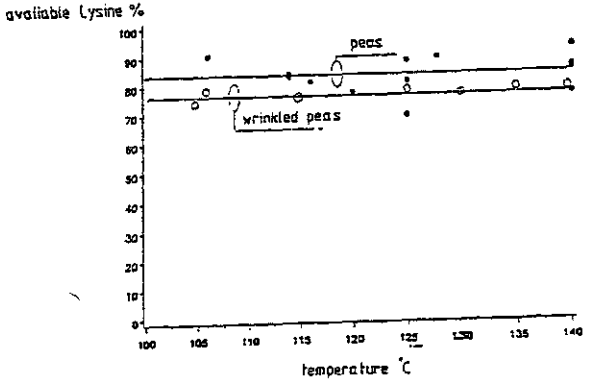


fig. 4

fig 5

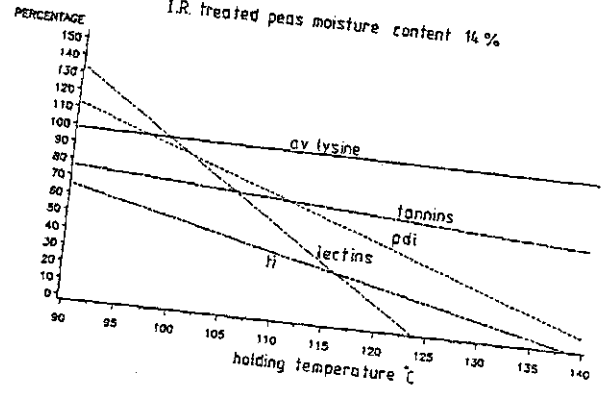


fig 6

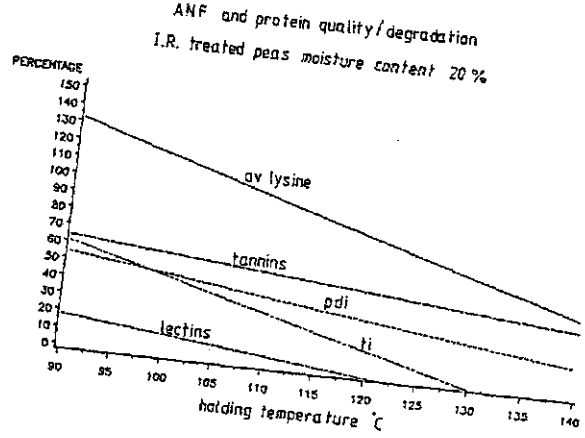


fig 7

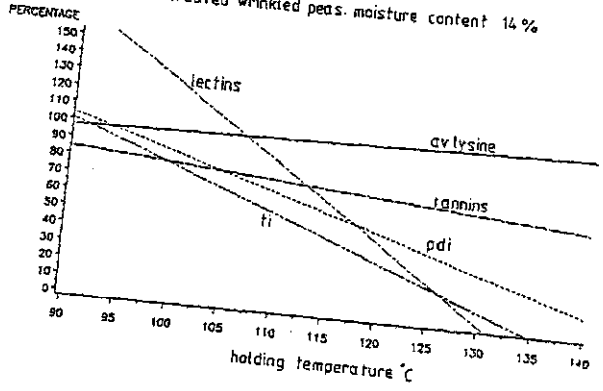
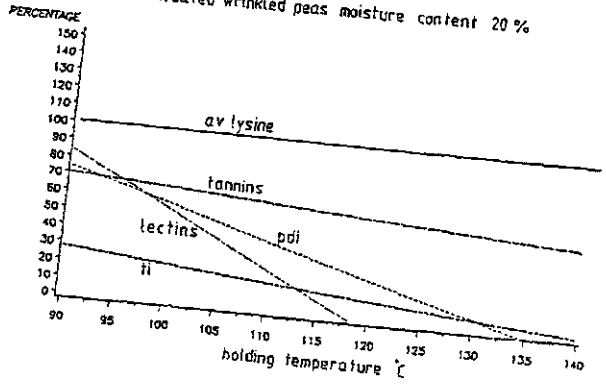


fig 8



4. Conclusions

Both treatments are suitable, infrared-radiation and extrusion cooking give a good reduction of ANF-factors like TIA and lectins. Tannins are only partly broken down. The temperatures needed for TIA inactivations are more than sufficient for lectine-inactivations. For the infrared treatment there is a better effect in keeping the available lysine content as original, compared with the extrusion cooking data. More research should be done with the extruder cooker since it seems that the process conditions investigated proved to be severe for the task asked for. Furthermore, the relation between ANF-reduction and in vivo nutritional value has to be established to establish optimal process criteria in optimizing conditions for HTST-inactivation of antinutritional factors in legume seeds.

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OPTIMALIZATION OF DEHULLING TECHNIQUE AND ENZYMATIC HYDROLYSIS OF VICINE/CONVICINE TO ELIMINATE ANF'S OF FABA BEANS

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SUMMARY

Tannins are mainly located in the seed coats of faba beans. Dehulling will eliminate most of the tannins present in the beans. The influence of the roller distance, air velocity and dosage on the dehulling process was studied. Breaking the beans by a rollermill with roller distances of 3.25 and 3.00 mm and air classifying the beans in a zig-zag windsifter with air velocity of 5.56 m/sec and dosage 200 kg/h, gave a high yield of dehulled beans (82.4 %) with only 0.5 % hulls.
Vicine and possibly convicine have adverse effects on laying hens. B-glucosidase from almonds showed to be able to hydrolyse vicine and convicine in phosphate (pH=6.8) and rapidly in HCl (pH=4.2).

1 INTRODUCTION

The protein of faba beans is known to be poorly digested by monogastric animals. Condensed tannins which are mainly located in the seed coats of faba beans appear to be responsible for these anti-nutritive effects (Griffiths & Jones, 1977). Removal of the seed coat will substantially increase the digestibility of the seeds as a result of the simultaneous removal of tannins and crude fibre (50 % of the hull, Marquardt et al., 1975). The objective of this study was to examine the parameters that influence the technological dehulling efficiency of faba beans in a trial to remove the tannins.

Some of the other adverse effects of feeding faba beans especially to laying hens can be attributed to vicine and possibly convicine (Olaboro et al., 1981; Muduuli et al., 1982). Vicine and convicine were not hydrolysed in the presence of homogenates from liver, kidney, intestinal wall or caecal wall, digesta from the large intestine or by enzymes present in faba beans. These compounds, however, were slowly hydrolysed by the low pH in stomach and rapidly hydrolysed by micro-organisms present in the caeca of the chick (Frohlich & Marquardt, 1983). B-glucosidase is known to convert vicine and convicine into their aglucones and glucose. Unlike their glycosides, the aglucones are very unstable (Arbid & Marquardt, 1985; Liener, 1980). Vicine and convicine might be hydrolysed in the digestive tract of the animal by adding B-glucosidase to the feed. Then B-glucosidase must be active under conditions as occur in the digestive tract. The objective of this study was to determine the hydrolysis of vicine and convicine by B-glucosidase in-vitro under conditions of low and neutral pH.

2 MATERIALS AND METHODS

2.1 Sources of faba beans, chemicals and analytic procedures

Faba beans (Vicia faba L. var. Alfred) were obtained from CEBECO-Handelsraad. B-glucosidase (EC 3.2.1.21) extracted from almonds and vicine were from Sigma Chemicals. Convicine was kindly donated by

A. Nieuwenhuis (IBVL, The Netherlands). Vicine and convicine were determined by HPLC (cyano-radial- Compression-column; elution solvent distilled water 0.8 ml/min; UV-detector 273 nm. A 1.5 ml sample was mixed with 11.5 ml distilled water, the proteins in the extract precipitated with 0.1 N HCl at pH 4.2, centrifuged for 10 minutes at 7000 rpm and the supernatant filtered through a 0.45 um filter.

2.2 Technological dehulling

The faba beans were broken by a rollermill with two roller pairs with roller distances of 3.25 and 3.00 mm respectively. The broken beans passed through sieves of 5.0 and 1.7 mm respectively. The fraction greater than 5.0 mm was broken again in the rollermill with a roller distance of 2.0 mm and sieved. The fraction greater than 1.7 mm but smaller than 5.0 mm was air classified in a zigzag windsifter (dosage of 540 kg/h) with an air velocity of 5.56 m/sec. The channel of the windsifter had a cross-section of 100 by 200 mm. The yields of the hulls (sieved fraction greater than 5.0 mm and lightest air classified fraction) and of the dehulled beans (sieved fraction smaller than 1.7 mm and heaviest air classified fraction) were determined. The residue of cotyledons in the hulls and the residue of hulls in the dehulled beans were determined through separation by hand.

2.3 Enzymatic hydrolysis of vicine and convicine

B-glucosidase solution was prepared by adding 250 mg B-glucosidase to 10 ml phosphate buffer (pH=7.0). Ten grams of faba beans were ground, homogenised, and extracted with 50 ml 1N NaOH. The extract was centrifuged for 10 minutes (7000 rpm). Ten millilitres of the extract were mixed with 15 ml phosphate buffer (pH=6.2), and 10 ml of the extract were mixed with 15 ml 0.1 M HCl (pH=1.4). Five millilitres of the B-glucosidase solution were added to both extracts and the solutions were incubated at 37 °C for 1h. Samples were taken every 15 minutes and analysed for vicine and convicine. The enzymatic reaction was stopped by heating the sample for 5 minutes at 100 °C.

3 RESULTS AND DISCUSSION

3.1 Technological dehulling

Points of study were the roller distances in the rollermill with one or two roller pairs and dosages and air velocities of the windsifter. The process parameters as used in this study were obtained from earlier experiments on other materials (Ogink, 1988). The results of the dehulling experiment, yields and residues, are summarised in table 1 and table 2. The whole beans contained about 14.3 % hulls.

Table 1. The rollermill experiment, yields and residues.

Rollermill roller distance (mm)	Yield dehulled beans (%)	hull residues in dehulled beans (%)	Cotyledon residues in hulls (%)
3.50, 3.00	81.8	0.7	24.2
3.25, 3.00	82.5	0.6	20.6
3.25, 2.75	81.9	0.6	23.2
3.00	82.9	0.6	19.7

The dehulled beans which were broken at different roller distances show about the same content of hull residues. To minimize the loss of cotyledons in the hull fraction, roller distances of 3.25 and 3.00 mm with two roller pairs or a roller distance of 3.00 mm with one roller pair were found the best.

Table 2. The air classification experiment, yields and residues.

Air velocity (m/sec)	Dosage (kg/h)	Yield dehulled beans 200	540	1300	Hull residue in dehulled beans 200
		(%)	(%)	(%)	(%)
4.45		83.2	83.9	85.1	1.0
5.56		82.4	82.8	84.3	0.5
6.67		73.4	75.3	78.3	0.3

In the air classification experiment only the residue of hulls of the dehulled beans air classified at a dosage of 200 kg/h was determined. The hulls in the dehulled beans obtained with an air velocity of 6.67 m/sec and a dosage of 200 kg/h were stuck to the cotyledons and could not be removed with a higher air velocity or lower dosage. A dosage of up to 540 kg/h did not influence the yield of the dehulled beans which were air classified with an air velocity of 4.45 and 5.56 m/sec. The beans air classified with an air velocity of 6.67 m/sec (dosage of 200 kg/h) had a low yield of dehulled beans but also the lowest content of hull residues. The beans air classified with an air velocity of 4.45 and 5.56 m/sec (dosage of 200 kg/h) gave a high yield of dehulled beans. However, the dehulled beans air classified with air velocity of 5.56 m/sec contained two times less hulls than the dehulled beans air classified with an air velocity of 4.45 m/sec.

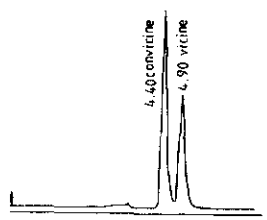

Figure 1. HPLC chromatogram of vicine and convicine.

3.2 Enzymatic hydrolysis of vicine and convicine

HPLC analysis gave a good separation of vicine and convicine as is illustrated in figure 1. In figure 2 the hydrolysis of vicine and convicine by B-glucosidase at pH of 4.2 and 6.8 is shown. The hydrolysis of convicine was faster than that of vicine. Since the pH optimum of B-glucosidase is about 4, the hydrolysis of vicine and convicine in HCl (pH=4.2) was faster than in phosphate buffer (pH=6.8). Vicine and convicine were hydrolysed almost completely in HCl (pH=4.2) within 25 minutes so they might be converted entirely in the stomach of monogastric animals when B-glucosidase is added to the feed.

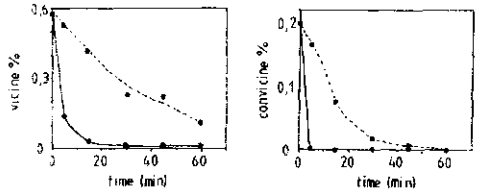

Figure 2. Vicine and convicine hydrolysation by B-glucosidase in HCl (pH 4.2) (——) and in phosphate buffer (pH 6.8) (- - -).

4. CONCLUSIONS

The efficiency of the dehulling is defined by a high yield of dehulled beans which contain a minimum of hulls. The loss of cotyledons was minimal at roller distances of 3.25 and 3.00 mm or of 3.00 mm. When the beans were broken by a rollermill with roller distances of 3.25 and 3.00 mm followed by air classifying in a zigzag windsifter with an air velocity of 5.56 m/sec and a dosage of 200 kg/h a high yield of dehulled beans (82.4 %) with only 0.5 % hulls was obtained. Further studies will be made on the feasibility of the dehulling process in the animal-feed industry in a trial to remove the tannins.

B-glucosidase from almonds was shown to be able to hydrolyse vicine and convicine in phosphate buffer (pH=6.8) but much more rapidly in HCl (pH=4.2). Further studies should show whether the aglucons (divicine and isouramile) are broken down as well.

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KINETIC METHODS FOR RESEARCH INTO INACTIVATION OF ANTINUTRITIONAL FACTORS

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Summary

Processes to inactivate ANF in animal feeds cannot be evaluated well due to the lack of appropriate methods of ANF analysis. Though this problem is gradually being solved, isothermal process research, based upon such methods, is laborious and thus expensive. Differential Scanning Calorimetry (DSC), based upon measurement of transition energies of chemical changes, seems to be a promising tool in addition to isothermal process research. From our experiments it follows that DSC can be used to study factors influencing the inactivation of a soybean trypsin inhibitor, and possibly other ANF as well. However, it is expected that DSC cannot replace isothermal experimental work on ANF inactivation completely due to its limitations. The main limitation of DSC is the rather high minimum content of ANF necessary to be able to detect the transition.

Introduction

Leguminous seeds such as soya, beans and peas are important feed ingredients for pigs and poultry in Europe. These seeds however, often contain various amounts of antinutritional factors (ANF). In practice reduction of ANF activity is mainly achieved by heat treatment, which is applied on a large industrial scale. To comply with a growing need to incorporate higher quantities, especially in pig and poultry feeds, the development of cheaper and better inactivation processes is needed. Enzymatic, technological and combinations of treatments offer realistic possibilities for eliminating the harmful action of ANF (Liener, 1980).

Though based upon extensive research, both actual and new processes applied to inactivate ANF in raw materials cannot be evaluated well sofar and thus may not be optimal from a nutritional point of view. This is due to the lack of appropriate methods of analysis, especially for lectins and tannins. This implies that these processes cannot be well controlled. The actual progress in the development of such methods of analysis and the availability of new experimental techniques to study physico-chemical changes both open new and promising opportunities for research into optimizing inactivation processes. In addition to isothermal process research, thermal analysis is considered to be an interesting tool for systematic research into inactivation of ANF.

Differential Scanning Calorimetry (DSC) is used to study transition phenomena of materials by scanning the heat flow to a sample. Data are obtained in the form of differential heat input versus time or temperature for a constant heating rate. DSC has numerous applications in food technology. Most of the studies published however, refer to starch gelatinization and protein denaturation (Lund, 1983). A limitation of this technique is that measurements on components which are present in very low concentrations are inaccurate or even impossible.

We carried out preliminary research into both an isothermal process approach and thermal analysis as potential methods to study process parameters which are important for the inactivation of ANF.

Treatments for inactivation of ANF

The aim of treating ANF-containing raw materials is to change the functional group(s) and/or structural conformation of the ANF in such a way that the negative influences on the animal are eliminated. At the same time it is very important that the nutritional value of the raw material is not negatively affected.

The processes and treatments to be studied can be described as: thermal (heat), physical (shear), chemical (acid, base, salt, water, etc...), enzymatic, and combinations thereof.

In addition to heat treatment, shear can be applied to inactivate ANF. A recently developed and suitable combination of heat treatment and shear is realized using an extruder. Extrusion (150 °C, 16 s) of small red beans appeared to be as effective in eliminating ANF as an isothermal heat treatment (121 °C, 15 min) (Meyer and Froseth, 1983).

It is generally believed that the main function of both shear and enzymatic treatment is to increase the accessibility and sensitivity of the ANF to chemicals and heat. However, enzymes can possibly play a more direct role as well in inactivating ANF in animal feed.

In some specific processes for food preparation heat treatment is combined with the use of certain chemicals. Among others, Friedman and Gumbmann (1986) have investigated the influence of chemicals such as sodium sulphite, cysteine and N-acetyl-cysteine. They appeared to increase the sensitivity of ANF to heat. These chemicals cleave the disulfide bonds of proteins resulting in a disruption of the three dimensional structure and thus the stability of the protein.

Isothermal (kinetic) experiments

A common method for studying the effects of heat treatment on unstable components (e.g. vitamins) is to carry out isothermal experiments. Varying treatment time and temperature and determination of the amounts of the component in samples, the kinetic parameters of the inactivation can be calculated. Comparison of the kinetic parameters of both ANF and potentially unstable nutrients (e.g. lysine) should give an insight into

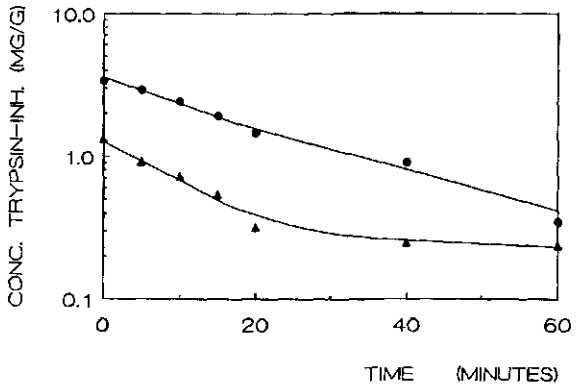

Fig. 1. The effect of isothermal heat treatment at 104(●) and 120(▲) °C on the activity of the trypsin inhibitor (mg trypsin inhibitor/ g sample).

the direction in which processes can be optimized. Results of such isothermal experiments are given in figure 1.

This figure represents the effects of autoclaving at two temperatures (104°C and 120 °C) on the most important ANF in soya beans, namely trypsin inhibitor. Figure 1 suggests that the trypsin inhibitor in soya beans consists of at least two types with different stability towards heat treatment.

Isothermal experiments are very laborious, time consuming, and expensive due to the amount of analytical work involved, when so many process variables are to be studied. Therefore we looked into the possibilities of DSC as well as a potential means of pre-screening possible treatments, combinations of treatments and process conditions. Its main advantage is that no chemical analysis is needed to study the inactivation process, so the amount of experimental work is considerably reduced. On the other hand research with DSC will probably have to be carried out with model systems containing more ANF than occurring naturally. This not only puts a question mark to the validity of results in practice. It could also mean a limitation of its application to evaluation of treatments which can be carried out on a sufficiently small scale, e.g. thermal and chemical treatments. Therefore, in practice, isothermal experiments will be needed both to validate results obtained by DSC and to complete experimental data if DSC cannot be applied.

Thermal analysis

In order to demonstrate the potential of using DSC, we studied the denaturation of commercially available trypsin inhibitor (Kunitz) from soybeans as a model. Figure 2 is a typical DSC scan of a sample of trypsin inhibitor, showing its transition energy required on inactivation.

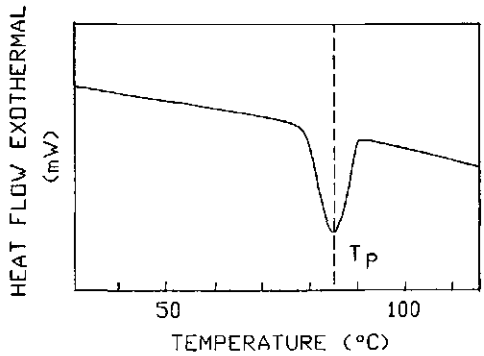

Fig. 2. A typical DSC scan of a sample of pure trypsin inhibitor (Kunitz); Tp represents the peak temperature of transition.

The amount of water added strongly influences the position of the peak temperature of denaturation. Figure 3 shows that on adding water from 10% to 80% the peak temperature shifts from 105 °C to 75 °C. We conclude that, as the water content of the samples increases, denaturation will occur at a lower temperature.

Besides the temperature, the processing time is an important parameter in DSC. In figure 4 the processing time necessary to reach 90% inactivation of trypsin inhibitor is given for three temperatures. Figure 4 shows that the processing time is strongly influenced by both temperature and water content. This is especially clear between 80 and 90 °C and water contents between 20 and 40 %.

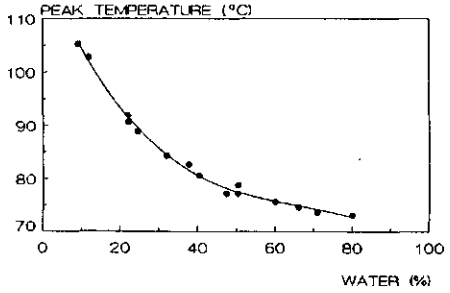

Fig. 3. Effect of water content on the peak temperature of denaturation of trypsin inhibitor; DSC heating rate is 10 K/min.

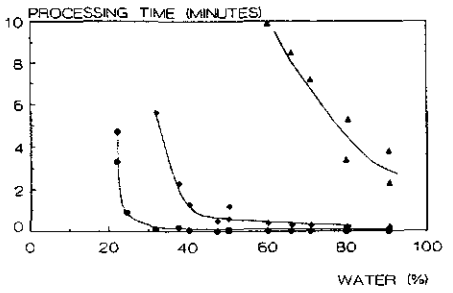

Fig. 4. Processing time for 90 % denaturation as a function of water content and processing temperature (▲= 70, ♦= 80, and ●= 90°C); DSC heating rate is 10K/min.

These effects would be expected to be even greater at temperatures above 90 °C and water contents less than 20 %. Other parameters, such as pH, added salt, and chemical additives, are under investigation.

A next step will be to study application of DSC to products enriched with trypsin inhibitor. Also the possibility of studying the denaturation of ANF as they occur naturally in the matrix of the raw material will be investigated. In the future, DSC experiments will be carried out using other naturally occurring ANF (e.g. lectins) and nutrients (e.g. lysine).

Conclusions

From our experiments it follows that DSC is a useful technique, in addition to isothermal process experiments, for studying factors influencing the denaturation of a soybean trypsin inhibitor and possibly other ANF as well. As we expected, the DSC measurements show that a higher water content will allow a lower processing temperature or a shorter processing time. However, we expect that DSC cannot replace isothermal experimental work on ANF inactivation completely due to its limitations. The main limitation of DSC is the rather high minimum content of ANF necessary to be able to detect the transition. This means that extracted ANF are to be used either as pure substances or to prepare samples with artificially increased ANF contents. It is uncertain whether such purified ANF have the same inactivation behaviour as the naturally occurring ones.

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EFFECT OF THE EXTRACTION OF α-GALACTOSIDES FROM TOASTED OR RAW SOYBEAN MEAL ON DIETARY NITROGEN AND FAT UTILIZATION IN THE YOUNG PIG

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Summary

Two soybean meals, one prepared in the usual way (ETSM) and the other one without intensive heat treatment (ERSM) were submitted to a water-extraction treatment at pH 4.5 in order to remove the α-D-galactosides. They were incorporated in iso-energy, iso-nitrogen and iso-fat diets, for early-weaned pigs, in comparison with a regular toasted soybean meal (TSM). Growth performance, feed efficiency, nitrogen digestibility and retention were not affected when ETSM replaced TSM, but were significantly depressed when ERSM replaced ETSM or TSM. These results could be explained by the high levels of ANF in the ERSM diet. Energy digestibility was slightly improved (2.5%) by the extraction of -D-galactosides (ETSM vs TSM diet) and all the difference could be accounted for by a significant increase in fat digestibility. The same effect was observed with ERSM first but disappeared later.

Introduction

The production of soybean meal requires a heat-moisture treatment to inactivate heat-sensitive ANF (protease inhibitor, lectins). However, at the same time a marked denaturation of protein, parallel to their insolubilisation, is known to occur. For a number of technological and/or nutritional reasons it is advisable to minimize the denaturation of proteins and to preserve their functional properties. On the other hand, legume seeds such as soybeans contain oligosaccharides of the raffinose family, α-D-galactosides (AG), responsible for gas producing fermentations (flatulence) in the caecum. These two aspects of soybean-meal quality were studied in the present experiment by testing two soybean meals from which the AG had been extracted, one of them prepared without intensive heat-treatment, in diets for young weaned pigs.

Material and methods

Twenty four piglets from 8 litters were weaned at 20 days of age and reared in metabolism crates for 18 days (4 days of adaptation and 2 weeks of measurements) in order to compare three diets within each litter. These diets were formulated to supply equal amounts of crude fiber, fat and protein (3.3, 10 and 24% respectively). Refatted skimmilk powder provided 22.5% of the protein content and the rest

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came from a mixture of barley with one of the tested soya products. A regular toasted soybean meal (TSM, 50.6% protein) contributed 26.8% of the control diet (diet 1). Another toasted soybean meal (ETSM, 64.0% protein) and a raw one (ERSM, 61.9%) differed markedly in their protein dispersibility index (PDI, Table 1). Both had been submitted to a water extraction treatment at pH 4.5 (Smith and Circle, 1972) in order to remove the AG. They were incorporated in diet 2 and 3 at 19.8 and 21.2% respectively. Dietary AG contents were assessed by HPLC (table 1).

Table 1. Physicochemical characteristics of the tested products and ANF contents in diets (per 100 g dry matter)

Soybean meal	TSM	ETSM	ERSM
PDI* value, %	-	27	72
Diet	1	2	3
. Trypsin inhibitor, 10^3 units**	60	80	830
. Raffinose***, g	0.20	0.05	0.10
. Stachyose***, g	0.95	0.10	0.20
. Verbascose***, g	0.06	0.01	0.01
Total α-D-galactosides, g	1.21	0.16	0.31

* According to AOCS (1970), no data available for TSM
** According to Kakade et al (1974)
***Ethanol extraction and HPLC analysis- Stationary phase : Rosil NH_2 (3μm)- Mobile phase : $CH_3CN:H_2O$, 64:36 - Flow rate : 1 ml/min. -Pressure 1100 psi.

Results

As shown in table 2, growth rate, feed efficiency and feed intake were not affected by the extraction of AG from ETSM (diet 2) as compared to TSM (diet 1). ERSM (diet 3) induced a marked decrease in growth rate on both weeks. But, when compared to that of diet 1, feed intake of diet 3 was less depressed on week 1 than on week 2 (+1% vs -12%) while the contrary was true for feed efficiency (-48% vs -25%).

Energy digestibility was not significantly increased by the extraction of AG from TSM (diet 2 vs diet 1). However, partly due to a slightly higher raw energy content, digestible energy (DE) content was significantly higher in diet 2 than in diet 1. ERSM induced a significant decrease in energy digestibility and DE content of diet 3 as compared to diets 1 and 2. Energy digestibility of diet 3 decreased markedly according to time. The extraction of AG from both raw and toasted meals induced a significant increase in fat digestibility of diet 2 and 3 as compared to diet 1. But, the beneficial effect of ERSM was no longer significant on the second week (Table 3).

The response of nitrogen digestibility to dietary components was strictly parallel to that of energy digestibility. Daily nitrogen retention or NPU were not significantly improved by the extraction of AG from TSM. On the contrary, the utilization of nitrogen was significantly depressed when ERSM replaced ETSM or TSM (diet 3 vs diets 1 or 2) (Table 4).

Table 2. Growth performance and feed efficiency

Diet	Week	1	2	3	Sx**	P***
. Weight gain g/day	W_1	220	227	119	20.6	>0.25
	W_2	340	350	231		
	W_1+W_2	281	289	175	14.9	<0.01
. Feed intake g/day	W_1	197	232	207	13.1	0.07
	W_2	326	345	288		
	W_1+W_2	262	289	248	10.8	<0.01
. Feed efficiency g/g	W_1	1.25	1.11	0.65	0.086	0.18
	W_2	1.06	1.02	0.80		
	W_1+W_2	1.16	1.07	0.73	0.046	<0.01

Table 3. Energy utilization.

Diet	Week*	1	2	3	Sx**	P***
Energy digestibility, %	W_1	82.8	85.6	82.0	0.82	0.08
	W_2	83.9	85.0	79.6		
	W_1+W_2	83.4	85.3	80.8	0.67	<0.01
DE content, kcal/kg dry matter	W_1	3931	4109	3930	39.7	0.23
	W_2	3962	4080	3831		
	W_1+W_2	3946	4094	3878	33.0	<0.01
Fat digestibility, %	W_1	64.2	74.2	73.5	2.47	>0.25
	W_2	68.2	73.4	68.5		
	W_1+W_2	66.2	73.8	70.9	1.96	<0.01

Table 4. Nitrogen utilization

Diet	Week*	1	2	3	Sx**	P***
Nitrogen digestibility, %	W_1	86.0	87.8	76.8	0.96	0.22
	W_2	87.0	87.0	74.5		
	W_1+W_2	86.5	87.4	75.6	0.76	<0.01
Nitrogen retention, g/day	W_1	4.1	4.9	2.4	0.41	<0.01
	W_2	8.2	8.3	4.5		
	W_1+W_2	6.2	6.6	3.4	0.35	<0.01
Net protein utilization (NPU), %	W_1	50.1	58.0	31.4	2.55	0.23
	W_2	57.5	58.3	36.2		
	W_1+W_2	53.8	58.2	33.8	1.95	<0.01

* W_1 = Week 1, W_2 = Week 2, W_1+W_2 = average value of 2 weeks
** Standard error for comparison of means on the same line
*** Level of significance for diet x week interactions (W_1, W_2) or differences between diets (W_1+W_2)

Discussion

These data provide additional evidence that heat treatment of the soybean meal greatly improves its nutritional value for pigs in terms of nitrogen digestibility and retention (Delort-Laval and Charlet-Lery, 1971). In previous papers from our laboratory it was shown that apparent fat digestibility could be beneficially influenced when dietary proteins were kept undenatured (Sève and Aumaître, 1983, Sève et al, 1985, Bertrand et al, 1988). In the present experiment, both depressions of appetite and digestibility on the second week might reflect a general disfunction of the digestive system due to heat labile ANF such as trypsin inhibitor or lectins. This could explain the lack of improvement of fat digestibility with ERSM as compared to ETSM (diet 3 vs diet 2).

These results also imply that an increase of only 0.8% of the proportion of dietary energy as AG (diet 1 vs diets 2 and 3) induced a significant reduction of 2.3% in total DE. This reduction was totally accounted for by the decrease in apparent digestibility of fat. Drochner (1984) reported that the addition of 5% crude wood fibre or isolated wood cellulose to a wheat-soybean meal diet also decreased apparent fat digestibility. Precaecal fat digestibility was highly depressed with crude wood fibre but, in both cases, microbial fat synthesis increased 70% over control. If this last hypothesis could be confirmed in the conditions of the present experiment, it would mean that AG suppress energy salvage from cell-wall components through conversion of undigested residues into microbial fat, in addition to possible losses in the form of gas.

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DISCUSSION ON TECHNOLOGY

Chairman: Z. Nitsan
Reported by: D.J. van Zuilichem

The workshop session on technology was opened by the presentation of a main paper which served to give an overview of the general effects of processing on antinutritional factors. Furthermore, the speaker emphasized the fact, that thermal processing need to be studied both in protein and carbohydrates for amino acid availability and digestion of protein and energy may change to a different extent after processing.

Although in the main paper and following short papers much attention is paid to the relation of heat-processing variables and levels of ANF's, it must be reported that there is no complete uniformity in the listing of ANF's. Many authors differ in their opinion about the relevancy of certain members in the ANF family e.g. functional lectins vs lectins in total, trypsin (TI) and chymotrypsin inhibitors vs TI alone.

As to the influence of process-variables like temperature and added moisture it is noticed that trials are performed giving results about the dependence of trypsin levels from moisture content and temperature levels which are already known in literature out of the late seventies and early eighties.

This literature covers results from the food process engineering and takes mostly into account the quality of the food and feed with respect to heat labile amino-acids like lysine, vitamines like thiamin and the state of the protein like PDI's and NSI indexes next to deficiency factors like ANF's. It should be stated that results from trials giving only the effect on ANF's are not complete and will not explain clearly the results of feeding trials, where clear processing data about the heat impact on the product in question could have given much more information. This means that it is advisable to describe more carefuly the process conditions and the process equipment used. Time has gone completely to mention that a certain product is extruded, since we know that the construction of the equipment may have a dominant and "one-sided" influence on the product properties. It is also insufficient to report that a product is micronised or infrared heated without mentioning the conditions under which the product is kept after the heat treatment since we know that the effect of cooking and holding is considerably.

The effects of simple pelleting are discussed and found to be of minor importance since there is hardly any effect in capability to reduce ANF'S levels apart, however, from other positive effects. Here the comment should be given that combinations of pretreatment by heating, prior to pelleting is of interest.

As to the analytical methods is to mention, that there is a need for standardization in the measuring methods used for ANF levels; especially in relation with kinetic methods used in research into the reduction of ANF levels these analytical methods are very important. Other questions refer to the residence times of the product in and on the processing equipment used, since the residence time is one of the most important process variables.

Another need for standardization is in the field of starch gelatinization. Still there are users of the pancreatic enzym method and users of the amyloglucosidase method, which means that analytical data leads to confusion. As to the application of new types of extruder equipment the scale up of pilot plant sites was discussed.

Mentioned were some rules, pointing out that capacities of good constructed twin screw equipment, e.g. APV-Baker vs Cincinnati-extruders scale up with the rule

$$\frac{Q1 \text{ big extruder}}{Q2 \text{ pilot extruder}} \triangleq \frac{D1 \text{ screw}}{D2 \text{ screw}} \qquad \frac{kg}{kg} \qquad 1.8\text{-}2.2$$

For single screw extruder equipment the rule is like $\frac{Q1}{Q2} \triangleq \frac{D1}{D2}$

With this general formulas it is indicated that the extrusion technology is well developed but this knowledge is not widely known in the animal feeding group of producers.

Session plant breeding

POSSIBILITIES FOR THE REDUCTION OF ANTINUTRITIONAL FACTORS IN GRAIN LEGUMES BY BREEDING

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Summary

There is genetic variation among the grain legumes for most of the ANFs, some of which have already been reduced by selection. Priorities for breeding are reduced concentrations of tannin, glucosides and possibly trypsin inhibitors in faba beans; lower lectin levels in Phaseolus and alkaloid-free Lupinus mutabilis. Oligosaccharides should also be investigated in all grain legumes. However, most ANFs also have functions in the growth and protection of the plant and there is a need to ascertain whether lowered levels preserve these functions whilst providing improved nutritional value. Liaison between plant breeders and animal nutritionists is essential to define objectives correctly in both disciplines.
Keywords: faba beans, peas, Phaseolus, lupins, soya bean, tannin, trypsin inhibitors, lectins, phytates, oligosaccharides, alkaloids, glucosides, breeding.

Introduction

Until recently the main objectives in breeding grain legumes have been, with a few exceptions, improvement of yield and yield stability through changes in plant habit and resistance to disease. The surplus of cereals in the EEC and the need for alternatives to imported soya have now provided incentives for selection for improved seed quality.

Some breeders have sought to increase the protein content of beans and peas, but the major obstacle to the utilisation of temperate leguminous grain in animal rations appears to be the level of anti-nutritional factors (ANFs). Breeders are now turning their attention to the important ANFs.

There is evidence of genetic variation in most of the ANFs in one or more of the grain legumes and therefore it should be possible to achieve a reduction in their levels by breeding. However most of the ANFs have evolved to protect the plant against pests or diseases and therefore their reduction may increase susceptibility. Instances are not confined to legumes; the greater damage to low-tannin sorghum caused by birds is well known (Doggett 1976) and there is concern about the possible susceptibility of low-glucosinolate rapeseed to small mammals and insects (Jonasson 1982). ANFs may also have biochemical functions in the plant which could lead to a yield penalty even in the absence of pest or disease.

Thus the breeder has not only to select for reduced levels of ANFs; he must balance these objectives against the deficiencies in the plants that this may cause. Alternatively, he must seek protection against pests by other forms of genetic resistance or by chemical treatments.

Genetic variation in concentration of ANF, and progress in breeding

The approximate levels of importance of the main ANFs in five grain legumes are summarised in Table 1. Only certain ANFs are in sufficient concentration to warrant priority in breeding programmes, though most other ANFs are present in low amounts in all five crops. Exceptions are alkaloids which occur only in lupins (but have now been removed in all the important *Lupinus* crop species except *L. mutabilis*), and favism-inducing glucosides which are a problem only in *Vicia faba*.

Table 1. Approximate levels of importance of the main ANFs in five grain legumes.

ANF	FABA BEAN	PEA	PHASEOLUS	LUPIN	SOYA
Tannin	High	Low	Low-Med	Low	Low
Trypsin inhibitors	Low-Med	Low-Med	Low	Low	High
Lectins	Low	Med	High	Low	High
Phytates	Low	Low	?	?	?
Oligosaccharides	Med	Low	Med	Med	Low
Glucosides	High	Absent	Absent	Absent	Absent
Alkaloids	Absent	Absent	Absent	High	Absent

1. Tannins

1.1 Association of white flowers with absence of tannin

The presence of tannin in the seed coat is pleiotropic with the black or coloured spot on the wing petal in faba beans and peas. As most of the modern cultivars of peas have white flowers, and thus associated with very low levels of tannin in seed-coats (except cv. Stehgolt, see Christiansen *et al.*, 1985), the problem is mainly in faba beans where white-flowered cultivars for animal feed are only just becoming available.

Breeding for freedom from tannin by selecting for white flowers is simple because both characters are controlled by one gene. This has been utilised in broad beans (*V. faba major*) since the 1950s. However, before embarking on a large breeding programme in small-seeded types for animal feed it has been necessary to determine whether any deficiency other than tannin is associated with white flowers. At PBI Cambridge this was done by developing and comparing near-isogenic inbred lines.

The white-flower line germinated as well as the coloured but in some soil conditions established poorly (Bond *et al.*, 1986). This was rectified by treating the seeds with a fungicide, suggesting that tannin protects faba bean seeds against soil-borne fungi.

Yields of the white-flowered spring bean line, in trials where plant numbers were equalised or yields adjusted by covariance, were not significantly different from the coloured-flowered line, although it averaged only 93% (Table 2). A white-flowered winter bean yielded 25% less than its coloured-flowered counterpart, a significant difference.

Table 2. Yield of near-isogenic tannin-free and tannin-containing lines of *Vicia faba* (t/ha).

Trial No.		Location	Tannin +	Tannin -	Diff.	SED	P
1	Spring '84+	PBI	3.41	3.56	-0.15	0.21	NS
2	Spring '84+	HAS	3.46	2.49	+0.97	0.51	NS
3	Spring '85++	PBI	3.07	3.40	-0.33	0.18	NS
4	Spring '85	SCRI	3.11	2.21	+0.90	0.48	NS
5	Winter '85++	PBI	3.66	2.75	+0.91	0.13	0.001
6	Spring '86++	PBI(T)	3.28	2.91	+0.37	0.19	NS
7	Spring '86	PBI(U)	3.38(78)*	1.64(35)	+1.74	0.19	0.001
Mean trials 1,2,3,6(Spring)			3.31	3.09			

(T) = seed treated with fungicide (U) = untreated
* Percent establishment in brackets
\+ Yield adjusted for plant numbers by covariance
++ Plant numbers equalised
PBI = Cambridge, England; SCRI = Dundee, Scotland; HAS = Haslingfield, Cambridge

The main yield component responsible for the lower yield of the tannin-free lines was fewer seeds per pod, and this is thought to indicate less insect pollination in the white-flowered line (Bond *et al.*, 1986). Other defects noted in white flowered lines were greater susceptibility to *Aphis fabae* in spring beans and greater cover in winter beans by bindweed (*Polygonum convolvulus*) although susceptibility to chocolate spot (*Botrytis fabae*) was not significantly different (Table 3).

Table 3. Infestation of near-isogenic tannin-free and tannin-containing lines of *V. faba* with black aphid, chocolate spot and bindweed.

	COLOURED.	WHITE.	DIFFERENCE.	SED.	P
Black Aphid, (1 = 10% infestation) Spring 1984	1.49	4.39	-2.90	1.29	*
Chocolate Spot, (0 = None, 6 = Aggressive) Winter 1988	3.93	3.78	+0.15	0.73	NS
Bindweed, (0 = None, 9 = Complete cover) Winter 1985	2.80	5.90	-3.10	0.64	***

Such results were not unexpected in view of reports of susceptibility of tannin-free faba beans to Fusarium solani (Statler, 1970) and to Callosobruchus (Boughdad et al., 1986). Better resistance to disease in tannin-containing seeds is also found in other legumes; for example coloured seeds of Phaseolus vulgaris are often less susceptible than white-seeded types (Reddy and Pietson, 1985).

The results of one pair of near-isogenic lines cannot be taken as definitive. Van Norel (1985) has shown that there is scope for selection for improved establishment among small-seeded white-flowered lines. Nagl (1986) reported evidence of reduced infestation of seed beetle (Bruchus rufimanus), and to the fungi Pleospora herbarum and Alternaria spp in tannin-free selections with dark-grey seed coats compared with those with white or light-grey seed coats. Like Nagl (1986), we at PBI Cambridge were unable to confirm the result of Papadopoulus et al. (1985) who reported that dark grey and/or black hilum seeds of white-flowered lines had more tannin than white seeds. We found they were almost free of tannin provided they had white flowers. It appears that in the absence of tannin, selection can result in the accummulation of other compounds that give the plant protection against some pathogens. In fact, Raczynska-Bojanowska et al. (1984) have recommended that breeders select for higher levels of non-tannin polyphenols. Also, most of the pathogens to which tannin-free beans have so far been shown to be particularly susceptible can be controlled chemically.

Moreover, composite populations constituted from white-flowered inbreds could be expected, because of partial heterosis, to perform better than the means of their components. White-flowered, tannin-free cultivars are now giving performances that are better than would be expected on the basis of near-isogenic lines, and approach that of standard, coloured-flowered cultivars.

1.2 Variation in tannin content among coloured-flowered faba beans

If the defects of white-flowered faba beans prove to be intractable, selection for low tannin concentration among coloured-flowered material may be worth while. Genetic variation has been reported in faba beans (Martin-Tanguy et al., 1977, Gorski et al., 1985 and Cabrera & Martin, 1986) and in Phaseolus (Ma and Bliss, 1978). At PBI Cambridge we confirmed the relatively low concentration of condensed tannin in the Cordoba accession, VFM 23. We also showed that standard UK cultivars of winter beans have less tannin than spring cultivars (Table 4).

Table 4. Concentration of tannin (mg/g) in coloured and white-flowered genotypes of spring and winter beans.

	COLOURED FLOWER	WHITE FLOWER
Mean of spring beans	1.77 ± 0.14	0.07 ± 0.015
Mean of winter beans	1.01 ± 0.06	0.07 ± 0.010

We also found, like Raczynska-Bojanowska et al (1984), that determinate (ti) types (with a mean of 2.15 ± 0.085 mg/g) had more tannin than indeterminates (1.77 ± 0.138), but we were unable to confirm Cabrera & Martin's (1986) finding that genotypes with diffuse flower pigment (dp) had more tannin than those with normal flower colour. Plants taken from a population of cultivar 'Bulldog', that had standard petals with deep, pale or no anthocyanin pigmentation, gave very similar means for tannin content of the seed, demonstrating that tannin is associated only with the melanin spot on the wing petals.

The above associated characters should help selection for low tannins but for the most part future breeding will involve screening of lines or plants by chemical tests. At PBI total polyphenols were highly correlated with condensed tannins but the latter could be determined at a rate of about 72 per day and would therefore be suitable for a routine screening method. However, unlike the gene controlling tannin and flower colour, inheritance is additive (Cabrera et al., 1989), the F_1 between low and high tannin parents being intermediate (Gorski et al., 1985), and introgression of low tannin into high yielding populations likely to be slow. In Phaseolus on the other hand, low tannin genes have been reported as being dominant (Ma & Bliss, 1978).

2. Trypsin inhibitors

Soya bean cultivars vary in concentrations of trypsin inhibitors (Liener, 1975), but as they are normally inactivated during the removal of oil, there is little incentive to reduce them by breeding. Lupins and Phaseolus beans have low amounts of trypsin inhibitors, and comparisons of peas and faba beans have to specify varieties because present data indicate more variation within than between species, Maro and Progeta, for example, have about three times the concentration of trypsin inhibitors as other standard cultivars of peas in UK (Griffiths, 1984, Christiansen et al., 1985, Bond and Smith, 1986, not yet published).

The high trypsin inhibitor activity (TIA) of Progreta is also associated with reduced biological value and net protein utilisation (Christiansen et al., 1985). Breeding for reduced TIA in peas has therefore to take into account any Maro or Progreta parentage. Also, winter peas have higher TIA than spring peas (Valdebouze, 1980).

There is genetic variation within faba beans, and TIA responds to selection (Sjödin et al 1981). However, there is also an effect of storage time and of the environment of the crop producing the seed. When 20 varieties were grown at two locations for 3 years and tested for TIA, effects of years and of locations within years were detected. Despite this, some agreement among the ranking of the varieties over the four trials could be discerned (Eight of the 20 varieties are shown in Table 5).

No associated characters have been detected and progress in selection for TIA may be slow unless the environment is standardised. The populations developed by Sjödin et al. (1981) from the Finnish cultivar Pirhonen were confirmed as contrasting in TIA by tests at Cambridge in 1987 but there were no associated differences in tannin content (Table 6).

Table 5. Mean TIA of faba bean cultivars at 2 locations in 3 years (mg TIA per g).

	1985		1986		1987	
	CAMBRIDGE	DUNDEE	CAMBRIDGE	DUNDEE	CAMBRIDGE	DUNDEE
Line C	2.2	3.0	2.2	3.4	2.6	3.3
Troy	2.6	2.6	3.7	2.8	2.1	2.8
Ticol	2.9	3.4	3.7	3.5	1.9	2.8
Kristall	3.2	3.5	3.7	3.6	2.5	2.9
Alfred	3.6	3.4	4.1	4.2	2.5	2.4
Minica	3.1	3.3	4.7	3.7	2.9	2.7
Whiteflower	3.8	3.9	4.5	4.0	3.4	4.0
M. Bead	3.5	4.1	4.7	4.6	2.8	3.9
LSD	0.6	0.9	0.2	0.4	0.4	0.5
CV	13.1	15.7	3.8	6.4	10.0	8.7

Table 6. TIA and tannin contents of populations selected by Sjödin et al. (1981) for high and low TIA.

	TIA	Tannin		
	Whole seed	Whole seed	Testa	Cotyledon
High TI	4.02	1.00	6.29	0.11
Low TI	2.74	1.35	6.82	0.0
Alfred	3.62	1.72		
LSD	0.17			

Tannin extracted from seed coats can inhibit trypsin (Griffiths, 1981) but the TIA of PBI Cambridge lines which are near-isogenic except for tannin did not differ in TIA, (Table 7) and a white-flowered accession that was almost free of tannin had as much TIA as standard tannin-containing varieties (Table 5).

Table 7. TIA of lines near-isogenic except for tannin.

	FLOWER COLOUR	TIA 1985
Spring isogenic	White	1.86
" "	Coloured	1.65
Winter isogenic	White	1.00
" "	Coloured	1.00

It cannot be concluded that white-flowered, tannin-free cultivars inhibit less trypsin.

As with tannins, trypsin inhibitors may have functions in deterring insect pests, an effect on Callosobruchus in cowpea(Vigna) having been shown by Gatehouse et al. (1979). Also, as trypsin inhibitors are themselves rich in methionine and cystine, their reduction might aggravate an already critically low level of these essential amino acids in the protein of beans and peas.

3. Lectins

Although small amounts of lectin have been detected in most grain legumes, it is in Phaseolus beans where lectins have caused most problems in animal and human nutrition and where plant breeding could contribute most. However it is not a major problem when the beans are heated, as in the normal method of processing Phaseolus and soya beans. Also, as total haemagglutinating activity may involve different lectins (each with different functions, like interaction with seed components (Bond et al., 1985), and perhaps controlling seed maturation and germination) it is not surprising that a lectin-free Phaseolus bean can differ from its lectin-containing counterpart in a number of other traits (Osborn & Bliss, 1985). Other functions of lectins in Phaseolus are thought to include Rhizobium recognition (Hamblin & Kent, 1973) and protection against bruchids (Janzen et al., 1976). Thus, although breeding work was already in progress in 1976 to reduce lectins in Phaseolus (Evans, 1976), low-lectin beans are not commonly in cultivation. Another problem in soya beans is that lectins have been recently detected in cultivars previously considered to be lectin free, (Tsien et al., 1983).

Lectin concentration responded to selection in faba beans (Sjödin et al., 1981) but their 'high' and 'low' lectin populations differ in some morphological characters. The lectin concentration in faba beans is not high enough to give it priority amongst breeding objectives.

4. Phytates

Generally low levels of phytates occur in most grain legumes though the amount varies among species (Marquardt, 1983). Griffiths (1982) reported differences amongst cultivars of faba beans, while Carnovale et al. (1988) reported differences among cultivars of both faba beans and peas. However, little breeding work is in progress.

5. Oligosaccharides

Flatus-producing sugars are common in grain legumes but are least troublesome in peas. In soya beans there is not sufficient genetic variation to justify a breeding programme (Rackis, 1975). However, Lattanzio et al. (1986), after examining 15 cultivars of faba beans for contents of raffinose, stachyose and verbascose concluded that some genetic variation exists. Murphy (1973) described a non-flatulent variety of P. lunatus called Fordhook and a cultivar of Phaseolus vulgaris, Pika's Jacobs, that had half the normal flatulence activity. The level of stachyose plus verbascose in Lupinus mutabilis and L. luteus is about 10%, unacceptably high compared with 4% in soya (Williams, 1984) and in need of reduction by breeding.

Recent improvements in rapid chemical assays have widened the possibilities of screening for lower levels of these oligosaccharides. For example, Price (1986) described a simple method for the isolation of verbascose from peas. Thus, where genetic variation exists, there may be an expansion of breeding work for this quality factor.

6. Glucosides

Convicine, vicine and L-DOPA occur only in faba beans. The genetic variation and high heritability values that have been reported (Bjerg et al., 1985) offer prospects for breeding both to reduce favism in humans and to enhance the nutritional value of beans for animals. Faba bean accessions differ in the level of each glucoside but it is rare to find a line that is low in both convicine and vicine. If reductions in both are necessary the scale of breeding programmes will have to be increased accordingly.

There is some evidence from in vitro studies that glucosides, particularly L-DOPA, may confer resistance to Botrytis cinerea and Ascochyta fabae (Bjerg et al., 1984). However this has yet to be confirmed in the field, and white-flowered varieties, which lack L-DOPA (Rivoira et al., 1979), do not seem to be any more susceptible to Botrytis fabae (Table 3).

If glucosides are shown by nutritionists to be partly responsible for the poor nutritional performance of faba beans compared with other grain legumes, breeders are ready to utilise the genetic variation that exists.

7. Alkaloids

Reduction of alkaloids in lupins in the 1930's is an example of the successful use of breeding to reduce ANFs. The use of two mutant alleles (Harrison and Williams, 1983) in Lupinus albus and L. angustifolius resulted in sweet cultivars with alkaloid levels below 0.25 per cent. Recently a recessive mutant for low alkaloid content has been isolated in L. mutabilis and breeding is proceeding to produce sweet cultivars of this species (Williams, 1984). This should provide a legume with grain containing good quality oil with residue suitable for animal feed. Though sweet lupins are more susceptible to aphids, mites and viruses (Pate et al., 1985), these pests are easily controlled chemically and this has been no obstacle to the change from bitter to sweet types.

8. Saponins

About 1.9% has been found in soya beans and less than 1.7% in the four crop lupins (Williams, 1984). These amounts are too small to affect animal nutrition to the extent of justifying breeding. A saponin in faba beans is hypocholesterolemic and may benefit human diets (Marquardt, 1983).

Conclusions about future breeding possibilities in each crop

(1) Faba beans

There is little doubt that an increasing number of small-seeded, white-flowered, tannin-free cultivars will be submitted for national trials and that some will be recommended for cultivation and thus extend

the use of faba beans for feeding pigs and poultry. The extra value which tannin-free faba beans might attract in EEC aid and/or fetch on the market should be nearly the same as the advantage that peas enjoy currently over faba beans, about 6%. The question of whether this will be sufficient to outweigh the defects of white-flowered beans will be answered by trials in the next few years. The evidence to date is that the problems associated with white flowers are such that genetic variation in concentration of condensed tannin in coloured-flowered faba beans should, at least, be further investigated in order to be in a position to breed low-tannin, coloured-flowered cultivars. Winter beans will be more advanced in this respect than spring beans.

Despite the demonstrated genetic variation in trypsin inhibitor levels, the effect of environment and uncertainty of the nutritional significance of lowering TIA from say 4 to 2%, make breeding for reduced TIA a secondary priority.

Selection for low glucoside levels is still at the strategic research stage but if it is confirmed that some of the poor animal performances can be attributed to glucosides, this could be an important breeding objective. If achieved, it would have the added advantage of offering safer beans for export from northern Europe as a food item to countries where favism is encountered.

(2) Peas

This crop probably has the least requirement for reduced ANFs, but improvements could be sought in TIA taking care to avoid parents that are high in TIA like Maro, Progreta and winter peas.

Coloured-flowered, tannin-containing peas are still used in some farming systems where the grain is fed mainly to cattle or the whole plant is used for fodder. Thus it is not always necessary to remove tannin, especially where attributes such as disease resistance can be more easily obtained from coloured-flowered parents. The rapid expansion of the use of peas for animal feed in Europe was with white-flowered varieties because types developed for food, being of similar seed size, could be easily transferred to the animal feed category. This contrasts with Vicia faba in which the large-seeded, white-flowered broad beans could not be so easily transferred and a breeding effort was needed.

(3) Phaseolus beans

Reduction in lectins is a clear priority in breeding most of the P. vulgaris commodities (e.g. navy beans and kidney beans). It would not only provide a safer product for human consumption but would widen the market for beans that for other reasons lack quality as human food and need to be sold for animal feed. Genetic variation exists, but lectins are compounds with complex biological functions; breeders have to be cautious about associated effects when lowering their levels. Phaseolus beans could also benefit from a reduction in oligosaccharides. Some variation exists and modern methods can detect it.

(4) Lupins

Having produced, some years ago, sweet cultivars in L. albus and L. angustifolius breeders are now engaged in reducing the alkaloid content

of L. mutabilis. Oligosaccharides also need to be reduced in L. mutabilis but the extent of genetic variation is not yet established. There are also other problems associated with the cultivation of lupins in northern Europe, e.g. diseases and speed of pod maturation. These need to be tackled by breeding before the benefit of the reduction of ANFs can be reaped in this region.

(5) Soya beans

Although TIA and lectins are quite high and some variation could be utilised, the processed soya bean meal that is imported to Europe has very little of these ANFs, and therefore they are only breeding objectives where soya beans are grown for direct feeding to animals. Such use is outside the scope of this review.

General Conclusion

Given sufficient incentives and resources, plant breeders could produce lines that vary significantly in the major ANFs. This should help nutritionists to evaluate the importance of ANF in domestic animals and man. However, plant breeders need advice, based upon existing knowledge, from animal nutritionists and chemists on (1) priorities for breeding objectives among the various ANFs and on (2) tests for ANFs enabling rapid screening of large numbers of breeding lines each with a small quantity of material.

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THE GENETICS OF TANNIN CONTENT IN FABA BEAN (VICIA FABA L.)

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Summary

Two high-tannin content lines and two low-tannin content lines differing in flower colour and seed coat colour were crossed. Tannin in the seed coat was estimated using vanillin-HCl procedure. The additive-dominance model was applied to parental, F1, F2 and backcrosses generations. Additive genetic variance was found to govern the inheritance of tannin in the seed coat. Selection in positive and negative directions was successful. The effect on tannin content of major genes controlling flower pigmentation and seed coat colour are discussed.
Keywords: tannin, tannin genetics, selection, flower colour, seed coat colour, Vicia faba

Introduction

In commom with other crop plants the study of tannin content in Vicia faba involved the analytical problems of these secondary products of plant metabolism and the influence of major genes on the synthesis of these compounds. Dickinson et al. (1957) were the first to notice the relation between white flowers and absence of tannins. Bond (1976) suggested that it is feasible to breed tannin-free varieties by selection from white-flowered material. However, white-flowered plants of faba bean are more susceptible to pest and diseases (Statler, 1970, Boughdad et al., 1986) than coloured-flowered varieties because of the lack of tannins. Thus, it will be desirable to develop varieties having low tannin content in the grain without eliminating these compounds in the rest of the plant. However, selection for low- and high-tannin lines from a coloured faba bean population has been unsuccessful (Sjödin et al., 1981).

The main objectives of the present study were to determine the genetic basis of the production of condensed tannins in the seed coats of faba beans, to create populations differing in tannin content by positive and negative selection and to study the relationships between tannin content and pigment characters in flower and seed coats.

Material and methods

Two high-tannin content lines (VF59 and VF34) were crossed with two low-tannin content lines (VF44 and VF23) of faba bean. Each line had had at least five generations of selfing. The lines differ in origin, flower colour and seed coat colour (Table 1).

Crosses (including reciprocals) were made between the high-tannin lines and low-tannin lines. F1, F2 and backcrosses were obtained in a glasshouse, free of pollinators. The genetics study was carried out by growing the plant in open field under the same enviromental conditions. The plants were allowed to mature fully and were harvested dry. F2 plants were assessed for flower colour and seed coat colour.

Table 1. Origin and characteristics of Vicia faba lines used in this work.

Line	Origin	Flower colour	Seed coat colour	Seed weight x ± Sx	% of tannin x ± Sx
VF23	Córdoba	yellow spotted	normal	0.41 ± 0.01	3.37 ± 0.14
VF44	Bond (U.K.)	normal	red-brown	0.33 ± 0.01	2.13 ± 0.09
VF34	Duc (F)	normal	yellow	1.28 ± 0.02	4.64 ± 0.08
VF59	Córdoba	solid brown	normal	0.40 ± 0.01	5.20 ± 0.23

Condensed tannins were quantitatively estimated using the vanillin-HCl method, as suggested by Broadhurst & Jones (1978). Tannin content was expressed as percentage of seed coat weight. The additive-dominance model illustrated by Mather & Jinks (1971) was applied to all generations. Data were transformed using arc sin transformation. Selection for low and high tannin content was realized from F2 populations. Analyses of the two selected populations were realized using the tannins index procedure (Ford & Hewitt, 1979).

Results and discussion

In F1, F2 and backcrosses no difference in tannin concentration was found between reciprocal crosses, so the data were pooled within each cross. The estimates of the three parameters (m= mean, d= additive and h= dominance) of the additive-dominance model are given in Table 2. The tests showed that the additive-dominance model is adequate for the four crosses performed. Clearly, additive effects are always significant. Only in a cross was the dominance effect significant.

Table 2. Joint-scaling test for tannin content in the seed coat of Vicia faba.

Parameters	VF34 X VF23	VF59 X VF23	VF34 X VF44	VF59 X VF44
(m)	4.14 ± 0.07 ***	4.36 ± 0.10 ***	3.28 ± 0.05 ***	3.47 ± 0.09 ***
(d)	0.56 ± 0.07 ***	0.90 ± 0.09 ***	1.21 ± 0.06 ***	1.41 ± 0.09 ***
(h)	0.08 ± 0.13 ns	-0.15 ± 0.20 ns	-0.43 ± 0.09 **	-0.21 ± 0.15 ns
X^2	5.11 ns	0.08 ns	6.72 ns	0.18 ns

*** : P<0.001; ** : P<0.01; * : P<0.05; ns : not significant

In contrast to the results obtained by previous authors (Sjödin et al., 1981), the positive and negative selections were sucessful, as expected after the presence of additive variance. The average values in the two selected populations clearly are different from each other in the two selection cycles (Fig. 1).

Besides the two major genes controlling the lack of pigments in the plant, other major genes are influencing tannin content in faba beans. When we look at the mean of tannin content in F2 plants with different colour and distribution of pigment in the flower we find that the presence of the recessive genes controlling solid distribution of pigment on the flower (sdp sdp) is positively correlated with high tannin content (Table 3). We found the same results previously in a study of a wide Vicia faba collection (Cabrera & Martín, 1986).

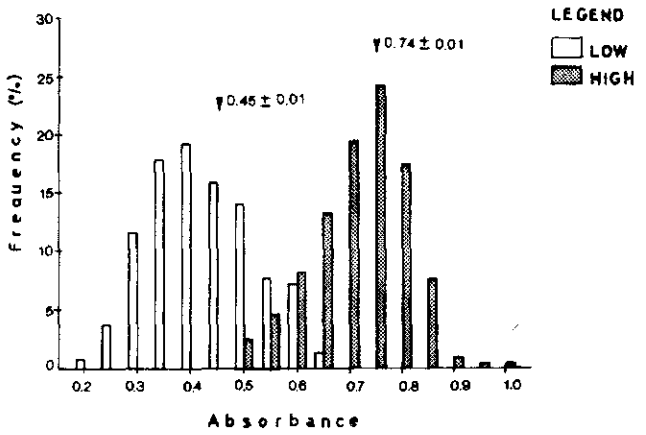

Fig. 1. Selection for tannins in faba bean.

Table 3. Mean tannin content (%) of Vicia faba F2 plants differing in flower colour.

Flower colour	Genotype	$\bar{x} \pm S\bar{x}$	N
Normal	Yf_ Sdp _	3.64 ± 0.03 a	989
Yellow spotted	yfyf Sdp_	3.52 ± 0.06 a	285
Solid brown	Yf _ sdp sdp	4.48 ± 0.08 b	222
Solid yellow	yfyf sdp sdp	4.36 ± 0.17 b	18

Mean with different letters are statistically different (P<0.01)

Table 4. Mean tannin content (%) of Vicia faba F2 plants differing in seed coat colour.

Seed coat colour	Genotype	$\bar{x} \pm S\bar{x}$	N
Red-brown	Sc_ rr	2.56 ± 0.08 a	83
Red	scsc rr	2.59 ± 0.15 a	45
Brown	Sc_ R_	3.04 ± 0.04 b	366
Normal	scsc R_	4.14 ± 0.04 c	768
Yellow	Yg_	4.18 ± 0.04 c	252

Mean with different letters are statistically different (P<0.01)

We found previously that there was no relationship between seed coat colour and tannin content in a wide collection of faba bean lines (Cabrera & Martin, 1986). Nevertheless, in the present study we find that there is a significant difference in tannin content between F2 plants with different seed coat colour. Plants which are homozygous recessive for the gene controlling red seed coat colour (rr) had significantly lower tannin content compared to plants which have the gene present in the dominant form (R_). On other hand, plants with normal or yellow seed coat colour have the higher tannin content compared with the rest of seed-coat colour (Table 4). We think that the homogeneity of the genetic background in the present study, that was absent in the first one, has made possible the detection of this relationship between seed coat colour and tannin content.

The two selected populations with high and low content of tannin were tested for flower colour and seed coat colour. It was observed that the two populations differed for these characters. The low tannin population had 96.5 % of plants with spotted flowers (normal black or yellow spots) and 3.5 % with solid pigmentation, whereas in the high tannin population

the latter percentage had risen to 40.

With respect to seed-coat colour, we found that 64 % of the low tannin-content population had brown coat and 15 % had red or red-brown seed-coat colour. The rest of the plants were normal or yellow seed-coat coloured. However, in the high tannin-content population 92.75 % had normal and yellow seed-coat colour; red or red-brown seeds were never found.

The results of the selection, which agreed with the results showed in Table 3 and Table 4, suggest a strong relationship between genes controlling tannin and pigment compounds in the plant. Tannin and anthocyanin pigments share the same biosynthetic pathway (Kristiansen, 1984), and the results obtained by us are probably a reflection of the competition for the precursor (leucocyanidin) of anthocyanidins and procyanidins. This hypothesis, if proved, would facilitate the selection for low tannin content in faba bean. On the basis of the results obtained, it is concluded that breeding for low tannin content in faba bean is feasible and that selection can be based on plants with normal flower pigmentation and red seed coat colour.

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TANNIN-FREE VICIA FABA L. AND DISEASE RESISTANCE: CONFLICTING BREEDING OBJECTIVES ?

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Abstract

Three pairs of near-isogenic lines of Vicia faba L. were bred that differed in anthocyanin-based pigmentation of the petals, one line within a pair having pigmented flowers, the other one white flowers. Condensed tannins, as determined by the vanillin-sulfuric acid method, were virtually absent in seed of the white-flowering genotypes. The isogenic lines, four parental genotypes and a commercial variety were compared with respect to resistance to soil-borne pathogens by exposing them to diseased soil in a controlled greenhouse. Plant resistance of white-flower lines or varieties was equal to or higher than that of anthocyanin-pigmented genotypes. Emergence under conditions of cold-stress was significantly lower for one of the white-flower lines, but not so for the other two. Poor emergence was alleviated by a combined seed dressing of thiram and benomyl.

The present results seem to justify the conclusion that the disease resistance of white-flower, tannin-free faba beans depends on the genetic background and that tannin-free genotypes can be bred that have adequate levels of disease resistance under stress conditions.

Introduction

Condensed tannins present in the seed coats of Vicia faba L. have been designated as the main antinutritional factors that decrease the value of the faba bean as a protein crop for animal feed. Plant breeding is a feasible approach to obtain tannin-free seeds (Bond, 1976; Cabrera & Martin, 1986). A number of recessive genes (designated as sp-genes) have been found to control the white-flower characteristic and at least one of them ($sp\text{-}b^1$) has been shown to exert pleiotropic control over the absence of tannins in the seed coat (Picard, 1976; Chapman, 1981). White-flower faba beans invariably had low tannin levels (Bond, 1976; Cabrera & Martin, 1986).

However, poor emergence of white-flower faba beans under unfavourable conditions has been reported as a serious problem connected with their potential use (van Norel, 1985; Bond et al., 1986). This study was undertaken to explore whether the use of different genetic backgrounds for introducing sp-genes could eliminate this problem. Three sets of isogenic lines were produced to study the effect of the absence of tannins on susceptibility to soil-borne diseases.

Material and methods

Three pairs of isogenic lines were studied. They were derived from the following three crosses (the second parent in each cross donated the white-flower gene): SVP-78999 x Staygreen (I10-lines); Pavane x Metissa (I4); Herz Freya x Rowena (I3). These lines were produced by continued

selfing of heterozygous F6 plants in the successive generations, in which I1 means the first generation after selfing and so on. The six (near-) isogenic lines, the four parents from the latter two crosses and the widely grown commercial coloured-flower variety Alfred were sown in a light clay soil taken from a field with a 10-year history of continuous cropping of faba beans. In a previous study (Oyarzun & van Loon, in prep.), several soil-borne fungi known to be pathogenic to faba beans (Fusarium spp., Pythium spp.; Salt, 1983) had frequently been isolated from diseased plants of the variety Alfred in the initial flowering phase.

In a first greenhouse-test, the damage to the root system of plants at the onset of flowering was examined for the 11 genotypes. Eight seeds not treated with chemicals were sown in 3 liter pots filled with diseased soil. There were 5 replicates for each genotype, which were arranged as randomized blocks. Soil moisture was kept at field capacity (water content of 25%). The plants were grown in a controlled greenhouse at 20/15 ^{o}C day/night with natural daylight under long-day conditions. After emergence the number of plants was reduced to four. The moment at which roots were inspected for symptoms of cortical rot was determined physiologically, i.e. at the onset of flowering. Plant age at which flowering occurred differed between genotypes from 36 to 48 days after sowing. Root systems were cleaned from soil with running tap water and a disease index was assigned on a visual scale of 0 to 10, reflecting the amount of damage. Zero on this scale means a completely healthy root system without brown-reddish or black discolourations, while 10 corresponds with a totally rotten root system.

In a second test, emergence under cold stress was assessed. This test was performed on the 6 isogenic lines and Alfred. Seed was sown in the same soil as used in the first test. The seed was split in two equal lots, one lot received a combined treatment with two fungicides: thiram (2 g/kg) and benomyl (4 g/kg) and the other lot was untreated. The design of the test was identical to the first test (8 seeds per pot, replicated 5 times). Cold stress was applied by exposing pots to 5 ^{o}C in a dark climatic room for 14 days. Afterwards they were transferred to a controlled greenhouse in which the temperature was set at 15/10 ^{o}C day/night. Soil moisture level was kept at saturation. Emergence was scored at 20 days after sowing.

Tannin analyses

Tannin analyses were performed on whole seeds that had been finely ground. The colorimetric vanillin-method of Swain & Hillis (1959) was employed to quantify condensed tannins.

Results

White-flower isogenic lines and parents were found to have equal or higher levels of resistance to attack by soil-borne pathogens as compared with lines and parent varieties having coloured flowers (Table 1). Significant differences occurred between parent varieties and between the isogenic lines from the cross between Pavane and Metissa. Susceptibility of Alfred was equal to or higher than of the white-flower lines.

In general, small non-significant differences in emergence were found between isogenic lines and between untreated and treated lots (Table 1). However, the difference between the untreated I10 isogenic lines was significant, the untreated white-flower line displaying a lower emergence. In this case, fungicidal treatment significantly improved emergence. Interaction between genotypes and fungicidal treatment was

absent. These results have been reproduced with larger seed quantities.

Tannin analyses demonstrated that all white-flower genotypes contained negligible amounts of tannins (0.05 % by weight) in contrast to coloured-flower ones (0.51 - 1.56 %).

Table 1. Disease indices of 6 near-isogenic lines (c - coloured flowers, w - white flowers) and 4 parental varieties and the standard variety Alfred of Vicia faba plants at the onset of flowering (test 1) and emergence of seed under cold stress, either after treatment with fungicides or untreated (test 2).

Genotype	Disease index		Emergence (%)	
	mean	SD	treated	untreated
Herz Freya (H)	7.6	0.19	-&	-
Rowena (R)	3.5	0.70	-	-
H x R I3 c	5.1	1.26	100	90
H x R I3 w	4.8	0.78	95	97.5
Pavane (P)	5.6	0.52	-	-
Metissa (M)	2.9	0.77	-	-
P x M I4 c	5.5	1.43	97.5	97.5
P x M I4 w	3.0	1.50	100	92.5
SVP 78999 (V)	-		-	-
Staygreen (S)	-		-	-
V x S I10 c	3.3	0.45	90	87.5
V x S I10 w	3.6	0.94	90	75 *
Alfred	5.7	0.78	97.5	97.5
LSD (5%)	1.12			

&: - means not tested. *: significant difference between treatments and within the untreated samples significantly lower than all other untreated genotypes ($p < 0.05$; based on 95% confidence intervals).

Discussion

So far, research on the effect of sp-genes when introduced into different genetic backgrounds has been very limited (van Norel, 1985; Bond et al., 1986). The present findings show significant differences between lines with different genetic backgrounds. This indicates that in evaluating the potential of white-flower faba beans, genotypes with different genetic backgrounds should be studied. The results reported here need, however, further confirmation under field conditions, where stress may be different or higher than in the greenhouse experiments described here. Van Norel (1985) reported a large variation in field emergence between 300 white-flower F3 lines, with a small number of these lines performing very well.

Tannins have been put forward as important resistance factors against

pathogenic fungi and insects (Levin, 1971). It is conceivable that in faba beans other defence mechanisms than those based on tannin content of the seed coat are present that are by themselves adequate to ensure a satisfactory success of germination, emergence, establishment and production under field conditions with stress due to cold, soil pathogens, excess of soil moisture and their combined actions. The involvement of pathogenic fungi in causing poor emergence is indicated by the remedial effect of fungicide application. Post-emergence resistance, as expressed in the disease index, seemed not to be influenced by the absence of tannins in the testa. This is in agreement with the field observations of Bond et al. (1986) that showed no apparent defects in white-flower lines once established.

It is concluded that opportunities exist to develop faba beans without tannins that do have adequate levels of resistance to soilborne diseases under conditions of cold stress. Both breeding objectives need not be in conflict.

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SEARCH OF GENETIC VARIABILITY FOR VICINE AND CONVICINE CONTENT IN VICIA FABA L.. A FIRST REPORT OF A GENE WHICH CODES FOR NEARLY ZERO-VICINE AND ZERO-CONVICINE CONTENTS

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Summary

A collection of 918 genotypes of Vicia faba L. has been explored for the genetic variability in two glucosides contents of mature seeds, vicine (V) and convicine (C). A continuous variation has been found for V (0.22 to 1.01% of seed dry matter), C (0.07 to 0.96% of seed DM) and total glucosides (V+C) (0.41 to 1.5% of seed DM) with independant variation for V and C contents. No relationship of glucosides content with seed size, protein content and presence or absence of tanins in the seed, was found. Additionnaly one genotype named 1268 (4)(1) was discovered, which expresses a heritable character of nearly zero glucosides content in seeds (V= 0.04% of seed DM, C= 0.004% of seed DM) as well as in green pods. The genetic determinism of this seed character appears monogenic and additive with determination by the mother plant genotype. This gene was named (vc-).

An attempt of calibrating near infrared reflectance spectroscopy (NIRS) on HPLC measurements of V, C, V+C contents has been done on seed flour. It resulted in best equations of prediction using 5 to 6 wavelengths for which the predictive values were low (best correlation coefficient between HPLC determination and NIRS prediction of V+C content was 0.7). Consequently, it seems that NIRS could only be used to measure tendancies in glucosides contents and to screen the more extreme contents. This technique could be useful but not sufficient in a breeding program using the (vc-) gene.

Keywords : Vicia faba L., breeding, glucosides, vicine, convicine, (vc-)gene.

Introduction

World production of fababean seeds (Vicia faba L.) is close to 4 millions of tons (F.A.O. source 1981). A major part of it (more than 90%) is used as human food and the remaining part, mainly in Europe, is used as a protein source in animal feed. If generally it represents a good quality food for human, however some individuals who have an inherent deficiency of glucose-6-phosphate dehydrogenase can express hemolytic crisis, called favism, after ingestion of fababeans. The consumption of fresh green pods causes more problems of this kind than the use of cooked dry seeds. The aglycones of pyrimidine glucosides, vicine and convicine, are believed to have some responsability in these problems (Belsey 1973, Liener 1979, Mager et al. 1980).

When fababean seed is used in diets of laying hens, egg production rates are reduced when more than 25% of fababean is incorporated in the feed (Campbell et al. 1980). This depressive effect has been attributed to vicine and/or convicine (Olaboro et al. 1981) with more evidence for

vicine, as convicine does not seem to be absorbed by the chick (Frohlich et al. 1983). However, in the case of rat feeding experiments, convicine singly added to a standard diet, gave higher reduction of biological value for the feed than vicine did (Bjerg et al. 1984).

Since these glucosides are thermostable products (Marquardt et al. 1983) and are mainly located in cotyledons of the seeds (Pitz et al. 1981, Jamalian 1978) removal or destruction of the causative agents by processing is difficult. However, for dishes preparations, Hegazy & Marquardt (1983) propose soaking dry seeds at temperature between 20-40°C in weak acid solution prior to cooking.

Consequently, it appears of importance to use genetic breeding to remove these products. Previous workers have explored large collections of Vicia faba L. and have found genetic variability for the total of these two glucosides content in the seed (percentage of dry matter) : 0.62 to 1.25 (Pitz et al. 1981), 0.69 to 1.26 (Gardiner et al. 1982), 0.16 to 1.79 (Bjerg et al. 1985) but to our knowledge, a glucoside-free genotype has never been reported. With such objective, we explored the genetic variability available in our fababean collection and we tested the near infrared reflectance spectroscopy (NIRS) which could facilitate the level of these products.

Material and Methods

Throughout four years (1984 to 1987), 919 genotypes of Vicia faba L. have been grown at INRA Station - Domaine d'Epoisses (Bretenières 21110 Genlis, France). They were different accessions in our collection, previously submitted to 1 to 5 generations of selfing. For each genotype, a selfing-progeny of 20 plants was grown in a row of a nursery at a density of 25 plants/m^2. Seed yield, 100 seed weight, seed protein content were measured on these genotypes. Seeds harvested on 20 plants per genotype were milled using a Cyclotec-sample-mill (Tecator) with a 1mm screen to be analysed for glucosides content. F1 seeds, and individual harvests on F1 and F2 plants from crosses with 1268 (4) (1) genotype were similarly treated.

Vicine and convicine contents were determined by high pressure liquid chromatography according to the technique described by Quemener et al. (1982) and modified in 1986 (Quemener, in press). Standards used were obtained from Dr. R.R. Marquardt (University of Manitoba, Canada). Results of vicine (V) and convicine (C) contents are expressed in percent of seed dry matter (% SDM).

The near infrared reflectance spectrum has been determined twice for each flour sample of 1985 and 1986 harvest using a reflectance spectrophotometer Infraalyzer Technicon IA500. The data have been analysed with the computer program Technicon-IDAS.

Results

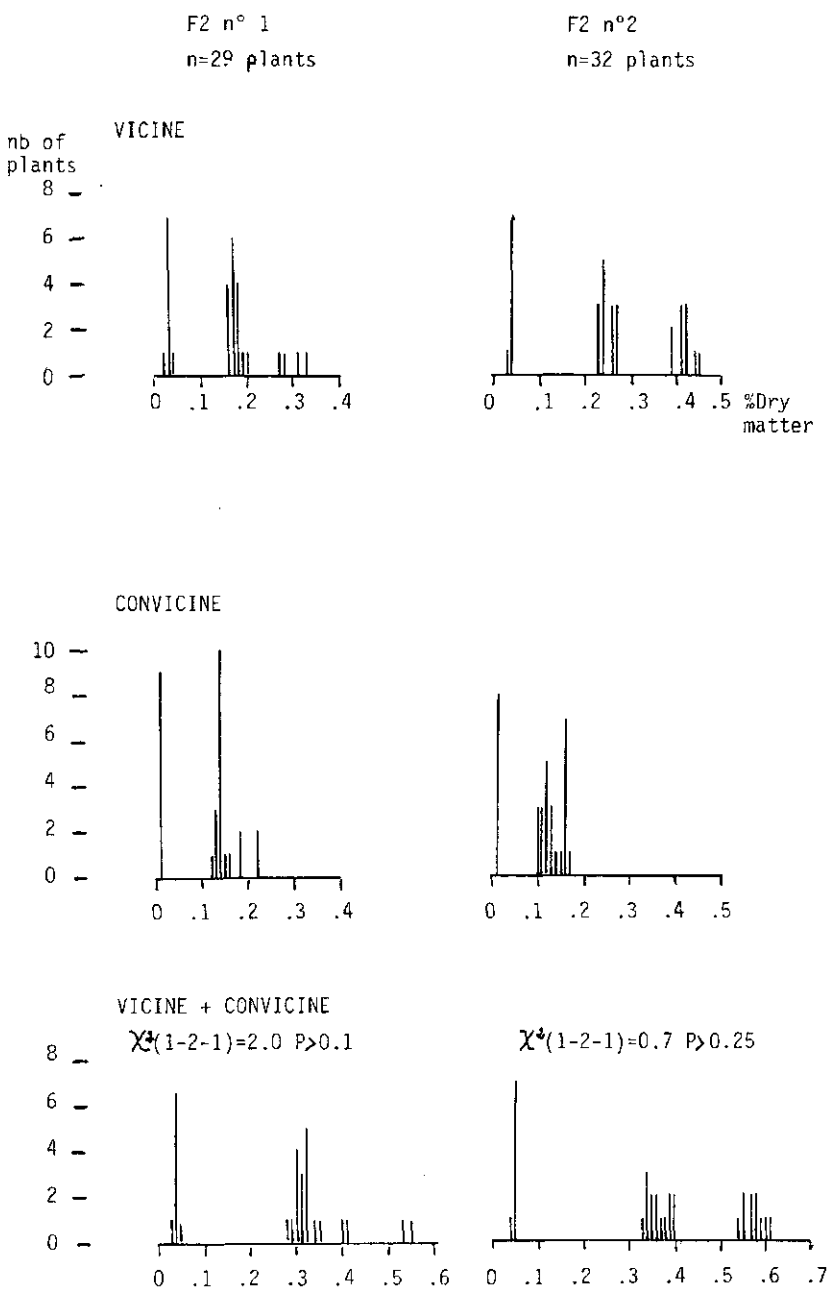
HPLC determinations of glucosides contents were of good precision since the coefficients of variation found for 15 analyses of the same flour sample were respectively 3, 4 and 2.8% for V, C and V+C contents. Excluding one particular genotype, the data showed continuous variation for V, C or V+C contents of 918 genotypes. The observed range (Table 1) was 0.22 to 1.0% SDM for V, 0.07 to 0.96% SDM for C, 0.41 to 1.5% SDM for V+C, 0.5 to 9.3 for V/C and the respective means were close to 0.6%, 0.35%, 0.95% and 2.2 in 1986-1987. Correlations between V and C contents were low and only significant in 1984 (Table 1). This result, as well as the broad range of V/C ratios (Table 1), suggest independancy in the variation of V and C contents. The detection of

Table 1. Measured genetic variability for vicine (V), convicine (C), vicine + convicine (V+C) contents expressed in percentage of seed dry matter (Min= Minimum, Max= Maximum, SE = Standard Error, n = number of samples).

		Min	Max	Mean	SE
1984					
n = 190					
	V	0.25	0.70	0.42	0.08
	C	0.08	0.41	0.22	0.06
	V+C	0.41	0.97	0.64	0.09
	V/C	0.80	8.00	2.20	1.30
	Correlation V-C (188df) = 0.22 S 1%				
1985					
n = 147					
	V	0.29	0.65	0.45	0.08
	C	0.07	0.41	0.17	0.07
	V+C	0.41	0.99	0.62	0.10
	V/C	0.80	8.40	2.40	1.40
	Correlation V-C (145df) = -0.10 NS				
1986					
n = 325*					
	V	0.22	1.01	0.61	0.13
	C	0.12	0.96	0.32	0.12
	V+C	0.47	1.50	0.93	0.18
	V/C	0.50	6.70	2.30	0.90
	Correlation V-C (323df) = 0.07 NS				
1987					
n = 257					
	V	0.29	0.88	0.58	0.10
	C	0.08	0.74	0.37	0.11
	V+C	0.47	1.37	0.95	0.13
	V/C	0.70	9.30	2.10	1.10
	Correlation V-C (256df) = 0.00 NS				

* excepted data for genotype 1268 (4) (1)

Fig 1. Segregations in two F2 progenies of crosses 1268(4)(1)Xglucosides containing genotypes.



genotypes exhibiting C content higher than V content should be noticed, as the opposite is a general situation. We did not find any clear relationship of the content in these products with seed yield or other characteristics of the seed such as the seed weight, the protein content or the presence/absence of tanins. Correlation between measurements on the same genotypes in two years (1984-1986) had values of 0.42 (S 5%) for V, 0.64 (S 1%) for C, 0.42 (S 5%) for V+C which gives a first measurement of the heritability of these contents appearing higher for C.

One particular genotype, 1268(4)(1), studied in 1986 is an introduction from a collection of the Department of Plant Genetic Resources (Radzikov - Poland), and it is an exception to the previously described continuous variation. Its contents in V, C, V+C are respectively 0.042, 0.004, 0.046% SDM which is 10 times lower that the minimum of V+C content previously reported. We call it a "zero-VC" genotype. It has been submitted to two generations of selfing and the resulting progenies observed in field and in glasshouse, either considered at the individual plant level or in the aggregate, expressed identical values for V and C contents. We conclude this character is highly heritable.

F1 seeds from crosses between 1268(4)(1) (used as male or female) and glucosides containing genotypes (cv. Troy and cv. Alfred) appeared of maternal phenotype for V and C contents. This result shows the mother plant genotype and not the embryo genotype determines the phenotype of seeds.

From the absence of difference in the harvest phenotype on reciprocal F1 plants (Table 2) we can eliminate in our crosses any extranuclear genetic determinism on the glucoside character. The intermediate phenotype of F1 plants, when compared to the parental values (Table 2), indicates an additive genetic determinism.

Segregations in F2 progenies from the cross 1268(4)(1) ("zero-VC") x 1268(2) (glucosides containing genotype) appeared to fit a (1-2-1) ratio (zero V+C-medium V+C-high V+C contents). This result supports a nuclear monogenic additive determinism of the "zero-vc" character. We named this gene (vc-).

The coincidence in the distribution of plants for V, C and V+C values (correlation between V and C : r= 0.98) shows that the same gene (vc-) must code for zero-V and zero-C characters.

Analysis (Fig. 2) of V and C contents in seeds and shells of pods of two genotypes (1268(4)(1) and a glucosides-containing genotype) at two stages (young pods of 4cm and mature pods) shows 1- that shells of both genotypes have in young pods higher content in C than in V and that these contents are strongly reduced at maturity ; 2- that seeds of both genotypes have at the two stages higher content in V than in C and that these contents are reduced at maturity ; 3- that 1268(4)(1) has V and C contents close to zero at young stages of pod development as well as in mature pods, in shells as well as in seeds.

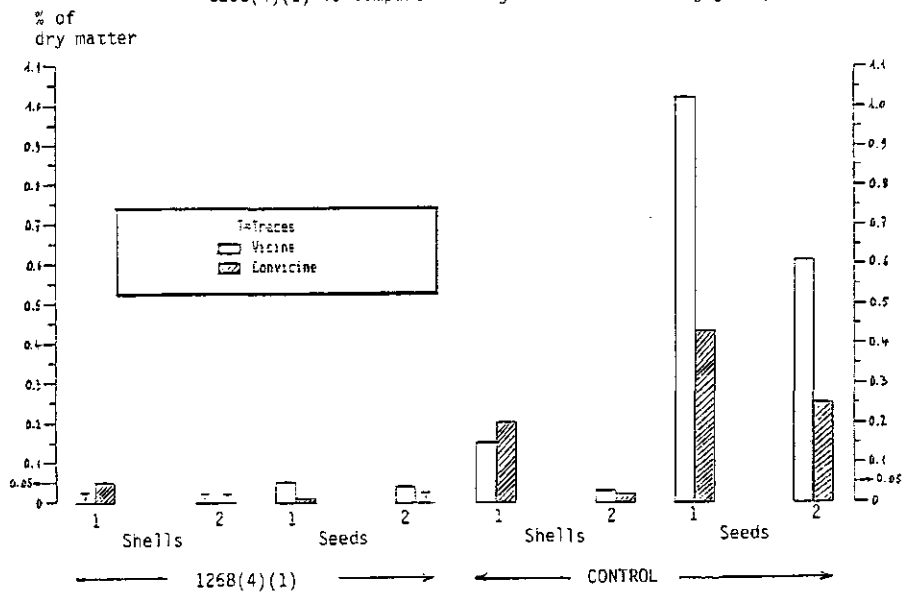
As a first approach, the (vc-) gene does not seem to have deleterious effect on the plant. Over three years of observation in Dijon revealed no particular disease susceptibility, a seed protein content of 32-34%, a 100 seed weight of 50-60g, a seed yield in nursery plots very close to the controls (cv. Ascott and Blandine).

Calibration of NIRS on HPLC total glucosides determinations was investigated on 1985 and 1986 harvests. A set of 57 samples in 1985 and 69 samples in 1986, representing the genetic variability for the glucoside contents, was used for the calibration. Resulting equation of prediction (linear regression on absorbance in defined wavelengths) was

Table 2. Parental and F1 values for vicine and convicine contents (% DM) in crosses with 1268(4)(1) genotype.

		V	C	V+C
Parents				
	1268(4)(1)	0.043	0.004	0.0047
	Alfred	0.74	0.31	1.05
	Troy	0.96	0.18	1.14
F1				
	1268(4)(1) x Alfred	0.32	0.16	0.48
	Alfred x 1268(4)(1)	0.31	0.15	0.46
	1268(4)(1) x Troy	0.43	0.13	0.56
	Troy x 1268(4)(1)	0.39	0.13	0.52

Fig 2 Vicine and convicine concentrations in shells and seeds of pods at two stages 1- young pod of 4cm, 2- mature pod. 1268(4)(1) is compared to a glucoside containing genotype.



then applied on the remaining sample to test its predictive value. Best correlations of calibration were obtained when using the second derivative on spectrum data with 6 selected wavelengths 2242, 1686, 1790, 1970, 2150, 1962nm in 1985 and 1204, 1952, 1508, 2192, 1776, 1572nm in 1986 with respective coefficients of correlation r= 0.81, r= 0.86. The respective estimated standard errors were 0.072, 0.12. Testing these equations on a set or prediction from 1985 and 1986 separately gave lower correlations, r, ranging from 0.56 to 0.69 (64 DF) and higher standard error ranging from 0.10 to 0.17. Testing calibration on V and C contents singly did not improve the quality of the prediction by NIRS.

A new equation was developed with 6 wavelengths on the individual harvest of F2 plants from the cross 1268 (4)(1) x 1268(2). When tested on a set of prediction in this F2 (Fig. 3), it correlates at level of 0.7 with a SE = 0.12. Consequently it seems that NIRS technique could reduce by half the number of HPLC determinations necessary to detect vc-/vc- plants in F2 progenies but cannot replace HPLC measurement in a breeding program using the (vc-) gene.

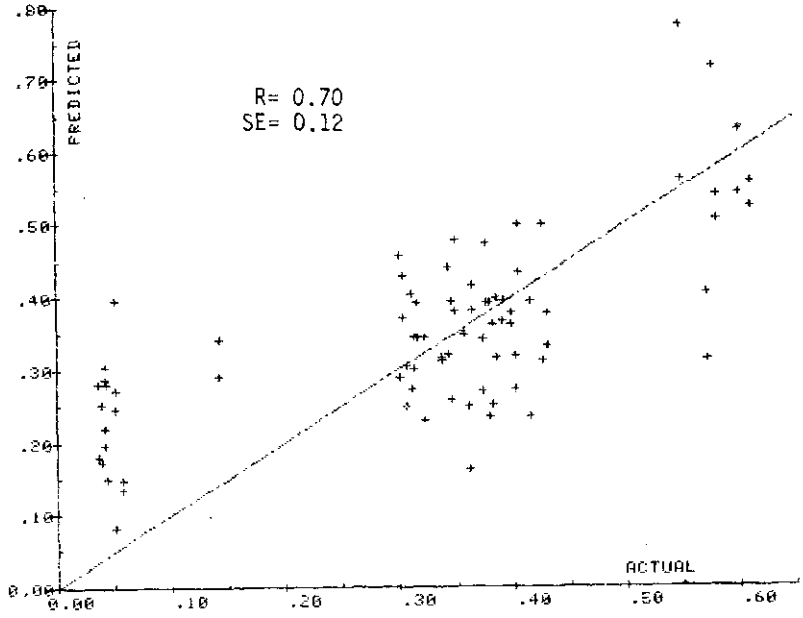
Discussion

Beside a continuous and independant variation in genetic variability in Vicia faba L. for V and C content very similar to the measures of Jamalian 1978, Pitz et al. 1981, Gardiner et al. 1982 and a sligthly lower variability found by Bjerg et al. 1985 (V+C content varied from 0.16% to 1.79% of SDM), we report on a genotype 1268(4)(1') which presents nearly zero V and zero C contents. We called it "zero- VC". V+C content in this genotype (0.046% SDM) is 10 times lower than our minimum measurement and 35 times lower than our maximum measurement. It still contains traces of V (0.042%) higher than traces of C (0.004%). This observation on mature seeds was also true for young green pods, which indicates this new genotypes has also a potential to reduce the antinutritionnal character of Vicia faba when it is used in human food at a green-stage. Consequence of the "zero-vc" character on the DOPA content (L-3,4-dihydroxyphenylalanine) is presently under investigation.

Even if V and C are contained in the cotyledons of the seed, no effect of the embryo genotype on the VC seed phenotype was detected. This result shows these two products must be transfered from the mother plant to the seed without biosynthesis of them into the seed.The fact zero-V and zero-C characters are controlled by the same gene suggest the action of this gene at a common step in the biosynthesis pathway of the two products.

From the monogenic additive determinism of the "zero-vc" character we can expect a simple use of 1268(4)(1) as genitor in a breeding program aiming at reducing vicine and convicine contents in Vicia faba. The fact that no deleterious effect of the (vc-) gene on plant growth was detected to date is another argument to support its use in a breeding program. NIRS technique did not seem to be very well adapted to the measurement of the continuous genetic variation in V, C or V+C contents and even to characterize the homozygous state for (vc-) gene. However it could reduce by half the number of HPLC determinations of VC contents when used in a breeding program involving the "zero-vc" character.

Figure 3. Correlation between actual values and predicted values by NIRS technique of V+C content in a F2 progeny segregating for the"zero-VC"character.



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DISCUSSION ON PLANT BREEDING

Chairman: Z. Nitsan
Reported by: D.J. van Zuilichem

As to the plant breeders comments it should be mentioned that there is a gap in the information stream between breeders and nutritionists. Since it is possible to produce crop-lines that vary significantly in the major ANF's, it should be logic to evaluate the effects of the combined levels of ANF's in the animal. Furthermore, the variability between batches used in studies can be overcome by the use of isogenic lines; these lines can be also very important in relation with livestock feeding trials. The possibly conflicting breeding objectives for lowering ANF levels and a consequently lower disease resistance stress the need for estimating threshold levels for ANF in the animal. Information flow from the nutritionists to the breeders must be improved.

Advises were given not only to breeding goals for low levels of ANF's but also to concentrate on patterns of sulphuric amino acids and types of storage proteins.
This effect can also be reached by making mixtures of totally different crops, getting the missing supplementary patterns without using artificially produced additives.

Finally the comment was recorded that there is a strong need for more confirmation data of breeding varieties by performing field trials under different and severe circumstances due to cold, soil pathogens, dryness and combined effect.

Sessions different Nutritional aspects of ANFs

COMPARISON OF EFFECTS OF ANTINUTRITIONAL FACTORS (ANFs) IN DIFFERENT ANIMAL SPECIES.

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Summary

Four experiments with piglets, rats and chickens were carried out in which the effects of ANFs present in Phaseolus vulgaris and Pisum sativum on zootechnical characteristics were compared.
Weight gain in piglets was much more negatively affected than in rats and chickens.
The negative effects on weight gain were not related to a deficiency of amino acids, but to a toxic factor. It was found that the difference in sensitivity to ANFs in the Phaseolus vulgaris between rats and piglets is not related to differences in physiological age.
Feeding Phaseolus vulgaris caused a decreased weight of the pancreas and the spleen in piglets, this was not observed in rats and chickens.
The results demonstrate that effects of ANFs present in Phaseolus vulgaris and Pisum sativum obtained in rats and chickens cannot be extrapolated to the piglet, indicating that nutritional aspects of ANF research should be studied in target animals.

Keywords: Antinutritional factors, Phaseolus vulgaris, Pisum sativum, piglets, rats, chickens, weight gain, pancreas, spleen.

Introduction

Many seeds contain substances wich are referred as Antinutritional Factors (ANFs) (Liener, 1980, 1989). The main ANFs in the legume seeds like beans and peas are trypsin inhibitors, lectins and tannins (Huisman et al, 1989). Reports in literature and the contributions of this workshop demonstrate that ANFs cause their negative effects in various animal species (Liener, 1980 and papers of this Workshop). However, the mode of action of ANFs is mainly studied in rats and chickens. For a good assesment of the detrimental effects of ANFs it is necessary to quantify these effects and to study their consequences for nutritional value. It is also important to know to which level the ANFs have to be reduced in order to neutralize their negative effects. Most of the studies in which the effect of technological treatments on ANF activity were studied, have been carried out with rats using PER, NPU, N balance, weight gain as parameters. Based on these results conclusions are made about the optimal treatments for inactivation of ANFs. An important question is whether results obtained in rats and chickens can be extrapolated to the pig. There is also hardly any information available regarding threshold levels in target animals. From these points of view it is important to know whether results obtained in rats and chickens can be extrapolated to other animal species like the pig.

Until now there are hardly any data published regarding this question. Studies of Combs et al. (1967) and Yen et al. (1977) suggest that the rat and the piglet respond differently to ANF in raw soyabeans. Visitpanich et al. (1985) found that the rat and piglet respond differently to feeding chickpeas. Liener and Kakade (1980) summarised data regarding the effect of trypsin inhibitors on the pancreas in different animal species. They showed that in the smaller animals like the rat, mouse and chicken pancreas hypertrophy occurs due to the trypsin inhibitors, but not in the larger animals like the pig and the calf. However, although no pancreas hypertrophy occur in the larger animals it may not be concluded that the negative feedback mechanism is not affected which is responsible for an increased secretion of pancreas enzymes. It may also be possible that the animal can compensate the effect of trypsin inhibitors to a certain extent in such a way that weight gain is not influenced (Huisman and van der Poel, 1989b).
An important point is that there is a gap in knowledge regarding the sensitivity of the animal species to ANF. It possible that the tolerance to ANFs is different in the various animal species. To get insight into this question four experiments with piglets, rats and chickens were carried out in which the effects of ANF present in Phaseolus vulgaris and Pisum sativum were compared.
In the first experiment a comparison between rats and piglets fed diets containing 20% Phaseolus vulgaris beans was made. These diets were balanced for total protein and amino acids.
In the second experiment the effect of inclusion 20% Phaseolus beans in diets with a different content of digestible protein and amino acids were studied in piglets, rats and chickens.
In the third experiment the effect of feeding a diet containing 20% Phaseolus beans was studied in pigs of different ages.
In the fourth experiment the sensitivity of piglets, rats and chickens was tested in diets containing 30% peas.

EXPERIMENT 1

Material and methods

In this experiment a comparison between rats and piglets fed diets containing 20% Phaseolus beans was made. Three diets were formulated, a control diet (C) containing no beans (main protein source soya and fishmeal) and two test diets containing 20% Phaseolus beans. In one of the test diets raw Phaseolus beans (rPh) was included and in the other test diet Phaseolus beans which had been toasted for 40 minutes (tPh). The diets were balanced for total protein, lysine, methionine + cystine, net energy, Ca and P. The contents in the control and test diets were: crude protein 18.4 and 18.2%, respectively, lysine 1.0%, methionine + cystine 0.65%, net energy 10 MJ/Kg, Ca 0.99% and P 0.70%. The diets were pelleted without steam. Detailed information is given in Huisman and van der Poel, 1989a. A batch with a rather high lectin content was selected. The chemical composition of the bean was: dry matter 89.2%, ash 4.8%, crude protein 22.7% and crude fat 2.0%. The ANF contents in the Phaseolus beans are given in table 1.

Table 1. Contents of ANF, protein solubility and urease activity in the raw and the toasted beans.

Criteria	raw beans	toasted beans
Haemagglutinins (HA)*	30	1.92
Trypsin inhibitors**	4.7 mg	<0.3 mg
Protein dispersibility index (PDI)***	36	22
Urease activity****	0.02 mg	0.0 mg

* Haemagglutination of rabbit red bloodcells. 1 HA= 1:1000 dilution steps. (Valdebouze et al., 1980)
** mg inhibited trypsin per g product.
*** Analysed according to the method Ba 10 - 65 of the American Oil Chemists Society.
**** Release of ammonia-N (mg) during one minute from a solution of 30 C caused by addition of 1 gram product.

Each diet was fed to 15 piglets of the crossbred Dutch Landrace x Dutch Yorkshire and to 15 Wistar rats. The main criteria were weight gain and the weight of pancreas and spleen.
Weight gain in piglets was measured in the period of 4 - 7 weeks of age and in rats from 5 - 8 weeks of age. On the day following the termination of the growth period, 8 piglets and 8 rats of each treatment were taken at random for dissection and collection of organs. After anaesthesia the abdomen was opened, and the organs were removed quickly and weighed immidiately. Just before dissection the animals were weighed; the organ weights were calculated as a percentage of body weight.
The daily allotment of feed to the piglets was restricted to 4% of body weight. Feed consumption of the rats was restricted to 80% of the feed consumed by an extra group of rats fed the control diet ad libitum. Water was freely available from nipple drinkers.

Results and discussion

Weight gain measured in the rats and piglets is presented in table 2. Different organs were weighed. Only the data of the pancreas and the spleen are presented here (tables 3 and 4).

Table 2. Weight gain measured during 21 days.

Treatment	Piglets			Rats		
	g/day	SD	%*	g/day	SD	%*
C diet	137.7^{a}	19.3	100	6.68^{a}	0.24	100
rPh diet	-36.0^{b}	5.8	-26	4.86^{b}	0.20	73
tPh diet	111.8^{c}	20.7	81	6.32^{c}	0.46	95

* Weight gain as a % of control
Data in the same column with a different superscript differ significantly ($P<0.05$).

Table 3. Weight of the pancreas (% of live weight).

Treatment	Piglets		Rats	
	mean	SD	mean	SD
C diet	0.21[a]	0.04	0.34[a]	0.43
rPh diet	0.10[b]	0.03	0.61[b]	0.07
tPh diet	0.29[a]	0.03	0.36[a]	0.06

Data in the same column with a different superscript differ significantly ($P<0.05$).

Table 4. Weight of the spleen (% of live weight).

Treatment	Piglets		Rats	
	mean	SD	mean	SD
C diet	0.34[a]	0.09	0.24[a]	0.07
rPh diet	0.14[b]	0.05	0.23[a]	0.07
tPh diet	0.32[a]	0.10	0.25[a]	0.06

Data in the same column with a different superscript differ significantly ($P<0.05$).

The results show clearly that weight gain in the piglets was distinctly more depressed than in the rats fed the same diet. The piglets fed the rPh diet could not even maintain their body weight, whereas the rats were still gaining weight, although at a lower level (-27%) compared to the control rats. Weight gain in the rats was not as markedly reduced as has often been reported in literature when raw Phaseolus beans are fed. However, in many reports the dietary inclusion level of beans was higher and in some reports the beans were the only protein source.
Marked differences were observed in effects on pancreas and spleen weight between both animal species. The rats showed an increased weight of the pancreas when the rPh diet was fed, but in piglets the pancreas weight was decreased.
The increased weight of the pancreas in the rats may be related to the presence of trypsin inhibitors in the raw beans. It is not clear why the weight of the pancreas in the piglets was decreased. In the same piglets we found a severe gutwall damage. One can speculate that the decrease in pancreas weight may be related to a damage of the CCK-PZ producing endocrine cells in the gutwall. A tendency for lower pancreas weight due to feeding of raw Phaseolus beans was also observed by Meyer et al. (1982). They also found that the activity of the pancreas enzymes was decreased. These results seem also to agree with the results of King et al. (1983) who observed degenerative changes in the pancreas cells of pigs fed raw Phaseolus beans.

The weight of the spleen of the rats was not different between the treatments. In the piglets fed the rPh diet, however, spleen weight was significantly lower compared to the control animals and the piglets fed the tPh diet. Greer (1983) found a reduced spleen weight in rats fed diets containing 5% casein protein and 5% protein from Phaseolus beans. In our experiment the diets contained about 4.5% protein from the Phaseolus bean and about 14% protein from other sources. In fact our diets contained relatively lower levels of bean protein than in the study of Greer (1983). This may be the reason that Greer found a reduced spleen weight in the rats and whereas we did not.
Summarising the results it can be concluded that the weight gain in the piglets was much more negatively affected due to feeding of Phaseolus beans than in the rats. Moreover, the effects on the weight of the pancreas and the spleen were distinctly different between the rats and the piglets. The reason for this species difference is not known. A possible factor that may have played a role was that in this experiment the protein digestibility of the rPh diet was distinctly different between piglets and rats (data not presented here). For the tPh diet the difference in digestibility was only small. It may not be excluded that a part of the differences between both animal species was related to the differences in protein digestibility. This hypothesis was tested in experiment 2 with piglets, rats and chickens fed diets differing in amounts of digestible protein.

EXPERIMENT 2.

Materials and methods

In this experiment a comparison was made between piglets, rats and chickens fed diets differing in content of digestible protein. Prior to the experiment the digestibility of the protein of the toasted Phaseolus beans was measured to be 60% in piglets. This digestibility coefficient was used to formulate the diets. Four diets were formulated, a control diet (C) containing no beans (main protein source casein), two diets containing 20% raw Phaseolus beans (rPh) and one diet containing 20% toasted Phaseolus beans (tPh). The diets were balanced for 15.5% digestible protein, 1.27% digestible lysine, 0.65% digestible methionine + cystine, 0.73% digestible threonine and 0.21% digestible tryptophan and further for net energy and minerals. Extra methionine and arginine were added to the chick diets.
In the tPh diet and one of the rPh diets (rPh60) the protein digestibilty of the Phaseolus vulgaris was set at 60%. In the other rPh diet protein digestibility was assumed to be 0% (rPh0). In this diet extra casein was incorporated to compensate the assumed lower digetibility of the bean protein. The diets were pelleted without steam. A commercial batch with a medium high level of lectins was selected. The chemical composition of the raw Phaseolus beans was: Dry matter 89.4%, ash 4.9%, crude protein 22.4%, crude fat 2.0% and crude fibre 7.1%. The ANF contents were: haemagglutinins 40 HA and 4.7 mg inhibited trypsin per gram product in the raw Phaseolus beans and 0.8 HA and <0.3 mg inhibited trypsin inhibitor in the toasted beans.
Each diet was fed to 12 piglets (crossbred : Dutch landrace x Dutch Yorkshire), 15 Wistar rats and 60 Hybro chickens.

Weight gain was measured during 14 days in the piglets and 21 days in the rats and the chickens. The age of the animals during the test periods was: 4-6 weeks for the piglets, 5 - 8 weeks for the rats and 1 - 4 weeks for the chickens. Main criteria were weight gain and organ weights. On the day following the termination of the growth period from each treatment 7 piglets, 7 rats and 12 chickens were taken at randomly for collection of organs. All three animal species were fed according to 2.2 times maintenance for energy.

Results and discussion

Weight gain in the piglets fed the rPh diets was again very markedly reduced (table 5). Extra addition of casein in the rPh0 diet did not increase the weight gain. This result indicates that the negative effects on weight gain are related to a toxic factor and not to a deficiency of protein or amino acids.
Weight gain of the piglets fed the tPh diet was about similar to the C diet. This result indicates that toasted beans can be incorporated in pig diets when an adequate correction is made for the lower protein digestibility. The negative effect in the rats fed the rPh diets was smaller than in the previous experiment. This may be related to the fact that in this experiment the diets were balanced with respect to digestible protein and amino acids. In chickens the negative effects on weight gain were also small.

Table 5. Weight gain measured during 14 days in piglets and 21 days in rats and chickens.

Treatment	Piglets			Rats			Chickens		
	g/day	SD	%*	g/day	SD	%*	g/day	SD	%*
C diet	151.6^{a}	32.9	100	3.0^{a}	0.6	100	11.7^{a}	0.7	100
rPh0 diet	-2.1^{b}	26.3	-1	3.1^{a}	0.4	103	12.1^{b}	0.3	103
rPh60 diet	-3.5^{b}	23.7	-2	2.9^{a}	0.5	97	10.6^{c}	0.4	91
tPh diet	145.8^{a}	37.0	96	3.1^{a}	0.4	103	11.9^{a}	0.5	102

Data with a different superscript in the same column differ significantly ($P<0.05$)

In rats and chickens fed the rPh60 diet a pancreas hypertrophy was observed, but not in piglets (table 6). In piglets there was a tendency for decreased weight of the pancreas when the three Ph diets were fed. However, this effect is not as dramatic as found in the previous experiment.

Table 6. Weight of the pancreas (% of live weight).

Treatment	Piglets mean	SD	Rats mean	SD	Chickens mean	SD
C diet	0.18[a]	0.03	0.51[a]	0.15	0.29[a]	0.04
rPh0 diet	0.16[a]	0.02	--	--	--	--
rPh60 diet	0.16[a]	0.04	0.69[b]	0.23	0.39[b]	0.05
tPh diet	0.16[a]	0.02	0.65[b]	0.22	0.30[a]	0.03

Data with a different superscript in the same column differ significantly ($P<0.05$).

Spleen weights of the piglets fed both rPh diets were markedly decreased. In rats and chickens no effect on the spleen was observed (table 6).

Table 7. Weight of the spleen (% of live weight).

Treatment	Piglets mean	SD	Rats mean	SD	Chickens mean	SD
C diet	0.27[a]	0.09	0.24[a]	0.03	0.10[a]	0.02
rPh0 diet	0.15[b]	0.04	--	--	--	--
rPh60 diet	0.15[b]	0.04	0.23[a]	0.03	0.10[a]	0.03
tPh diet	0.21[c]	0.04	0.25[a]	0.03	0.11[a]	0.03

Data with a different superscript in the same column differ significantly ($P<0.05$).

In conclusion it can be stated that the piglet respond differently to feeding diets containing 20% Phaseolus bean than the rat and the chicken.
A discussion point that remains is the fact that, although the rat and piglet used in these experiments had about the same age, their physiological age may be different. It is possible that an older pig may be less sensitive to ANF than the young pig and may be more comparable with the young rat. Grant et al. (1985) studied the age dependency to ANF in rats. He found that there was no age dependency to effects of ANF in Phaseolus beans. In experiment 3 observations regarding the age dependency of pigs to ANF in the Phaseolus beans were carried out.

EXPERIMENT 3

Material and methods

In this experiment the effect of feeding a diet containing 20% raw Phaseolus beans was tested in pigs of different ages. Two diets were formulated, a control diet containing no beans (C diet) and a test diet containing 20% raw Phaseolus beans (rPh diet). Both diets were balanced for digestible protein, lysine, methionine+cystine, threonine and tryptophan and further for net energy and minerals. The contents in both diets were: digestible protein 14.0%, dig.lysine 0.90%, dig. methionine + cystine 0.55%, dig.threonine 0.65% and dig.tryptophan 0.19%. Net energy was 9.6 Mj/kg. The diets were pelleted without steam. The experiment was carried out with 144 pigs of the cross Dutch Landrace x Dutch Yorkshire. The pigs were housed in 36 pens of 4 animals each. At three times (P1, P2 and P3) pigs were changed from the control diet to the test diet. In P1 the live weight of the pigs was about 18 kg (about 8 weeks of age), in P2 about 35 kg (about 12 weeks of age) and in P3 about 55 kg (about 16 weeks of age). During each P period 6 pens remained on the control diet and 6 pens were changed to the test diet. Because the effects were so clear the weight gain was only measured during 2 weeks. The daily allotment of feed to the pigs was based on 3.2 times maintenance for energy. The diets were fed as a dry meal and water was freely available from nipple drinkers.

Results and discussion

In all three age periods there was a strong negative effect due to feeding the raw Phaseolus beans (table 8).
The results demonstrate clearly that in pigs there was no age dependency in the period up to 4 months of age regarding the effects of feeding a diet containing 20% raw Phaseolus beans. In conclusion it can be stated that differences in sensitivity between piglets and rats cannot be explained by differences in physiological age in the periods measured.

Table 8. Weight gain (g/day) measured during periods of 14 days.

Treatment	Weight gain of the pigs					
	P1: live weight 18 kg		P2:Live weight 35 kg		P3:Live weight 55 kg	
	g/day	SD	g/day	SD	g/day	SD
C diet	448[a]	32	626[a]	36	801[a]	53
rPh diet	-65[b]	22	-154[b]	54	-128[b]	94

Data with a different superscript in the same column differ significantly ($P < 0.001$).

Experiment 4.

Materials and methods.

In the previous experiments the sensitivity of different animal species to ANF was tested by using Phaseolus beans as a model for a lectin containing seed. In Europe this bean has only limited use in pig nutrition due to the low protein nutritional value (Van der Poel, 1989). Peas are more commonly used. Therefore a test was also carried out with diets containing Pisum sativum.
Two diets were formulated, a control diet containing no Pisum sativum (C diet) and a test diet containing 30% Pisum sativum (P diet). The digestibility of the protein of the batch peas had been previously measured to be 85% in piglets. By using this figure the diets were balanced for digestible protein and amino acids, net energy and minerals. The contents in both diets were: digestible protein 15.5%, dig. lysine 1.27%, dig.threonine 0.73%, dig.tryptophan 0.21% and net energy 10.0 Mj/kg. The diets were pelleted without steam. The contents of crude protein and ANFs in the Pisum sativum were: crude protein 22.9%, 0.9 mg inhibited trypsin/g product and haemagglutination activity 20 HA. Each diet was fed to 12 piglets (Dutch landrace x Dutch Yorkshire), 12 Wistar rats and 60 Hybro chickens. The diets were fed at two feeding levels, 2.2 and 3.2 times maintenance requirement for energy. Water was freely available.
Weight gain in piglets was measured in the period 4-6 weeks of age, in the rats in the period of 5-8 weeks of age and in the chickens in the period of 1- 4 weeks of age. On the day following the termination of the growth period a part of the animals was dissected and their organs were weighed. From the treatments fed at the 3.2 feeding level 7 piglets, 7 rats and 12 chickens were at randomly for collection of the organs. From both rat treatments fed at the 2.2. feeding level also 7 animals were dissected. After anaesthesia the abdomen was opened and the organs were removed quickly and weighed immediately.

Results and discussion

The results of weight gain (table 9) show that there was a negative effect on gain in the piglets when fed the P diet, but not in rats and chickens. In piglets the negative effect was more marked on the low feeding level.
The weight of the pancreas was not affected in the piglet. In rats and chickens the pancreas weight of the P diet fed animals was higher compared to the control animals.
The spleen weight was not affected in all three species (table 10).
Summarising the results it can be concluded that the piglet is more sensitive to ANFs in the pea than rats and chickens.

Table 9. Weight gain (g/day) measured in piglets, rats and chickens.

Treatment	Piglets g/day	SD	% *	Rats g/day	SD	% *	Chickens g/day	SD	% *
	Feeding level 2.2 maintenance								
C diet	124.4[a]	27.1	100	3.0[a]	0.5	100	20.9[a]	0.4	100
P diet	98.1[b]	22.3	79	3.3[a]	0.5	110	21.3[a]	0.9	102
	Feeding level 3.2 maintenance								
C diet	201.7[a]	52.7	100	5.8[a]	0.7	100	41.7[a]	1.6	100
P diet	187.2[a]	34.7	93	5.8[a]	0.5	100	43.4[a]	5.8	104

* Weight gain as a % of control
Data in the same column with a different superscript differ signififcantly ($P < 0.05$).

Table 10. Weight of pancreas and spleen (% of live weight).

Treatment	Piglets 3.2 feeding level mean	SD	Rats 2.2 feeding level mean	SD	Rats 3.2 feeding level mean	SD	Chickens 3.2 feeding level mean	SD
			Pancreas					
C diet	0.17[a]	0.06	0.46[a]	0.06	0.49[a]	0.11	0.18[a]	0.02
P diet	0.18[a]	0.03	0.60[b]	0.08	0.53[a]	0.08	0.21[a]	0.03
			Spleen					
C diet	0.24[a]	0.05	0.23[a]	0.03	0.22[a]	0.02	0.14[a]	0.06
P diet	0.21[a]	0.04	0.25[a]	0.04	0.23[a]	0.03	0.13[a]	0.06

Data in the same column with a different superscript differ significantly ($P<0.05$).

General conclusions

The results presented in this paper demonstrate clearly that there is a difference in sensitivity between animal species to ANF present in Phaseolus vulgaris and Pisum sativum. The piglets were much more sensitive to these ANFs than rats and chickens. It was demonstrated that the negative effects in pigs caused by ANFs present in Phaseolus vulgaris were not age-dependent in the period up to 16 weeks of age. From this result the conclusion can be drawn that the difference in sensitivity to ANF in the Phaseolus bean between rats and piglets is not related to differences in physiological age.

The results demonstrate also that effects of ANF obtained in rats and chickens cannot be extrapolated to the piglet. This leads us to the conclusion that the nutritional aspects of ANF should be studied in target animals.

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LEGUME SEED OLIGOSACCHARIDES

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Summary

The oligosaccharides of the raffinose family (eg. raffinose, stachyose, verbascose) are important constituents of a wide variety of grain legumes and vary in their concentration among leguminous species as well as in varieties of a given legume. These oligosaccharides possess an ability to induce flatulence in monogastrics, which is generally ascribed to the action of intestinal anaerobic microflora on galactose residues released as a result of α-galactosidase action in the lower intestinal tract. High levels of oligosaccharides can therefore impair the nutritional utilization of grain legumes, as well as causing distressing symptoms such as diarrhoea, nausea, cramps and discomfort in the animals.
Keywords: legumes, oligosaccharides, formation, elimination, flatulence.

Introduction

Seeds of many legume species provide an important source of concentrated dietary protein. Carbohydrate fractions of these legumes have also played a leading role as a source of food and energy. The legumes are also rich sources of galactosyl-sucrose oligosaccharides which are present mainly in the endosperm of the seeds.

Galactosyl-sucrose oligosaccharides, also known as α-galactosides of sucrose are members of the raffinose family of oligosaccharides. Raffinose, stachyose, verbascose and ajugose comprise the full series of the family. The structural relationship within these series is illustrated in Fig. 1. The α-galactose units are bound to the glucose moiety of sucrose through α (1-4) linkage. Galactosyl groups linked to other sugars and nonsugars are also found in nature, but whether these constituents can cause flatulence is unknown.

It is now well established that the raffinose series oligosaccharides present in legume seeds cause flatulence (Murphy 1969, Calloway and Borough, 1969, Rackis 1975). This property is associated more with some legume species than others. Most investigators (Rackis 1975) ascribe flatulence to the action of anaerobic microflora on the oligosaccharides. In man and monogastric animals (pigs and poultry), the intestinal mucosa lacks the enzyme α-galactosidase which is required to cleave the α-linked galactose units present in these oligosaccharides. These sugars are not absorbed into the bloodstream and consequently escape undigested to the lower intestinal tract, where they are degraded by the action of the bacterial α-galactosidase. The cleavage product is then rapidly converted to carbon dioxide, hydrogen and methane, resulting in flatulence, diarrhoea, nausea, cramps and discomfort in the animals.

Accumulation of Oligosaccharides in Seeds

Galactosyl-sucrose oligosaccharides are formed by the successive addition of the galactosyl moiety to a sucrose primer (Fig. 1) in the maturing seeds (Korytnyx & Metzler 1962). These are known to accumulate in many leguminous species during seed development. Korytnyx & Metzler (1962) hypothesized a stepwise formation of stachyose and raffinose from sucrose in developing Phaseolus lunatus seeds. Gould and Greenshield (1964) reported changes in the galactose containing oligosaccharides, di- and monosaccharides in the ripening seeds of Phaseolus vulgaris. These and other studies on Vicia Faba and other legume seeds (Bourne et al. 1965) suggested that raffinose series oligosaccharides do not appear in the tissue until the onset of ripening. Similar studies have also been conducted on soybeans using labelled $^{14}CO_2$, suggesting their incorporation of label into maltose took place during initial seed development and appeared in raffinose and stachyose during the later stages of seed maturation (Long, 1971). A similar sequence was also followed in soybeans by Amuti & Pollard (1977). Singh & Jamburatham (1982) observed a steady accumulation of raffinose and stachyose in developing chickpea (Cicer arietinum). Raffinose and stachyose first appeared in the tissues 21 days after flowering and reached its maximum at maturity. The sequence of formation of monosaccharides and raffinose series oligosaccharides in developing lupin seeds was reported by Saini and Lymbery (1983). Sucrose and glucose were the only sugars detected immediately after anthesis and deposition of the higher members of the series did not commence until the onset of the "drying" phase of seed ripening (Fig. 2). Raffinose and stachyose first appeared in the tissues towards the end of the yellowing phase. Verbascose was the last oligomer to be deposited in the seeds. In ripening beans, stachyose and raffinose did not appear until seeds had reached 50% of maximum fresh weight (Kandler & Hopf, 1980), probably after the end of the cell division. They then accumulated steadily. In a number of mature leguminous seeds the stachyose plus verbascose contents is higher than that of raffinose (Schweizer et al. 1978, Saini, 1988).

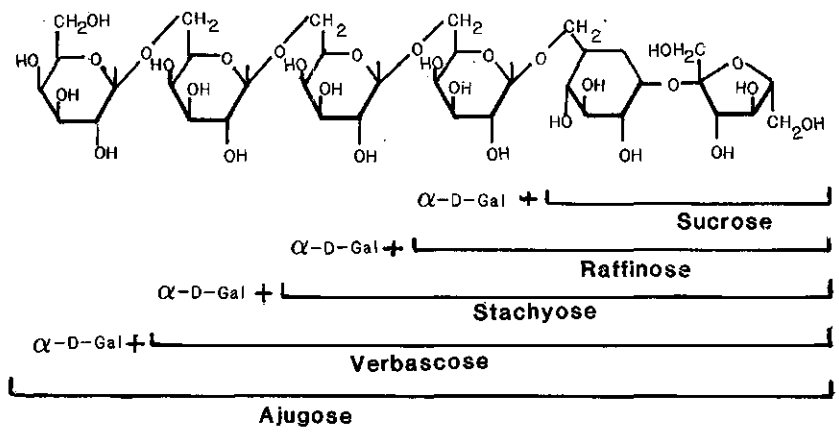

Fig. 1. Structural relationships of the raffinose family oligosaccharides

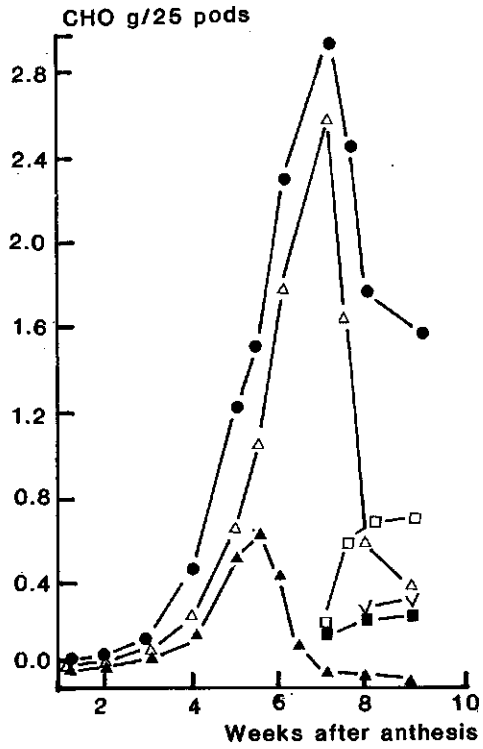

Fig. 2. Patterns of changes in mono-, di- and oligosaccharides of developing lupin seeds. (●) total; (△) glucose; (▲) sucrose; (□) raffinose; (■) stachyose; (v) verbascose.

Extraction and Estimation

The extraction and determination of α-galactoside contents of leguminous seeds has been the subject of many investigations (Hymowitz et al. 1972, Black & Bagley 1978, Macrae & Zand-Maghaddam 1978, Ford 1979, Kennedy et al. 1985, Saini & Gladstones 1986, Allen et al. 1986, and Knudson 1986). A variety of procedures have been employed for the extraction of α-galactoside of sucrose from leguminous seeds. Boiling the seed meals in aqueous ethanol (Hymowitz et al. 1972, Saini & Gladstones, 1986), extraction of the previously defatted seed meal under reflux in aqueous solvents (Macrae & Zand-Maghaddam 1978), heating in aqueous solvent at low temperatures (Allen et al. 1986, Knudsen 1986), or straight water extraction (Kennedy et al. 1985) are some of the most commonly employed procedures.

Following extraction, such oligosaccharides have been analysed by gas-liquid chromatography of silylated derivatives using a temperature programme (Ford 1979, Hymowitz et al. 1972). The application of HPLC using acetonitrile-water as elution solvent for the determination of soybean oligosaccharides was achieved by Black & Bagley (1972) and, more recently, by Knudsen (1986). An HPLC procedure for oligosaccharides of lupin seeds has also been described (Macrae & Zand-Maghaddam, 1978).

Kennedy et al. employed HPLC with distilled water as the elution solvent for the separation of oligosaccharides from soybeans.

Paper chromatography (Lineback & Ke, 1975) and thin-layer chromatography (Tanaka et al. 1975) have been extensively used. Although these are useful qualitative methods, the results are often difficult to quantify. The gel-permeation chromatography has also been employed for the quantitation of oligosaccharides in the extracts of legume seeds (Saini & Gladstones 1986, Saini 1988).

As a result of these differences in the extraction and estimation of legume seed oligosaccharides, some anomalies are occasionally noted in the oligosaccharide levels within the same variety and species of a given legume (Saini & Gladstones 1986, De Almeida et al. 1986, Allen et al. 1986). These anomalies occur for total oligosaccharide contents as well as for individual components. Saini (1988) compared four methods of oligosaccharide extraction employed by various investigators and concluded that extraction procedure can contribute to the incomplete isolation of oligosaccharides.

Levels in Mature Seeds

The oligosaccharides are important constituents of a wide variety of grain legumes and vary in their distribution among leguminous species (Hymowitz et al. 1972, Ortega & Consuelo 1976, Naivikul & D'Appolona 1978, Rao & Belavady 1978). Recent investigations also indicate that the quantities of raffinose series oligosaccharides can vary among varieties of a given legume species (Ortega & Consuelo 1976, Macrae & Zand-Moghaddam 1978). The content of the raffinose family of oligosaccharides in various types of food legumes is given in Table 1.

Lupins contain the highest levels of raffinose and stachyose, whereas most other legumes have the highest levels of verbascose. Soybeans are devoid of verbascose when compared with other legumes. The oligosaccharide content of lupin species and varieties was determined by Saini & Gladstones (1986) in order to establish whether these carbohydrates could be eliminated genetically (Table 2). The distribution of the sugars among the species was variable, but in all cases more oligosaccharides were present than disaccharides. Oligosaccharide contents of the various accessions ranged, on a dry basis, from 6.8 to 18.7%. Stachyose was the predominant sugar in most of the species examined. Verbascose ranged from 0.0 to 33.4% of total oligosaccharides. Similar variations in the oligosaccharide content of varieties and strains of soybeans has previously been reported by Hymowitz et al. (1972) and more recently by Kennedy et al. 1985 (Table 3). Rao & Belavady (1978) observed differences in soluble sugar contents in four common pulses grown in India. The oligosaccharide composition in the seeds of six lupin species produced in south of Brazil has recently been reported by Trugo et al. (1988) and the results corroborated the findings of Saini & Gladstones (1986). Schweizer et al. (1978) examined a number of leguminous seeds representative of different countries and reported high but variable levels in oligosaccharides. Stachyose was the most prominent sugar in all the legumes examined. High but variable levels in these oligosaccharides have recently been reported by Saini (1988) in a range of commonly grown leguminous seeds.

Table 1. Oligosaccharides in leguminous seeds[a].

Legume/species	Oligosaccharides (% of dry matter) Raffinose	Stachyose	Verbascose
Chick peas	1.0	2.5	4.0
Mung beans	0.8	2.5	3.8
Cow peas	0.4	4.8	0.5
Haricot beans	0.5	2.1	4.0
Peas dry	0.6	1.9	2.2
Lentils	0.9	2.7	1.4
Soybeans	1.9	5.2	0.0
Lupins:			
L. albus[b]	1.9	11.0	1.8
L. luteus[c]	3.3	11.8	4.0
L. angustifolius[d]	4.4	9.2	1.3

[a] Sources: Rackis (1975); Matheson & Saini (1977).
[b] Ultra. [c] cv. Weiko III. [d] cv. Unicrop.

Oligosaccharides in the seed coats and embryo separately, of the cultivated species of lupins has also been recorded (Saini & Gladstones, 1986). These indicated lower levels in the seed coats than in the embryos, but the spectrum of sugars was broadly the same (Table 4). There was a complete lack of verbascose in the seed coats of L. angustifolius cultivars, and therefore seed coat removal seems unlikely to improve carbohydrate composition from the viewpoint of flatulence risk, although it will undoubtedly improve total digestibility in monogastric animals through reduced acid detergent fibre content.

In comparing the contents of total oligosaccharides as well as the contents of raffinose, stachyose and verbascose with the literature values, it must be remembered that different samples and methods are often involved. However, among all the investigated seeds, lupins and soybeans contain essentially the highest amounts of raffinose and stachyose and in case of soybeans, virtually no verbascose.

Elimination of flatulence factors

Various processing techniques, such as those used for the manufacture of isolates and concentrates are required to eliminate these factors. These techniques include hot water treatment, aqueous alcohol extraction, cooking, germination, fermentation and enzymatic treatments.

Enzymatic processes that hydrolyse oligosaccharides have been developed (Sherba 1972, Sugimoto & Van Buren 1970). An immobilized α-galactosidase continuous flow reactor has been used to reduce the raffinose in beet sugar molasses (Reynolds 1974). About 70% of the raffinose, plus stachyose, was removed from soybeans by a combination of various treatments that involved enzymic treatment (Kim et al. 1973). Cotyledons of lupins and soybeans have been shown to hydrolyse raffinose series oligosaccharides, apparently due to the presence of an enzyme, α-galactosidase in the seeds (East et al. 1972, Matheson & Saini 1976).

α-Galactosidase hydrolyses olgosaccharides to remove galactose residues from stachyose and raffinose with a corresponding release of sucrose and galactose. A number of leguminous seeds have been found to contain a constant but variable level of α-galactosidase activity (Saini 1988). In lupins, germination led to a rapid decline (Fig. 3) in raffinose series olgisaccharides with a corresponding increase in sucrose after 24 hours of germination (Matheson & Saini 1976). In germinating soybeans, stachyose and raffinose decreased rapidly during the first three days and disappeared after five days of germination.

Rao & Belavady (1978) observed a 50-75% decrease in stachyose and verbascose in four pulses after 24 hours of germination. Similar observations have been made by a number of other investigators (Reddy & Salunke 1980, Labaneiah & Luh 1981, Bianchi et al. 1983).

Table 2. Total and percent levels of oligosaccharides in lupins species & cultivars.

Species and cultivar/line[a]	Country of origin	Total oligo.	% of total di- and oligosaccharide components			
			Suc.	Raffin.	Stachy.	Verbas.
Lupinus albus						
Hamburg	Germany	10.3	29.4	17.1	53.5	tr
Ultra	Germany	11.3	31.4	8.5	50.6	8.0
L. angustifolius						
Marri	W. Aust.	10.6	27.2	17.6	51.6	3.6
Unicrop	W. Aust.	11.5	31.8	20.2	42.0	6.0
P22884	Morocco	8.5	19.1	12.2	53.2	15.3
P22765	Spain	11.9	21.4	9.4	51.6	17.6
L. atlanticus						
P22925	Morocco	10.0	30.3	13.2	56.6	tr.
P22930	Morocco	7.4	36.2	11.3	52.5	tr.
L. cosentinii						
P22916	Spain	11.8	18.3	18.2	53.4	10.1
P22915	Tunisia	11.4	19.6	14.8	55.4	10.2
L. hispanicus						
P23019	N. Spain	14.5	14.6	9.8	75.6	tr.
P23007	Spain	12.6	16.8	5.2	76.0	tr.
L. luteus						
P22915	Portugal	14.0	4.6	11.3	50.5	33.4
Weiko III	Germany	15.9	12.2	15.2	54.0	18.2
L. micranthus	Morocco	18.6	17.5	14.1	55.8	12.7
L. pilosus						
P22937	Israel	6.8	26.6	11.3	44.8	17.4
P20957	Israel	7.3	28.4	18.6	39.5	12.9

[a] Commonwealth plant introduction number assigned by the CSIRO Plant Introduction Section, Canberra.

Table 3. Oligosaccharide content of soybeans.

Cultivar	Percent meal dry weight			
	Total	Sucrose	Stachyose	Raffinose
Williams.	10.72	5.64	4.14	0.94
Forrest.	10.75	5.95	3.93	0.87
Big Jule.	12.42	7.67	3.86	0.89
Beeson.	7.63	4.00	2.96	0.67

Kennedy et al. 1985.

Table 4. Distibution of soluble sugars in seed coats and embryos of lupin cultivars[a].

Species and cultivar	Seed coat (% of seed)	Total soluble sugars		Component mono, di and oligosaccharides of seed coats[b]				
		Seed coat	Embryo	Gluc.	Suc.	Raff.	Stach.	Verb.
L. albus								
Hamburg	22.3	15.7	14.3	tr	23.6	14.2	52.5	7.1
Ultra	19.0	10.3	18.0	tr	21.9	13.2	53.5	10.9
L. Bean	18.0	10.9	19.3	tr	23.2	16.7	59.5	0.0
L. angustifolius								
Marri	25.0	9.6	16.3	-	30.0	20.0	50.0	0.0
Unicrop	23.0	9.5	19.1	tr	31.3	20.6	46.9	0.0
Uniharvest	26.6	8.8	20.0	tr	31.4	21.4	46.2	0.0
L. luteus								
Weiko III	26.0	12.3	20.3	-	16.9	15.6	44.6	22.7

[a] % dry matter basis. [b] Determined after separation on Biogel P-2.

Effect of soaking, cooking and fermentation processes on the reduction of oligosaccharides content of a number of legumes has also been investigated (Rao & Belavady, 1978, Reddy & Salunke 1980, Bianchi et al. 1983). Reddy & Salunke (1980) reported a 25% reduction in the content of black gram oligosaccharides after 40 minutes cooking at 116°C (Table 5). Bianchi et al. (1983) reported a similar decrease in oligosaccharides of soybean in a Brazilian variety after 30 minutes cooking and almost 80% reduction occurred after 90 minutes using 1:10 ratio of soybeans to water. Contrary to these reports, Rao & Belavady (1978) reported an increase in the oligosaccharide levels of four pulses after cooking for 15 minutes. Adding the remaining water after cooking to the extraction medium perhaps contributed to this increase.

However, soybeans and mungbeans sprouts retained most of the flatulence activity of the intact seeds when tested in humans (Calloway et al.

1971). The germinated (24 hours) black gram seeds produced low flatulence in rats compared to cooked and fermented products (Reddy et al. 1980). This low flatus production may be attributed to a reduction (87%) in the oligosaccharides (verbascose and stachyose) on germination.

Table 5. Effect of cooking on total sugars and oligosaccharide content of black grain seeds.

Cooking time min.	Total sugars	Oligosaccharides, mg/g			Percent reduction
		Verbascose	Stachyose	Raffinose	
0	78.5	40.3	7.2	tr.	0.0
10	59.3	35.7	7.6	ND	8.8
20	56.6	34.8	7.5	ND	10.9
30	54.9	33.9	6.8	ND	14.3
40	52.6	29.6	5.8	ND	25.5

ND not detected
Reddy & Salunke (1980)

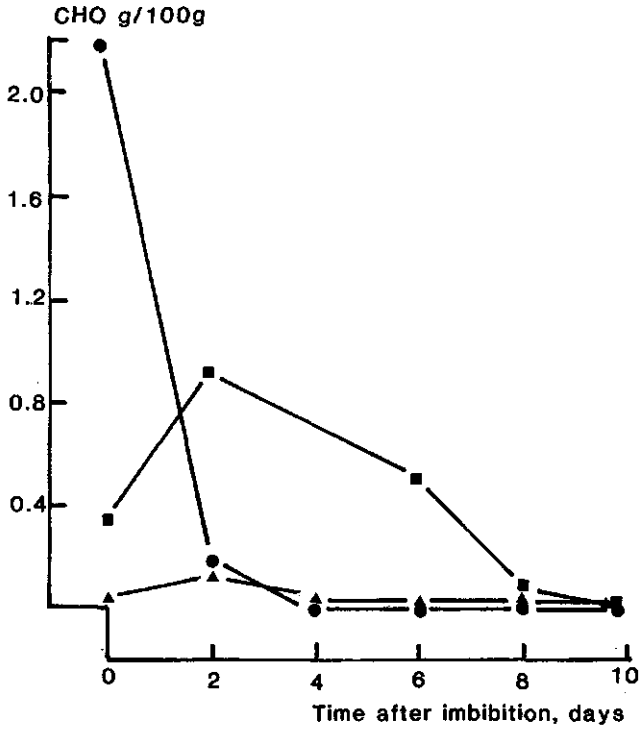

Fig. 3. Changes in the levels of mono-, di- and oligosaccharides in lupin cotyledons during germination. (●) oligosaccharides; (■) disaccharides; (▲) monosaccharides.

Although these processes have been demonstrated to be quite effective in the reduction of oligosaccharides, none of these processes has achieved a complete elimination of the oligosaccharides. Hymowitz (1972) reported a high degree of stability in the oligosaccharide content of soybeans and concluded that elimination of flatulence by breeding holds little promise unless new soybean strains are discovered which contain little or no oligosaccharides. However, Kennedy et al. (1985) showed that there was a genetic variation among lines of soybeans for total and component oligosaccharide contents and indicated a possibility of selection of particular lines combining different proportions of sucrose, raffinose and stachyose. Similar variability has also been recorded in lupins (Saini & Gladstones 1986, Trugo et al. 1988) and suggest that breeding programs may be used to breed legumes low in oligosaccharides.

Physiological effects of oligosaccharides

Production of flatulence is one of the primary physiological effects induced by increased ingestion of legume seeds (Rackis 1975). These legumes may also vary in their ability to induce flatulence. For example, oligosaccharides constitute approximately 50% of the flatulence activity of small white beans (Olson et al. 1975), and the same oligosaccharides contribute nearly all of the flatulence activity of the soybeans (Rackis 1975). Gitzelmann & Auricchio (1965) reported the absence of α-galactosidase activity in human intestinal mucosa. They demonstrated that a normal child and a galactosemic child were unable to digest raffinose and stachyose since there was no absorption of galactose in the blood. Ruttloff et al. (1967) also found no enzymic hydrolysis of raffinose in the intestinal mucosa of rats, pigs and humans. Other studies on the absorption and degradation of oligosaccharides containing α-galactosyl groups show that less than 1% of the administered dose was able to pass through the intestinal wall of man and animals (Taeufel et al. 1967). In the absence of α-galactosidase activity in the mucosa, these oligosaccharides remain intact and enter the lower intestines where they can be metabolised by existing microflora.

The increase in flatus is due primarily to increased amounts of two gases, carbon dioxide and hydrogen. Appreciable amounts of methane may also be produced. When flatulence is measured in human test subjects under controlled conditions, differences in degree of gas response are noted among members of the legume family (Table 6). Dry navy, kidney and pinto beans produce more flatulence than dry peas, peanuts, mungbeans or soybeans. In a series of experiments using dogs, Richard et al. (1968) demonstrated that anaerobic bacterial cultures isolated from the colon can metabolize raffinose and stachyose to produce large amounts of carbon dioxide and hydrogen, the major gases in flatus. In experiments with rats, Cristofaro et al. (1974) found that diets containing stachyose and verbascose exhibited the highest flatus activity. Carbon dioxide and hydrogen were the primary gases collected.

Evidently, the oligosaccharides breakdown to monosaccharides before the gas-producing mechanism can take place. Gas production has been related to the degree of enzymatic hydrolysis of stachyose and verbascose and the corresponding intermediate breakdown products, consisting mainly of di- and trisaccharides. As a result, the rate of gas production perhaps parallel the formation of monosaccharides.

Table 6. Flatus properties of different legumes*.

Species/variety	Ratio
Phaseolus vulgaris	
california small white	11.1
pinto	10.6
kidney	11.4
Phaseolus lunatus	
lima, Ventura	4.6
lima, Fordhook	1.3
Phaseolus mungo	
mungbeans	5.5
Glycine max	
soya, lee or yellow	3.8
Arachis hypogaea	
peanut	1.2
Pisum sativum	
pea, dry	5.3
pea, green	2.6
bland test meal	1.0

Ratio of flatus test meal of 100 g (dry wt.) for a three hour period measured from 4 to 7 hours after ingestion as compared to a bland test meal.

* From Murphy, E.L. (1964) and Calloway et al. 1971.

The mode of uptake of simple sugars including the raffinose series oligosaccharides from the small intestines of the fowl and swine is presumably similar to that established in mammals used in conventional laboratory experiments. The adverse effects of lactose on growth of chicks when fed at levels of 20% (w/w) or above (Rutter et al. 1953) might have been due either to incomplete hydrolysis of the disaccharide in the small intestine or to the toxicity of galactose, which can be tolerated by the hen only up to a level of 10%. Similar adverse effects will surely be induced by high levels of oligosaccharides (raffinose, stachyose and verbascose) which are known to produce galactose upon hydrolysis.

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ANTINUTRITIVE FACTORS IN PEAS

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Summary

Peas are an important constituent of both human and animal diets. The nutritive value and chemical composition of peas is similar to other grain legumes. The protein for example is markedly deficient in sulphur amino acids particularly methionine. The nutritive value of pea seed is limited by a range of toxic substances. However, most of them can be eliminated by cooking and processing. Experimental data strongly suggests that increased digestibility of pea protein on cooking is due to elimination of trypsin and chymotrypsin inhibitors.
Keywords: haemagglutinins, trypsin inhibitors, chymotrypsin inhibitors, amylase inhibitors, oxalates, phytic acid, tannins, phenolic acid, lipoxygenase.

Introduction

The proximate composition of peas (Pisum sativium L.) is similar to that of other grain legumes. The seeds contain high levels of protein and digestible carbohydrates and low concentrations of fat and fibre (Savage & Deo, 1988). Peas in common with many other legumes contain toxic factors which adversely affect the nutritive value of the seed. These toxic factors are a group of unrelated chemical compounds with varying effects on metabolic processes. A number of authors have published partial analyses of the toxic factors found in peas and these have been drawn together by Savage & Deo (1988), and are summarised in Table 1.

Haemagglutinins

Haemagglutinins are found in a wide range of legumes including peas. The arbitrary nature of the test used to assay this factor (Liener, 1955) can lead to considerable error and probably explains why the published data ranges so widely (Bender, 1983). Liener (1969) has extensively reviewed the metabolic effect of haemagglutinins. Manage et al., (1972), showed that haemagglutinins in peas are essentially non-toxic when incorporated in the diet at a level of 1%. Jindal et al., (1982) confirmed that haemagglutinins isolated from peas inhibited growth of rats. However, they observed a decrease in growth inhibition as the experiment proceeded indicating that animals can adapt to the adverse effects of moderate levels of haemagglutinins in the diet.

Tannous & Ullah (1969) showed that the haemagglutinin activity of peas (80 U/g) was completely eliminated by autoclaving at 121°C for 5 minutes. In green peas de Muelenaere (1965) showed that the haemagglutinin activity was considerably reduced by autoclaving at 121°C for 30 min (from 80 U/g raw to 4 U/g cooked), and a diet which

contained 65% raw peas was readily accepted by rats. Bender (1983) reported a 65% reduction in activity after soaking peas for 18 hours. Although haemagglutinins are present in peas, the seeds are essentially non-toxic (Grant et al., 1983). Net Protein Utilization (NPU) values of peas fed at 10% crude protein level in the diet ranged from 43 to 62%. This was comparable to a NPU of between 54 and 66% of a diet containing 5% pea protein and 5% casein protein.

Trypsin Inhibitors

Trypsin is a proteolytic enzyme secreted by the pancreas. Trypsin inhibitors are present in many legumes in varying amounts, they are generally measured by the original method developed by Kunitz (1947) but techniques based on Kakade et al., (1969) and Erlanger et al., (1961) are now more commonly used. When trypsin is inhibited, proteins are not digested adequately, and fewer amino acids are available for growth. In addition the pancreas may function abnormally when these inhibitors are present, and dietary amino acids may be directed to synthesis of additional pancreatic enzymes (Liener, 1976; 1979). Since pancreatic enzymes are rich in sulphur amino acids, hypertrophy of the pancreas may divert methionine and cystine from synthesis of body tissues to additional production of pancreatic enzymes. This division would further aggravate the sulphur amino deficiency in peas. Tannous & Ullah (1969) reported complete elimination of antitrypsin activity in peas by autoclaving seed at 121°C for 5 minutes. Griffiths (1984) showed that at temperatures at or below 80°C trypsin and chymotrypsin enzyme inhibitors were stable.

Table 1. Antinutritive factors found in pea seed

	Raw			Cooked
	Whole seed	Testa	Kernel	Whole Seed
Haemagglutinins U/g[1]	5100-15060	-	-	-
Trypsin inhibitor U/g[1]	150-10800	250-870	3000-12000	-
Chymotrypsin inhibitor U/g	740-10240	-	-	-
Amylase inhibitor U/g	14-80	-	-	-
Oxalate g/kg	6.67	-	-	-
Phytic acid g/kg	2.22-8.19	7.4	-	1.39-1.52
Tannins g/kg	0.2-13.0	-	-	-
Phenolic acid mg/kg	13-27	11.0	-	-

Based on data from: Jaffé et al., 1973; Bhatty, 1977; Bramsnaes & Olsen, 1979; Valdebouze et al., 1980; Davis, 1981; Elkowicz & Sosulski, 1982; Gad et al., 1982a; Gad et al., 1982b; Bender, 1983; Kumar & Kapoor, 1983; Sosulski & Dabrowski, 1984; Askbrant & Håkansson, 1984; Griffiths, 1984; Hlödversson, 1987; Manan et al., 1987.

[1] Data from different authors are difficult to correlate due to the enormous range of results in the literature.
- no values recorded in the literature.

However, at 100°C small but significant reductions in both inhibitors were observed. In contrast, autoclaving at 131°C for 10 minutes almost completely destroyed both inhibitors.

The trypsin inhibitor content of peas (Table 1) is one-tenth the level found in soya beans (Glycine max), and is similar to that of the field bean (Vicia fabia) (Hove & King, 1979; Valdebouze et al., 1980). The trypsin inhibitor content depends on the type of pea (Table 2). Wrinkled-seeded varieties have less trypsin inhibitor activity than smooth-seeded types and spring types (2,700-5,500 U/g DM) on average have less than winter types (5,700-11,700 U/g DM).

Valdebouze et al., (1980) stated that 90% of the trypsin inhibitor activity was found in the kernel and 10% in the testa, in direct proportion to the weight distribution of these factors in the whole seed. This does seem to conform with the range of values summarised in Table 1 which indicate that the testa has a lower trypsin inhibitor content than the kernel.

Table 2. Trypsin inhibitor (U/g DM) content of smooth and wrinkled pea cultivars (Valdebouze et al., 1980)

Cultivars	Smooth Seeds	Wrinkled Seeds
spring	4,200 - 5,500	2,700 - 3,700
winter	9,400 - 11,700	5,700 - 9,400

Deo (1987) showed that the trypsin inhibitor content ranged from 0 U/g for cultivar Rovar to 71 U/g for cultivar Whero (Table 3). In these experiments cooking completely destroyed the trypsin inhibition of all pea cultivars evaluated. The increase in True Digestibility in rats observed on cooking may well be due to the elimination of trypsin inhibitors which interfere with digestion. The Biological Value of each pea cultivar was, however, reduced in each case (Deo et al., 1986). Johns (1986; 1987) showed that the trypsin inhibitor content of similar New Zealand grown pea cultivars ranged from 88 to 716 U/g. From the results of Hove & King (1979), Deo (1987) and Johns (1986; 1987) it is clear that the trypsin inhibitor content of New Zealand grown peas is much lower than peas grown elsewhere (Tables 1 & 2).

Table 3. Trypsin inhibitor (U/g DM) content of New Zealand grown pea cultivars

Pania	Huka	Maro	Maple	Rovar	Whero	
88	145	716	216	-	-	(Johns, 1987)
26	49	-	-	0	71	(Deo, 1987)

- not determined

Johns (1987) also showed that there was a close, but non linear relationship between the trypsin inhibitor content of each pea variety and the pancreatic weight of broilers consuming diets containing 80% of each pea cultivar. Chickens offered diets containing cultivars Huka, Maro or Maple had considerably reduced weight gains as a result of reduced food intake when compared to cultivar Pania which contained the lowest levels of trypsin inhibitor. The addition of synthetic L-methionine to each of the pea containing diets markedly improved dietary intake in all cases. This would be expected as pea protein is deficient in methionine. It is also possible that methionine is directly involved in reducing the effects of the negative growth factors in peas. This effect has been shown in lentils by Savage & Scott (1988).

Chymotrypsin Inhibitors

Chymotrypsin is a proteolytic enzyme similar to trypsin. The mode of action of chymotrypsin inhibitor is therefore expected to be very similar to that of the trypsin inhibitors. Griffiths (1984) showed the chymotrypsin inhibitor content of nine pea cultivars ranged from 740 to 10240 U/g. Griffiths (1984) also showed that the chymotrypsin inhibitor content of pea cultivars was significantly higher than in field beans which contained 380 to 770 U/g. He showed that the effect of heat treatment on chymotrypsin inhibitor was similar to that on trypsin inhibitors. At temperatures at or below 80°C the inhibitor was stable and its inhibitory properties unaffected; at 100°C, a small but significant reduction in activity was observed. However, autoclaving for 10 minutes at 131°C almost completely destroyed all inhibitor activity.

Amylase Inhibitors

Inhibitors of pancreatic and salivary amylase are found in a wide range of legume seeds (Jaffé et al., 1973). The highest levels of amylase inhibitors were found in several varieties of kidney beans, while peas contain relatively low levels (Table 1); at least three samples of peas showed no detectable inhibitor activity. Amylase inhibitors are readily inactivated at 100°C (Jaffé et al., 1973) so they are unlikely to pose any problems in well cooked human food.

Oxalate Content

Oxalate can form salts with divalent metal cations such as magnesium and calcium and these salts are extremely insoluble, passing through the digestive tract unabsorbed. The oxalate content of peas is reported to be 6.67 g/kg (Gad et al., 1982a). They reported that both cooking and dehusking beans and lentils reduced the oxalate content. It is possible that this will also occur on processing peas.

Phytic Acid

Phytic acid is an important storage form of phosphorus in seeds and is also considered to be an antinutritive factor in peas. For instance, Barré (1956) demonstrated some *in vitro* inhibition of proteolytic activity by phytic acid.

Phytic acid is a powerful chelating agent for divalent cations and has the potential to interfere with mineral availability (Erdman & Forbes, 1977). Phytic acid can also affect digestibility by chelating with calcium or by binding with substrate or proteolytic enzymes. Kumar & Kapoor (1983) reported that rats receiving diets containing the lowest phytate : zinc molar ratio (13.7:1) had the highest Protein Efficiency Ratio (PER) 1.97 while rats with the highest phytate : zinc molar ratio (38.5:1) had the lowest PER (1.05).

The phytic acid content of peas ranges from 2.2 to 8.2 g/kg (Elkowicz & Sosulski, 1982; Kumar & Kapoor, 1983; Manan et al., 1987). Cooking peas gave a considerable reduction (82%) in the phytic acid content of Pakistani varieties without any loss of total phosphorus (Manan et al., 1987). Manan et al. (1987) showed that the nutritive value of the cooked peas was considerably improved compared to raw peas. They suggested however, that other water-soluble and/or heat-labile antinutritive factors might be more important than phytic acid in affecting the overall nutritive quality of seed measured by nitrogen balance techniques. Rosenbaum et al. (1966) showed that dried pea seed containing higher levels of kernel phytic acid took less time to cook. However, when calcium, which would bind with phytic acid in the pea, was added to the cooking water, there was no appreciable effect on the time taken to cook the seeds.

Tannins and Phenolic Acid

Tannins comprise a diverse group of polyphenolic compounds. The tannin contents of pea seeds range from 0.2 to 13.0 g/kg while the phenolic acid content in peas is reported to range from 13-27 mg/kg (Table 1). The phenolic groups of tannins bind to enzymes and other proteins by hydrogen binding to amide groups to form insoluble complexes. Polyphenols and tannins react with the α-amino group of lysine and polymerise into tannin-protein complexes, which will make large blocks of amino acids resistant to the digestive enzymes of monogastric animals (Sosulski, 1979). Since most of the tannins and the phenolic acid are contained in the testa, dehusking would reduce the tannin content of the seeds.

Generally, phenolic compounds are not toxic but if they are absorbed by mammals the detoxification of these polyphenolic compounds involves methylation which would put further stress on the limited methionine content of peas.

Lindgren (1975) using laying hens clearly showed that peas containing higher levels of tannins had a significantly lower metabolizable energy content and lower crude protein digestibility than low tannin containing peas. The regression coefficient linking tannin content and digestible crude protein in hens would suggest that a reduction in tannin content of 1% in peas would result in an increase of crude protein digestibility of 5% (Lindgren, 1975).

Hlödversson (1987) also showed that when peas were fed at 35% of the total diet to growing and finishing pigs lower digestibility coefficients were observed for the cultivar of peas which contained 13 g/kg tannins compared to two other cultivars containing 1 g/kg. The high tannin containing pea also contained high levels of total polyphenols.

Lipoxygenase Activity

Although the levels of lipids in peas are low, if they are not processed correctly lipoxygenase activity may become important. Deterioration in pea quality may be a result of microbiological, enzymatic and non-enzymatic changes. The last two are of particular importance in the preservation of fresh and processed peas. In both cases deterioration occurs mainly via lipid oxidation resulting in off-flavour development. Linoleic and linolenic acids are extremely susceptible to oxidation which is responsible for the development of rancid off-flavours. Eriksson (1967) showed that for peas 5 to 8% of the total lipoxidase content is located in the testa, 80% in the outer and 12% in the inner tissue of the kernel.

Conclusions

Peas contain a surprising range of antinutritive factors. Most of these cannot be regarded as toxic as such but it is clear that they do limit the utilization of the nutrients in peas. The lower utilization of nutrients in peas is possibly not important in human nutrition where they are consumed as part of a mixed diet. Antinutritive factors, however, do limit the total amount of peas that can be included in production diets for domestic animals.

One method for the improvement in the overall quality of peas involves selection of improved cultivars. In many cases selection criteria do not include a consideration of nutritional quality of peas. For example, newer pea cultivars developed in New Zealand do have lower levels of trypsin inhibitors but this was only discovered after their release.

Most of the antinutritive factors in peas can be eliminated by processing and cooking so they do not constitute a problem for human use. While it is clear that the overall quality of peas would be improved by heat processing, at present there are no conclusive studies with animals to show that this is an economic option.

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ANTINUTRITIONAL FACTORS IN PEA AND FABA BEANS
Required information levels, biochemical studies and analytical methods.

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Chemical and biochemical studies of several pea (**Pisum sativum** L.) and faba bean (**Vicia faba** L.) cultivars have been performed in connection with comprehensive investigations of their nutritive value and quality. The results obtained have been compared with corresponding results from investigations of soyabeans (**Glycine max** (L.) Merr.) and soyabean products.

Evaluation of the quality have been based on different levels of information obtained by various biochemical studies and results from analytical methods. The value of these informations in relation to quality improvements is obvious and has been proved in several cases. It gives the possibilities for explanation of the relations: STRUCTURE-PROPERTIES-FUNCTION. These relations are again the basis for development of the required methods.

Antinutritional compounds have well defined, but not always known structures. Their influence on the quality, acceptable concentrations, structure determination, properties, metabolism and appropriate analytical methods for these compounds are required. With the knowledge available, it is possible to obtain appreciable progress within this field, if the required support is obtainable. Illustration and discussion of these problems will be presented with background in the research performed on different legumes.

Keywords: Legumes, **Pisum sativum**, **Vicia faba**, **Glycine max**, antinutritional factors, quality subjects, information levels, proteins, proteinase inhibitors, tannin, phenolics, amino acids, pyridines, pyrimidines, purines, steroids, saponins, carbohydrates, glycosides, oligosaccharides, biochemical studies, analytical methods.

INTRODUCTION

Cultivars of pea (**Pisum sativum** L.), faba beans (**Vicia faba** L.) and soyabeans (**Glycine max** (L.) Merr.) are grain legumes of great importance for the supply of plant protein to feed and

food. The nutritive value of these plant protein sources depend on their quality. The quality of legume proteins is mainly determined by the generally limited content of methionine, threonine and in some cases tryptophan and abound content of lysine (Bjerg et al. 1988a; Bjerg et al. 1988b; Sørensen 1986; Eggum et al. 1986; Brandt et al. 1986). In addition, the grain legume quality embraces some proteins with antinutritional effects as well as several other subjects,- including various antinutritional factors.

Optimal utilization of legumes require that the various quality subjects are evaluated in relation to expected utilisation purpose for the produced legumes. The evaluations must be based on proper information levels which again depend on the applied analytical techniques.

Antinutritional factors are the reason to some of the quality problems. They are caused by too high concentrations in the plant products of some low molecular weight (LMW) compounds; non-protein amino acids, pyrimidines, pyridines, glycosides, oligosaccharides, phenolics ("tannin") (Eggum & Sørensen 1988; Bjerg et al. 1988a; Bjerg et al. 1984a; Bjerg et al. 1984b; Mortensen & Sørensen 1986), trypsin- and chymotrypsin inhibitors (Elnif et al. 1988) and saponins (Jensen et al. 1988; Sørensen 1988).

The aim of the present work has been directed at clarification of the above mentioned quality subjects and information levels required to solution of quality problems. Natural product chemistry and biochemistry have been used extensively in combination with animal trials including balance trials with young rats.

MATERIAL AND METHODS

The examined plant materials have comprised; **Pisum sativum** cv. Belinda(1), Bodil(2), Brandon(3), Countess(4), Danto(5), Fjord(6), Helka (7), Kelwo (8), Madria (9), Maro (10), Progreta (11), Solara (12), Stehgolt (13), WSB 1184 (14); various cultivars and lines of **Vicia faba** (Bjerg et al. 1985; 1986a); soyabeans (**Glycine max**)/ soyabean products (Sørensen 1987). The pea cultivars have been grown at five different places in Denmark 1986, 1987 and 1988.

Details on the applied analytical methods have been described elsewhere for: Trypsin, chymotrypsin and their inhibitors (Elnif et al. 1988); proteins, high molecular weight (HMW) and

LMW carbohydrates, tannin/phenolics, pyridines, pyrimidines and animal trials (Bjerg et al. 1988a, 1985, 1984a, 1984b; Sørensen 1987); amino acids (Eggum et al. 1988); saponins (Jensen et al. 1988).

RESULTS AND DISCUSSION

Comprehensive investigations on quality and antinutritional factors in faba beans have been performed in our laboratories (Bjerg et al. 1984a, 1984b, 1988a). Corresponding studies have also been performed on soyabeans/soyabean products (Mortensen & Sørensen 1986; Eggum et al. 1986; Brandt et al.1986), other legumes (Sørensen 1987) and recently studies of pea varieties have been initiated (Bjerg et al. 1988b; Sørensen 1988; Jensen et al. 1988). The content of both LMW and HMW compounds in these grain legumes showed appreciable variations - not only between the genera, soyabeans, faba beans, peas - but also within the cultivars of each of these three genera, and the nutritive value (quality) showed corresponding great variations.

Quality improvements of grain legumes used in feed and food require according to our observations knowledge to what quality embraces as well as knowledge to the required analytical methods. Optimal utilization of available resources and efforts used in relation to specific quality problems require use of analytical methods giving just the sufficient information level.

Quality embraces from a nutritional point of view several subjects which can be divided into few groups (Table 1).

(I) NUTRITIVE VALUE	(Ia) required nutritious matter
	(Ib) antinutritional compounds
(II) TOXIC COMPOUNDS	
(III) QUALITY OF PRODUCED PRODUCTS	

Table 1. Quality groups important for feed and food quality.

The levels of information obtainable from analytical me-

thods can be defined, discussed and denoted the **4xC** (Table 2). This is in analogy with corresponding terms used in chemistry and biochemistry.

(1.) **COMPOSITION**;	gross composition of the material (feed/food)
(2.) **CONSTITUTION**;	structure of the compounds in the material
(3.) **CONFIGURATION**;	stereochemistry of the compounds in the material
(4.) **CONFORMATION**;	"actual" spatial structure of the compounds

Table 2. Information levels used in quality evaluations of feed and food

The composition $[\ C_aH_bN_cS_dO_e\ ]_n$ can be obtained from determinations of the elements, and such analytical methods are generally used for quality parameter I_a. This information level is most often used in connection with evaluation of feed and food as shown in table 3.

		information level
Proteins;	crude protein from NX6.25	1
	amino acid composition	1→2
Lipids;	Stoldt fat	1
	fatty acid composition	1→2
Carbohydrates;	readily hydrolysable carbohydrates	1
	crude fibres	1
	soluble dietary fibre (SDF)	1->2
	insoluble dietary fibre (SDF)	1->2
	starch, amylose, amylopectin	1->2
Ash;		1
	individual minerals	2
Vitamins;	(table values)	2

Table 3. Information levels used for preparation of feed and food corresponding to quality group I_a.

Studies of the quality subjects I_b, II and III (Table 1) require as a minimum information level (2) (Table 2). We need

thus analytical methods which separate or determine the individual compounds according to their structure, configuration or conformation, e.g. HPLC, FPLC, GLC, enzymatic methods or corresponding efficient methods.

	Protein (Nx6.25)	Stoldt fat	RHC*	Starch	Crude fibre	Tannin	Ash
Pea	23-31	2-3	60-65	20-50	6	0.5-1.5	3
Faba beans	26-34	2-4	55-60	40-50	8	1-3	4
Soyabeans	40	20	13	-	6	3	5
Soyabean meal	50	2	8	-	7	2	6

*Readily hydrolysable carbohydrates

Table 4. Chemical composition of investigated pea and faba bean varieties compared to selected values for soyabeans/soyabean meal.

The result presented in table 4 illustrate the variations in the amount of quantitative dominating HMW pea and faba bean constituents. Informations on fat and protein quality can be obtained from determination of fatty acid composition and amino acid analysis (level 1->2 informations; tables 2 and 3). Additional information on fat quality e.g. on rancidity, phosphatides (lecithins), steroids and other fat constituents could be of value in some connections. The methionine and threonine content of grain legume proteins are a dominating quality factor, and often is a relatively low content of protein followed by a higher content of these essential amino acids (Bjerg et al. 1988a, 1988b).

Evaluations of the quality of HMW-carbohydrates require more than level 1 informations (RHC and crude fibre; tables 3 and 4). Determinations of starch, amylose, amylopectin, SDF and IDF (Table 3) can give valuable level 1->2 informations. Starch is a dominating fraction of faba beans and pea but only with limited effect on the nutritive value whereas IDF is more important in relation to quality of these grain legumes (Bjerg et al. 1988a; 1988b).

Among the LMW compounds, sucrose and the α-(1->6)galactopyranosylderivatives thereof, raffinose, stachyose and verbas-

cose, are quantitatively important grain legume constituents. Stachyose and sucrose are quantitatively dominating in soyabean meal, where only limited amounts of raffinose and verbascose occur, and the total amounts are in the range 6-12 % of DM. Verbascose is quantitatively dominating in faba beans followed by sucrose and stachyose and only a limited amount of raffinose and a total amount in the range 4-6 % of DM. Sucrose, verbascose and stachyose are quantitatively dominating in peas followed by minor amounts of raffinose and the total amount in the range 3-7 % of DM. These oligosaccharides can be of interest in relation to the grain legume quality e.g. owing to a possible relation to flatulence (Bjerg et al. 1988a, 1988b).

Saponins have also attracted special interest in relation to the pea quality (III, table 1) and analytical methods are now available for their isolation and HPLC determination (Jensen et al. 1988).

Tannins are quite often discussed as important for the grain legume quality (I_b and III, table 1) owing to possibilities for affecting the palatability due to astringent taste decreases feed consumption, and their ability to inhibit protein digestibility. This latter point of effect could be more or less correlated with trypsin and chymotrypsin inhibitor activity (Elnif et al. 1988). It is, however, also revealed that improved analytical methods of tannin determinations are required (Bjerg et al. 1988a).

Proteinase, trypsin and chymotrypsin inhibitors are of special interest in relation to grain legume quality (I_b, table 1). Depending on the applied methods of determination, and especially the animal source for the enzymes (Elnif et al. 1988), the reported estimates vary very much. The problems are well known for soyabeans, and it is found, that faba beans in general have a higher level of inhibitors than peas. The pea varieties Maro and Progreta, however, have a high inhibitor level when tested toward different types of proteinases (Elnif et al. 1988). It is not yet fully revealed to what extend HMW compounds and different types of LMW compounds (phenolics/tannin) contribute to the inhibitory activity.

With the different types of quality subjects now consi-

dered (vide supra), appreciable variations have been found as a function of growth conditions, year and growth places, for the pea varieties investigated. For the amino acids and heteroaromatics and some other LMW compounds the start of germination (only few days) have a very pronounced effect.

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GUT DYSFUNCTION AND DIARRHOEA IN CALVES FED ANTIGENIC SOYABEAN PROTEIN

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Summary

Calves reared on soya protein sometimes suffer from diarrhoea. Studies of this problem were made in experimental calves fed heated soya flour (HSF), ethanol extracted soya concentrate or casein. Results showed that feeding HSF led to mucosal tissue damage and characteristic disorders in gut motility. These disturbances appeared to be related to an adverse immune reaction to antigenic globulins rather than hyperosmotic effects of soya molasses in the small gut.
Keywords: calves, soya, antigenicity, diarrhoea

Introduction

Diarrhoea is a serious problem amongst calves weaned onto milk substitutes containing large amounts of soyabean flour. Previous studies of experimental calves fed heated soya flour showed that the diarrhoea was linked with dysfunctions of both digesta movement and secretory processes in the small intestine (Sissons & Smith, 1976). These disturbances in gut processses did not occur when the soya sensitive calves were challenged with soya concentrate which had been treated with hot aqueous ethanol. The alcohol treatment reduced the antigenicity of the soya protein, which has been implicated in tissue damaging reactions of the mucosa (Kilshaw & Slade, 1982), and removed oligosaccharides and sucrose, which the calf cannot digest, at least in the small bowel. However, it is unclear if the gut dysfunction and diarrhoea were due either to mucosal inflammation following an adverse immune response to antigenic protein or because of an osmotic condition arising from the presence of undigested molasses in the gut lumen. Here we describe studies of 'allergic' and 'hyper-osmotic' mechanisms involved in the aetiology of nutritional diarrhoea induced by feeding large amounts of soyabean flour to young calves.

Methods

Eight preruminant calves were surgically prepared with a simple abomasal cannula and either ileal re-entrant cannulas (n=4) for collecting digesta or wire electrodes on the gut wall (n=4) for recording myoelectric activity associated with contractions of smooth muscle. The calves were maintained on cows' milk, but at intervals of 2-3 days they were given, by direct infusion into the abomasum, liquid test feeds (2.7kg) containing protein (36g/kg liquid feed) derived from either casein, heated soya flour (HSF) or 'antigen-free' soya concentrate (AFSC) (extracted with hot aqueous ethanol). Soya molasses or sucrose were added to some of the casein and AFSC feeds. Values of trypsin inhibitor (mg/g protein) of 4.9 and 4.4 and of antigenic activity (log2 titre) of 15 and <1 for

glycinin and 13 and <1 for B-conglycinin were obtained for HSF and AFSC respectively.

Following abomasal infusion of a liquid feed (2.7kg) (dosed with phenol red as a marker), total collections of effluent flowing from the outgoing ileal cannula were made during a 21h period after the appearance of phenol red in the digesta. Electromyographic recordings from the wire electrodes were made using a multi-channel polygraph.

Results and discussion

The calves were sensitized to soya protein by infusing a succession of 4-5 HSF feeds into the abomasum. When they were later challenged with casein or AFSC feeds, measurements of digestive processes gave small gut transit times of 3-4h, ileal flow rates of 30-70g/h, net absorption of nitrogen of 0.74-0.81, regular initiations of migratory myoelectric complexes (MMC's) at intervals of 50-55min and no diarrhoea (see Table 1). These observations were similar to those recorded in earlier studies of milk fed calves (Sissons & Smith, 1976).

Table 1. Effect of giving cows' milk or a series of feeds prepared with casein, heated soyabean flour (HSF) or 'antigen-free' soya concentrate (AFSC) with or without the addition of 30g soya molasses or sucrose on digesta transport and nitrogen absorption in calves (n=4).

Order of giving feeds:	Small gut transit time (h)		Flow rate (g/h) of digesta		Net † nitrogen absorption	
	mean	SE	mean	SE	mean	SE
Cows' milk	3.6	0.3	31	6	0.81	0.03
Casein	3.7	0.4	45	9	0.81	0.03
HSF 1st feed	3.1	0.3	77	7	0.57	0.07
HSF challenge feed	2.0	0.3	141	17	0.34	0.13
AFSC	3.1	0.2	69	6	0.74	0.02
AFSC + 30g molasses	2.2	0.3	82	11	0.77	0.05
AFSC + 30g sucrose	2.3	0.2	61	15	0.83	0.04
HSF challenge feed	1.4	0.1	165	18	0.25	0.12
Casein	3.5	0.2	48	9	0.85	0.01
Casein + 200g sucrose	1.4	0.3	171	18	no data	

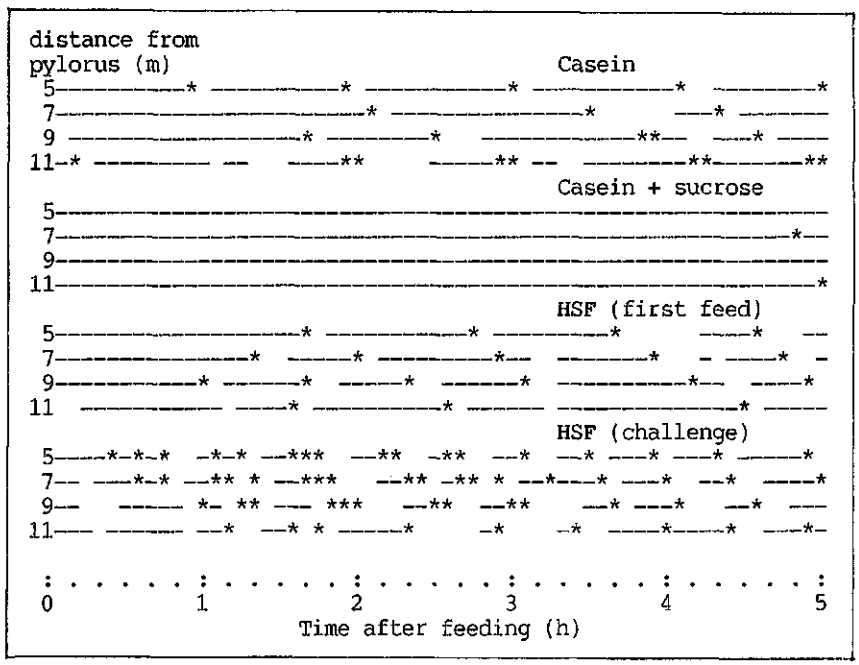
† proportion of nitrogen intake
Paired comparisons of values for HSF challenge or casein + 200g sucrose were significantly different ($P<0.05$) from results for casein or AFSC alone

Adding 30g of molasses or sucrose (equivalent to the amount of oligosaccharides in a HSF feed) to an AFSC feed increased the rate of passage of digesta, but did not affect the ileal flow rate or induce diarrhoea (see Table 1). But, addition of 200g of sucrose to a casein feed did result in diarrhoea following rapid intestinal transit and increased bulk of digesta. This 'hyper-osmotic' condition was linked with an inhibition in the initiation of MMC's which were replaced by

a prolonged phase of irregular spike activity (ISA) lasting several hours (see Fig 1).

Challenge feeds of HSF induced diarrhoea which was related to a markedly shorter time of small bowel transit and a substantial increase in the volume of ileal digesta compared with results for digesta transport after casein or AFSC feeds. Moreover, the intestinal motor pattern for these challenge HSF feeds was distinctly different from that observed for an HSF (first feed), casein alone or a casein feed with a sucrose load (see Fig 1). The disturbed pattern of myoelectric activity after HSF challenge was characterized by a 2-fold increase in the number of MMC's passing along the gut. Also in contrast to the 'hyper-osmotic' motor profile (induced by adding sucrose to casein), the HSF challenge reduced the ISA component and enhanced regular spike activity (RSA) i.e. the propulsive phase of a MMC. The numbers of RSA's recorded on the jejunum during a 6h period after giving calves (n=4) casein, HSF (first feed), HSF (challenge) or AFSC were (mean ± SE) 5.5 ± 0.2, 7.5 ± 0.2, 10.8 ± 0.7 and 7.5 ± 0.3.

Fig 1. Representation of typical recordings of myoelectric activity (— ISA, * RSA) made from the small intestine of a calf given feeds containing casein or casein with sucrose (200g) or HSF given on a first or fifth (challenge) occasion.



All of the calves showed high titres of systemic anti-soya antibodies after they had received 4 or more HSF feeds. Measurements of these antibodies showed that the calves possessed low levels of antibodies to soyabean constituents before receiving HSF feeds; for example, passive haemagglutination titres (log2) of 1 or 2 were

commonly observed. Subsequently, after giving four or more HSF feeds the titres rose to values of between 7 and 9.

Histological examination of mucosal tissue taken from calves within 2h after an HSF challenge revealed short villi, elongated crypts and increased numbers of mast cells compared with tissue from animals which received only cows' milk or from calves sensitized with HSF and then 2 days later given a feed of AFSC or casein (see Table 2).

Table 2. Effect of giving cows' milk or feeds containing casein, HSF or AFSC on height of villi, crypt depth and numbers of mast cells in mucosal tissue taken from the mid jejunal region of the small intestine of calves (n=3).

Order of giving feeds:	Height of villi (um)		Crypt depth (um)		Mast cells (no./mm^2)	
	Mean	SE	Mean	SE	Mean	SE
Cows' milk	993	177	393	37	67	24
HSF first feed	1040 †		366 †		no data	
HSF challenge feed	475	12	488	20	322	19
AFSC	839	65	363	33	120 †	
Casein	805	84	414	11	260 †	

† value for one animal
From variance analysis, values for HSF challenge were significantly different ($P<0.05$) from results for other treatments with 3 animals

It is concluded that diarrhoea in soya fed calves is caused primarily by an 'allergic' reaction to antigenic protein which enhances the propulsive activity of the gut wall and shortens the transit of digesta. The short time between giving a challenge feed of HSF and the onset of motor disorders (about 30 min) suggests that an 'immediate' type of hypersensitivity may be involved. Further studies are needed to elucidate whether the hypermotile state of gut smooth muscle is mediated by stimulants released from mast cells or through actions of systemic IgG anti-soya antibodies. A small osmotic effect of soya oligosaccharides may account for some water retention in the gut lumen and this could exacerbate the diarrhoea.

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DEGRADATION OF GLYCOPROTEIN II (PHASEOLIN), THE MAJOR STORAGE PROTEIN OF PHASEOLUS VULGARIS SEEDS

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Summary

Rats fed diets containing a highly purified preparation of Glycoprotein II (phaseolin, GII), the highly antigenic main storage glycoprotein of *Phaseolus vulgaris* seeds, rapidly lost weight. Total faecal N was elevated and, after correction for normal metabolic N loss, the true N digestibility was only 37%.

In contrast, the true extent of GII breadkown in the small intestine, excised one hour after the rats had been given a single dose of glycoprotein by gastric intubation, was estimated to be 57% by using immunological techniques. The overall digestibility of immunoreactive GII in the gastrointestinal tract, as established from faecal samples, was even higher and amounted to 74%.

The bulk of the N recovered from the small intestine and faeces was not related to GII. Thus, native GII and/or its fragments appeared to stimulate the secretion of endogenous N-containing materials, possibly mucins. Accordingly, based on conventional Kjeldahl N assays alone, *in vivo* N digestibility estimations in rats fed on diets containing legume seed storage proteins, such as GII, might give erroneously low values.
Keywords: Glycoprotein II (phaseolin), gut, digestibility, endogenous N, secretion.

Introduction

Storage proteins of legume seeds have been considered to be poorly digestible in their native form (Liener & Thompson, 1980). The partial resistance of native Glycoprotein II, (phaseolin, GII), the main storage protein of *Phaseolus vulgaris*, to degradation by pepsin, trypsin and chymotrypsin *in vitro*, has been demonstrated when the enzymes were used either alone or in sequential combination (Liener & Thompson, 1980; Deshpande & Nielsen, 1987). Molecular weight determinations and amino acid analyses indicated that, as a result of the limited proteolysis, the size of the native protein was reduced from 140 to 120 kDa (Deshpande & Nielsen, 1980). Under dissociating conditions, the subunits of the protease-treated GII had Mr values in the range of 22-30 kDa indicating that they had been produced by cleavage near the middle of the polypeptide chains of the original GII molecule.

Less information is available about the *in vivo* degradation of pure GII. Because this class of 7S seed reserve proteins is one of the major proteins found in all legume seeds, its digestibility and nutritional properties are of great importance. The purpose of the present study was therefore to investigate the true *in vivo* digestibility in the rat of a highly purified GII preparation obtained from kidney bean.

Materials and methods

GII Preparation

GII was isolated from seeds of *Phaseolus vulgaris* c.v. Processor by the procedure reported by Pusztai & Watt (1970). Residual lectin was removed by affinity chromatography on Sepharose 4B-fetuin (Pusztai & Palmer, 1977).

Rocket immunoelectrophoresis

The amounts of immunoreactive GII in the gut were estimated by rocket immunoelectrophoresis. The method was based on the observation that the reactivity of anti-native GII antibodies with the native glycoprotein or its core breakdown polypeptides obtained by digestion with endopeptidases, such as trypsin or chymotrypsin was quantitatively identical, while a more extensive further degradation by other proteolytic enzymes in the gut abolished this antigen-antibody reaction (Santoro et al., 1988).

In vivo studies

Nitrogen balance studies

These were carried out according to the procedure of Pusztai *et al.* (1981). Diets containing 10% protein (GII or lactalbumin) were formulated and supplemented with amino acids to target requirements for rats.

Short-term feeding experiments

Rats fasted for 16 h were given an intragastric dose of 300 mg of GII (45 mg N) in 2 ml of phosphate buffered saline, pH 7. Subsequently they were fed a protein-free diet *ad lib* for 3 days. Faeces were collected daily. N was estimated by the microkjeldhal method. G-II-derived materials in the faeces were estimated by rocket immunoelectrophoresis.

Acute experiments

Rats were given an intragastric dose of 150 mg of GII (22.5 mg N) and killed after 1 h. Stomach and small intestine were removed and the contents washed out with 50 mM phosphate buffer pH 7.4 containing 1000 KIU aprotinin/ml. The tissues were then homogenized in the same buffer. After centrifugation, Nitrogen and GII-related materials were estimated in the supernatants as before.

Results and discussion

GII preparation

Estimation of haemagglutinating activity using pronase-treated rat erythrocytes and SDS polyacrylamide gel electrophoresis indicated that the purified GII preparation contained less than 0.3% lectin (0.03% in the diet). When pure lectin was fed in diets at a concentration of 0.3% by weight, growth of rats was only slightly inhibited (Oliveira *et al.*, 1988). It is therefore unlikely that the performance of rats would be significantly influenced by the presence of 0.03% lectin in the diet.

Nitrogen balance

On diets containing GII, despite a food intake of 68 g per rat over ten days, rats lost weight over the trial period (Fig. 1a). The overall N balance (based on total Kjeldahl N) in these rats was poor in comparison with that of pair-fed lactalbumin controls (Table 1). After correction for normal

metabolic N loss from the faecal N content of rats fed on a protein-free diet (Table 1), the true N digestibility was only 37%.

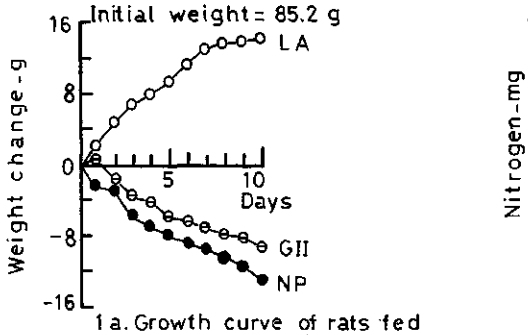
In contrast, by immunological methods it was found that, of the corrected total N (Kjeldahl) faecal output (648 mg) of rats fed on GII-containing diets only 240 mg was related to GII (Fig.1b). This indicated a true immunoreactive GII N digestibility of 74% and, accordingly, the bulk of the faecal N was probably of endogenous origin.

Short-term feeding experiments

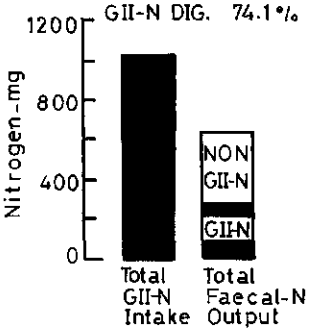
In short-term feeding experiments, in which a single dose of GII (45 mg of N) was given, the faecal N output over 3 days (corrected for normal metabolic output) was 50 mg (Fig. 2) of which only 12 mg was related to GII. This indicated that 74% of the GII had been degraded in the gastrointestinal tract. Moreover, the bulk of GII N appeared in the faeces during the first day whereas the maximum nitrogen output occurred on the second day.

Table 1. N balance (Kjeldahl N) in rats pair-fed on diets containing GII, or lactalbumin (LA) or a protein-free diet (NPC) for ten days.

	DIET		
	NPC	GII	LA
Food N (mg)	3 ± 0	1036 ± 43	1100 ± 10
Faecal N (mg)	147 ± 9	795 ± 7	167 ± 7
Urine N (mg)	42 ± 8	92 ± 6	120 ± 16
N balance (mg)	186 ± 1	149 ± 29	813 ± 22
True N digestibility %	-	37	98



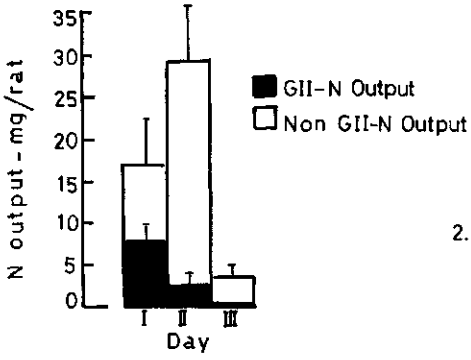
1a. Growth curve of rats fed Glycoprotein II (GII) or pai fed Lactalbumin (LA) or non protein (NP) diets for 10 days.



1b. GII N balance of rats for 10 days.

Apparently, the high faecal nitrogen output of rats given GII was primarily due to increased secretion of endogenous nitrogen rather than to poor digestion and absorption of dietary N.

In both feeding studies, however, proteolysis by bacteria in the large intestine may have contributed to the degradation of the protein without benefiting the animal. Therefore a series of acute experiments to estimate the digestibility of GII in the small intestine was carried out.



2. Faecal-N output from rats given a single dose of GII.

Acute experiments

The amount of N (23 mg) recovered from the small intestine after one hour was similar to that given by intubation (22.5 mg GII). However only 10 mg of this nitrogen was found to be related to GII. Thus 57% of the native GII was degraded and utilized within 1 h. The majority (6 mg) of the GII related protein recovered from the small intestine was strongly associated with the tissue, mainly the ileum, and could only be released by homogenisation. The reasons for this are not known. However adhesion of GII might aid the digestive process by extending the exposure time of the protein to the gut enzymes. Alternatively this effect might be linked with the finding that GII and/or its fragments could stimulate endogenous N secretion in the small intestine.

GII was not fully degraded in any of the three experiments described. This may be related to the microheterogeneity of this protein (Pusztai & Stewart, 1980).

In vitro studies, using rat small intestinal extracts (results not given) have indicated that small intestinal proteinases, other than trypsin and chymotrypsin, partially degrade GII and render it susceptible to further proteolysis by trypsin and chymotrypsin. This observation could possibly explain the large difference between the degradation found *in vivo* and the limited degradation (about 2%) obtained *in vitro* with pure endopeptidases (Romero & Ryan, 1978).

Conclusions

1. At least 57% of ingested Glycoprotein II (phaseolin) was degraded and absorbed from the small intestine within 1 hour. During the remaining time in the small intestine and the large intestine further degradation may occur.
2. The *in vivo* digestibility in the small intestine (57%) was far higher than that obtained with pure proteinases (2%). There were indications that, in the rat small intestine, proteinases unrelated to trypsin and chymotrypsin rendered GII partly susceptible to the action of these two enzymes.
3. Native GII or fragments derived from it greatly stimulated secretion of endogenous nitrogen, possibly mucins, in the small intestine. This constituted a major net loss of nitrogen to the animal and was the main reason for the weight loss found with rats given diets containing GII. Similar effects may occur with related proteins from other legume seeds.

Acknowledgements

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EFFECTS OF ZINC SUPPLEMENTATION ON VICIA FABA FED MICE

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Summary

Fortifying a faba bean diet with zinc slightly improves growth performance and immune response in mice, without achieving the control values. These processes would be due presumably by increasing the zinc bioavailability mediated by a lower phytate:Zn molar ratio, keeping in mind that other antinutritive factors (ANF) are involved.
Keywords: faba bean, growth, immune system, zinc, mice.

Introduction

Legumes are widely used as sources of protein in human and animal nutrition. However, the occurrence of some antinutritional factors (ANF) in these seeds can produce an impairment in growth, ussually accompained by other physiological alterations, when fed on the raw form (Martínez et al., 1985). Previous reports have shown that plant proteins contain considerable quantities of phytic acid, tannins, dietary fiber and other organic compounds, which may affect mineral utilization (Martínez et al., 1986). Also, recent studies have indicated that protein quality and zinc availability influence growth as well as cellular and humoral immunity (Bounous & Kongshavn, 1985; Verma et al., 1988). In that context, the antinutritive effects observed in animals fed on a bean diet are similar in some ways to those reported in subnutrition or zinc deficient conditions, which are associated with changes in growth and immunocompetence (Giuglia no & Millward, 1987; Martínez et al., 1987). Therefore, an experiment was conducted to evaluate if some of the undesirable effects caused by the intake of a faba bean diet are mediated by a reduction in zinc bioavailability affecting growth rate, organ weights and cellularity of the immune system (spleen and thymus) monitored through their nucleic acid content.

Material and methods

Male weanling mice, weighing about 14-16 g, were divided in three dietary groups of ten animals each. The mice were fed during 28 days on casein (Control), Vicia faba (Vf) or Vicia faba supplemented with zinc (Vf+Zn) as sources of protein, whose zinc content was 30, 30 and 60 ppm respectively. After killing the animal, thymus and spleen were carefully excised. Protein DNA and RNA were measured as described previously in a tissue homogenate (Millward et al., 1974). Phytate content of faba beans (1,2mg/100g Vf) was determined as published elsewhere (Martínez et al., 1985).

Results and discussion

Mice fed on the zinc supplemented bean diet slightly improved growth rate, which was statistically significant after 17 days ($p < 0.05$), however, the differences decreased with time (after 28 days). In both groups, these values were, in turn, significantly lower ($p < 0.001$) than those found in the control group (Fig. 1). These results are in good agreement with a previous report in rat (Martínez et al., 1985).

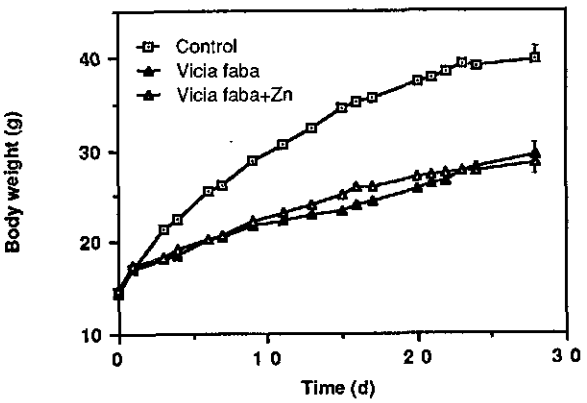

Fig. 1. Growth curves of animals ($\bar{X}\pm$SEM; n=10) fed on casein (Control), Vicia faba (Vf) or Vicia faba supplemented with zinc (Vf+Zn) as sources of protein.

Thymus and spleen weights were similar in all groups, although in a relative basis (%BW) a marked increase was found in both legume groups as compared to the control ($p < 0.01$). Cellular growth and protein biosynthesis are frecuently evaluated by nucleic acid and protein measurements (Giugliano & Millward, 1987). Thus, cell number, cell size and protein synthesis capacity are assessed from DNA, protein/DNA and RNA/protein values respectively (Martínez et al., 1985). Our results indicate that zinc supplementation prevents, in part, the hypertrophy and the reduction in cell number observed in the legume fed mice in the thymus, and more markedly in spleen (Fig. 2). The RNA/protein ratio in thymus and spleen were similarly reduced in both faba bean groups as compared to the control ($p < 0.01$).

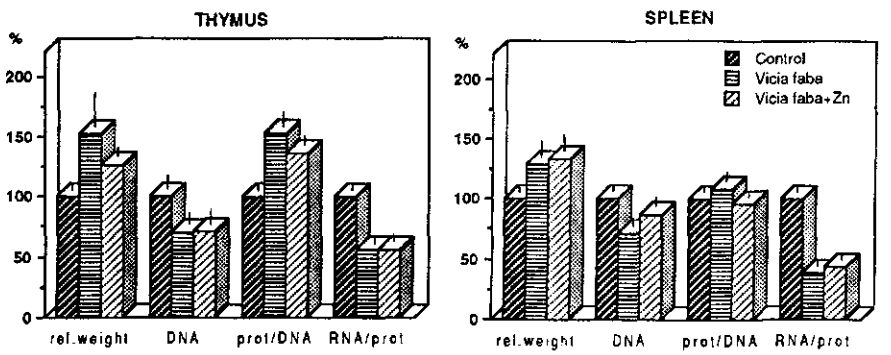

Fig. 2. Thymus and spleen weights with their protein and nucleic acid content from mice fed under the experimental dietary groups ($\bar{X}\pm$SEM;n=10).

Similar conclusions have been reached with a zinc deficient diet (Giugliano & Millward, 1987) which suggests that some of the antinutritive effects found in legume fed animals are partly due to the high phytate content of this bean, despite the fact that this diet contained adequate zinc levels (Mengheri et al., 1986).

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DEGRADATION OF PHYTATE BY WHEAT PHYTASE

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Abstract

Phytase in wheat can be used to degrade the phytate present in feed. Degradation of phytate by phytase was studied in vitro at 40 °C and pH 5.5. When milled wheat is incubated under these conditions most of the phytate is degraded within one hour. The degradation of phytate is interfered by high concentrations of calcium ions.

Introduction

Phytate is present in grains and seeds. It is a potential source of phosphorus which is liberated during the germination stage of the seeds. Breakdown of phytate into phosphate and inositol is caused by the action of phytase and related enzymes. High phytase activities have been reported in the literature for rye, triticale, wheat and barley. The availability of phytate-bound phosphate is very low in monogastric animals.

Moreover, phytate binds strongly to divalent metal ions resulting in a low biological availability of these ions (Wise, 1983). It seems attractive, therefore, to decrease the phytate level in certain feeds by means of phytase. In this connection the work of Pointillart et al. (1987) has to be mentioned.

Materials and methods

Phytic acid and purified wheat phytase were obtained from Sigma, St. Louis, USA.
Phytic acid was determined by ion chromatography (Slump et al.,1987).
Phytase activity was determined by incubating the milled samples in 0.1 M acetate buffer solution (pH 5.5) in the presence of added phytic acid (1 mM).

Results and discussion

The phytase activities observed in cultivars of several cereals have been summarized in Table 1. In some cultivars of rye a high activity was found.

Fretzdorff and Weipert (1986) found similar activities in rye. The availability of wheat in Europe is however much higher than that of rye. Therefore, the degradation of phytate by wheat phytase was studied in our laboratory.

Two phytate-degrading enzymes are present in wheat: a phytase having an optimum at pH 5.5 and a phytase with an optimum at a pH of about 7. The phytase activity at pH 7.0 was found to be about 80 % of the activity at pH 5.5.
When finely milled wheat with a phytase activity of 1000 units per kg is incubated at 20 °C and pH 5.5, the phytate is degraded almost completely into inositol and phosphate within 4 hours. At 40 °C this degradation is

complete within one hour. A chromatogram showing the lower inositol phosphates formed during the degradation of phytate by wheat phytase is shown in Figure 1.
When phytases are to be applied to animal feeds, the thermostability of these enzymes must be good, since most of the feeds are pelleted at about 80 °C to eliminate micro-organisms such as Salmonella.

Preliminary results indicate that the stability of wheat phytase is good up to 70 °C. Above 80 °C the wheat phytase is partly inactivated depending on the processing conditions.

High concentrations of calcium ions interfere with the degradation of phytate by wheat phytase at pH 5.5 (Table 2). Phytic acid is known to form strong complexes with calcium at this pH (Wise, 1983). It may be assumed that these strong complexes are the cause of the slow degradation of the phytate rather than a direct inhibition of the enzyme by calcium ions. The calcium in calcium hydrogen phosphate, in calcium lactate and in calcium citrate is strongly complexed. The calcium in these salts react only very slow with the phytate, and the phytate can thus be degraded by the phytase.
Animal feeds often have a high content of calcium salts, such as calcium carbonate or calcium hydrogen phosphate. These calcium salts may interfere with the degradation of phytate by wheat phytase at higher pH values.

Table 1. Phytase activities* in some cereals.

Cereal**		Observed activity (pH 5.5)	
		mean	(range)
wheat	(n=7)	1020	900–1200
barley	(n=6)	490	260– 700
triticale	(n=4)	1550	1350–1800
rye	(n=3)	2500	1400–3800

* The activities are given as liberated phosphate in μmol phosphate per min per kg.
** number of cultivars in parenthesis.

Table 2. Observed phytase activity in incubated finely milled wheat in the presence of calcium salts (40 °C ; pH 5.5)

Calcium content (% w/w)	$CaCl_2$	$CaCO_3$	$CaHPO_4$	Calcium lactate	Calcium citrate
1.0	40	20	240	120	500
0.5	90	40	250	340	540
0.2	430	110	360	550	470
0.1	570	410	440	560	520

* The phytase activity has been calculated from the degradation rate of phytic acid (μmol/min.kg)

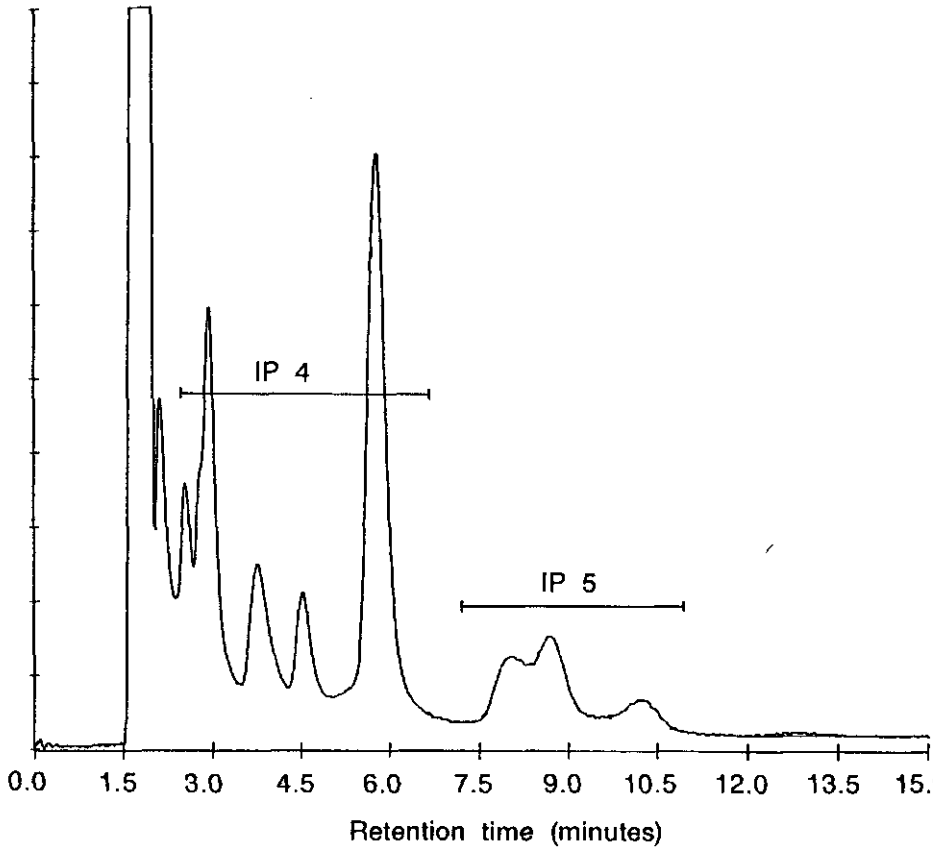

Figure 1. LC separation of a mixture of inositol tetraphosphates (IP4) and inositol pentaphosphates (IP5) derived from phytate by wheat phytase using an anion-exchange column (Dionex AS 3) with 0.09 M HNO_3 at a flow rate of 1.0 ml/min. Inositol hexaphosphate does not elute under these conditions.

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THE INFLUENCE OF ALPHA-GALACTOSIDES EXTRACTED FROM LUPIN SEED (LUPINUS ALBUS) ON THE DIGESTION OF DIETARY STARCH BY GROWING CHICKS

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Summary

An experiment has been conducted to investigate the effect of alpha-galactosides extracted from lupin seed meal on the degradability of starch in different intestinal sections of chicks. In the experimental trial the birds were fed diets containing lupin extracted meal without or with alpha-galactosides extract (0, 15 and 30 g/Kg).

No significant differences in body weight, relative lengths of duodenum, jejunum, ileum and caeca were observed among the three experimental diets. Starch degradability in the jejunum and ileum was not altered, in contrast with the caeca where starch degradability was significantly reduced in the birds fed 15 and 30 g/Kg of oligosaccharides extract.

Introduction

Most legume seeds, specially lupin, contain rather high level of alpha-galactosides (Mercier, 1979). Due to a lack of alpha-galactosidase enzyme in mammalian and other animals, these sugars may escape absorption from the small intestine (Cummings et al., 1986).

Legume starch has been shown to be less digestible than corn starch in rats (Thorne et al., 1983) and chicks (Guillaume, 1978). The nature of starch, protein-starch interaction, and antinutrients are some of the factores that may affect the intestinal degradation of starch (Jenkins et al., 1986; Thorne et al., 1983).

The purpose of this investigation was to determine the effect of alpha-galactosides extracted from lupin on the performance of birds and on the degradability of dietary starch in different sections of the small intestine and caecum.

Material and methods

Lupin seed meal (cv. Multolupa) was extracted by shaking 400 g of meal with 2 L of 80% (v/v) methanol for two hours followed by filtration through Wathman no I paper. The methanolic extract was concentrated under vacuum, then freeze-dried. The composition of lupin extracted meal and oligosaccharides dried extract appear in Table 1. Oligosaccharide contents were determined by high-presure liquid chromatography (Quemener and Mercier, 1980).

After a preparatory period of 14 days, during which time the chicks were fed a commercial starter diet, the birds were selected and randomly distributes into 3 groups of 8 chicks each and placed in metabolism cages. Birds were fed on experimental diets during a period of 2 weeks.

The composition of the experimental diets is given in Table 1. The dried alpha-galactoside extract was incorporated into the isocaloric and isonitrogenous experimental diets at the expense of glucose at the following concentrations (g/Kg): 0, 15 and 30. These concentrations of oligosaccharides in the experimental diets are approximately the same that those of diets containing 0, 200 and 400 g/Kg of lupin meal.

Table 1. Composition of experimental diets (g/kg)

Ingredients	A	B	C
Lupin extracted meal[1]	300.0	300.0	300.0
Isolated soybean protein	107.5	107.5	107.5
Glucose	248.0	233.0	218.0
Maize starch	300.0	300.0	300.0
Alpha-galactosides dried extract[2]	0	15.0	30.0
Constant ingredients[3]	44.5	44.5	44.5

1. Lupin extracted meal content (g/Kg): Crude protein, 411; Crude fibre, 112; Starch, 4; Soluble sugars, 4.6.
2. Alpha-galactosides dried extract (g/kg): raffinose, 288.7; stachyose, 417.6; verbascose, 39.4.
3. Constant ingredients were the following as g/kg: salt, 4.0; calcium carbonate, 13.0; dicalcium phosphate, 16.0; DL-methionine, 3.0; L-lysine HCL, 0.5; chromic oxide, 3.0; vitamins and minerals, 5.0.

When the chicks were 28 day of age, they were sacrificed by cervical dislocation and their intestines were removed immediately. The lengths of the small intestine sections and caeca were recorded. The contents of each segment were then removed into flasks and immediatelly freeze dried. Starch was analyzed by the enzymic method described by Karkalas (1985).

Results and discussion

The depressing growth rate produced in broilers by the inclusion in the diet of high concentrations of legume seeds, and the beneficial effects of dietary antibiotic led Guillaume (1977) to hypothesize an antinutritive effect caused by alpha-galactosides present in those seeds. It has been also suggested that the presence in the small intestine of rats of unabsorbable water-soluble sugars of low molecular weight, such as raffinose and stachyose, may result in an osmotic effect, leading to fluid retention and an increased rate of food passage that could adversely affect the absorption of nutrients (Wiggins, 1984). In contrast to these observations the results obtained in the present work showed that the addition of 15 and 30 g Kg^{-1} alpha-galactosides dried extract from lupin seed to the diet had no detrimental effect on the performance of birds (Table 2). Analysis of variance of the data indicated that there were not significant differences among chick groups in growth and food conversion efficiency. This agrees with the finding of Angel et al. (1988) who observed that the autolysis of alpha-galactosides of crude soybean meal did not measurably improve its nutritional value.

As can be seen in Table 2, there was a certain increasing trend of the relative length of the different intestinal sections with the concentration of oligosaccharides in the diet. This fact might be a consequence of the intestinal bacterial fermentation of these unavailable sugars (Cristoforo et al., 1974; Wagner et al., 1976).

Table 2. Effect of alpha-galactosides dried extract on live weight, food consumption, food to gain ratio and length of the intestinal sections of chicks fed from 14 to 28 d

	A	B	C	SEM
Final body weight (g)	698	709	672	17.14
Food consumption (g)	54.67	55.27	52.40	
Food to gain ratio	1.75	1.71	1.78	
Duodenum relative length (cm/100 g BW)	3.42	3.62	3.79	0.10
Jejunum relative length "	6.05	6.70	6.85	2.21
Ileum relative length "	5.68	6.16	6.41	0.03
Caeca relative length "	1.47[a]	1.56[b]	1.71[b]	0.04

[ab] Means in the same column with different superscript differ ($P < 0.05$)

Digestibility coefficients of starch, based on analyses of the contents of jejunum and ileum were not significantly affected by the inclusion of the alpha-galactoside dried extract in the diet (Table 3). However, the starch digestibility was reduced significantly ($P < 0.05$) in the caeca content of chicks fed on the diets containing the alpha-galactoside dried extract. This latter fact was probably due to the presence of these

Table 3. Effect of alpha-galactosides dried extract on starch digestibility (%) in several sections of intestinal tract of chicks fed from 14 to 28 d

	A	B	C	SEM
Jejunum	79.41	78.85	78.06	3.03
Ileum	92.18	94.91	94.89	0.79
Caeca	97.61[a]	92.94[b]	94.75[b]	0.47

[ab] Means in the same row with differen superscript differ ($P < 0.05$)

sugars which are more easily and rapidly fermented than the less soluble starch present in the caeca.

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DISCUSSION ON DIFFERENT NUTRITIONAL ASPECTS OF ANF

Chairman: L. Buraczewska
Reported by: M.W.A. Verstegen

Antinutritional factors (ANF) which are present in legume seeds and numerous other seeds limit the use of seeds as feedstuffs for different animals. These ANF can effect utilisation of nutrients and/or systemic processes at each step between ingestion and growth in various ways. These negative effects can be caused by a combination of many factors. One group of factors concerns the species and the physiological status of the animal.

This was discussed in the opening paper of this session by Huisman and Van der Poel. They found that piglets responded to raw phaseolus beans in the diet with a much greater negative effect on performance then rats. They compared also other characteristics like organ weights especially the pancreas and spleen were differently affected. From their experiments with phaseolus it was clear that nutritional value of phaseolus vulgaris beans for piglets was dramatic more depressed then for rats. Chickens reacted similarly as rats. They showed also that difference in physiological age can not be responsible for the differences in sensitivity between the animal species. Experiments with peas showed much less difference between the animal species. With piglets peas resulted in a somewhat reduced performance whereas in rats and chickens similar gain in control and pea diets were found. In the discussion it was concluded that the combination of various ANF in a seed will be responsable for the negative effects. Separate studies with specific ANF need to be made. This paper was followed by 3 papers and some posters in which it was discussed to what extent other seeds like peas, faba beans contain ANF and what their effect on animals are.

The presence of ANF in diets can decrease ingestion of feed as well as the digestibility. Also the transformation of digested energy to metabolizable energy can be affected. In addition utilisation of metabolizable energy for maintenance and/or production can be hampered as a result of ANF. The reason for this increased maintenance is not known as yet. Increased catabolism may be associated with this. Animals do not require specific feedstuffs but they need various nutrients that are present in feedstuffs like protein, amino acids, fat etc. Also energy is important. Each of these has a specific role in metabolism. Some of the storage proteins in legume seeds have been demonstrated to be poorly digested in their native form due to the presence of various factors (Santoro). The mode of action of ANF is different in various animals (Huisman and Van der Poel). Rats piglets and chickens may not react in the same way to ANF. For nutritional validation of feeds it is important to establish the effects in each target animal separately. Probably it is not sufficient to use laboratory animals like rats and mice for the evaluation of ANF in feedstuffs used for livestock feeding. However, it may be worthwhile to study the mode of action in a sensitive laboratory animal.

In this session some aspects of oligosacharides were discussed with regard to chicks (Brenes et al.) and other monogastrics (Saini). The ANF of the class oligosacharides may cause flatulence and can not be easily removed by heat treatment. Contents of these ANF in various legume seeds

can be very variable. Stage of growth can affect contents and contents of enzymes to break them down. A survey was given of various components in this group of ANF (Saini).

Levels at which ANF act or cause discomfort to the animals are not well defined. Therefore pointed threshold levels are a very important issue in each species and these need to be determined (Liener; general discussion).

Other investigations presented in this session have focussed on nutritional aspects of separate legumes like peas (Savage; Bjerg et al.; Grosjean and Gatel). ANF in peas were not considered to be very detrimental for pig diets. Their degree depends on what variety (winter or summer peas) are used (Grosjean and Gatel). Most ANF is found in the kernel (80%) and not in teste. Peas in diets for chickens sometimes resulted in reduced values for feed intake (Savage). Peas with tannins had reduced digestibility. By removing tannins protein digestibility was increased by as much as 5%. Peas can show a surprising large varietion of ANF. Selection of peas may also alter contents of trypsin inhibitors. Large variation in inhibitor activity between varieties of peas were found. Also oxylate was discussed as an ANF. Faba beans were discussed by various authors in this session. Mostly tannins seems to be responsible for negative effects and by dehulling they can be removed to a large extent. Tannins are a very diverse group of compounds (Bjerg; Macarulla; Jansman et al). Also the succesfull use of phytase for breaking down phytic acid was demonstrated (Bos). Phytic acid has a chelating activity.

The reasons for depression in performance of animals due to ANF are variable and are not very well defined. Part of it can be gut dysfunctioning due to trypsine inhibitors, lectine, tannins as shown by Sissons.

Effect of ANF on metabolism

ANF in legume seeds may reduce amount of nutrients absorbed into the animal systems. It is important to note that with ANF absorbed nutrients are sometimes less efficiently utilized for gain. Puzztai reported increased nitrogen output in the urine due to the ingestion of lectins. This may mean that nutrients from feed will not be used optimally and with lectins this may yield in a reducted synthesis or in an increased catabolism of body protein. The reduction in rate of gain in the body and in the various body components may be related to different types of ANF. There may be a decreased protein gain due to lectins. Also trypsin inhibitors and tannins may reduce protein gain (some times related to more endogenous N-secretion). It is not yet known to what extent metabolism in tissues with a very high turnover rate in protein, such as intestinal wall and liver is affected by ANF. It is clear (Huisman and Van der Poel and Pusztai) that some organs may become hypertrophic but other atrophic. It is not known if energetic efficiency of metabolizable energy in these organs is reduced by ANF allthough it was reported that ANF may increase catabolism (Pusztai, this symposium) thus suggesting a higher turnover. Specific studies in this subject have not been made sofar.

There is a special need to reduce the amount of nitrogen and other minerals in the animal waste. In view of this necessity to reduce the extra faecal and urinary output resulting from action of ANF it may be important to use heat treatment. Also in view of the beneficial effect of heating itself on protein digestibility heating may be important regardless of the ANF. In fact it is important to assess to what extent the heat treatment and other technological treatments may be needed to eliminate or to

remove ANF. In that respect, it is important to note that some ANF are specially present in specific parts of the seed like tannins which are present mostly in the hull rather than in other parts of the seed. Dehulling may thus improve nutritional value of total product. Various parts of the seed may be subject to treatments which are more efficient. It also enables part of seeds to be used for those kind of animals which are more tolerant for these specific ANF then other species (Van der Poel).
For a correct evaluation of the nutritional consequences of these treatments it is important to identify each of the ANF and to identify where it is located. In order to achieve this, analytical methods need to be standardized.

From various studies on ANF and nutrition it is clear that studies need to be made with target animals. In that respect it is also important to define conditions at which animals are kept.

SUMMARY OF GROUP DISCUSSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Chairman: L.A. Den Hartog
Reported by: I.E. Liener

The following is an attempt to summarize some of the highlights of the papers that were presented in this workshop and, based on the discussions that followed, to indicate where future research efforts should be focused. It seems convenient to divide the proceedings of this workshop into three main categories:

1. Digestive physiology and nutrition
2. Technology and plant breeding
3. Analysis and isolation

Digestive physiology and nutrition

Much of our current information regarding the effects of ANFs on animal digestion and nutrition has been based on experiments with small animals such as the rat and the chick, but considerable doubt was expressed as to whether these results can be extrapolated to other animal species of agricultural importance. Although the availability of a suitable animal model for studying the mode of action and possible threshold levels of ANF would be highly desirable, any extrpolation to other animal species should not be made without experimental verification in the target animal. In choosing an animal model, it perhaps should be one which shows the greatest sensitivity to the negative effects of ANF. This then would constitute a good starting point for studying their effects in the target animal. However, the allergic response of piglets and calves to soybeans provides a unique example of animal species specificity which is not revealed by the rat and for which we still have no satisfactory explanation.

Still unresolved is the question of why the protein of heat-treated beans, in which the protease inhibitors and lectins have been inactivated, still shows poor digestibility in comparison to that of a standard protein such as casein. It remains to be established to what extent relatively heat-stable factors such as tannins may contribute to this effect. It is also possible that the low digestibility of heated beans may be only apparent and that the the true digestibility is being obscured by the excessive secretion of endogenous protein, either from the pancreas or the intestinal mucosa.

The presence of several ANFs in any one legume makes it difficult to assess the contribution of each ANF to the overall negative effects exhibited by that legume. Ideally, purified preparations of ANF should be tested both singly and in combination in the target animal, but it was appreciated that, since extremely large quantities of purified ANF would be required for such studies in large animals, this may not be a very realistic approach. It was suggested that perhaps some surgical technique might be possible whereby small amounts of purified ANF could be applied directly to the organ or tissue of a living animal.

Although the protease inhibitors and lectins may be of principle concern in the case of soybeans and Phaseolus vulgaris beans, the tannins, phytates, and alkaloids would probably be of greater nutritional significance in other legumes such as faba beans, peas, and lupins. In any event, it is important that any legume under study should be characterized not only with respect to composition but also with respect to variety or cultivar, maturity, and even growing conditions.

The participants were unanimous in their opinion that there must be standardized analytical techniques for measuring the in vitro activity of ANFs if meaningful comparisons are to be made between the results obtained in different laboratories. (This point is discussed in further detail under "Analysis and isolation".) Techniques and protocols for evaluating the biological effects of ANFs should also be standardized so that all investigators use the same basal or control diet whether it be synthetic, semi-synthetic, or comprised of natural ingredients. The exact formulation of such diets, depending on the target animal, could be determined by an international committee of experts. It might even be desirable to have a central supply of the basic ingredients which could then be made available to different laboratories. Not only should the diets be standardized, but the choice of the test animal should be specified, or at least carefully described by each investigator, with respect to breed, age and sex. There should also be some recommendation as to the minimum number of animals that should be employed if statistically significant results are to be obtained.

One of the most important objectives of ANF research is to be able to establish threshold levels for various ANFs in different species of animals, i.e. the maximim level of ANF that can be tolerated by a given animal species without producing an adverse physiological response. In the final analysis this will require an evaluation of the biological response in a given animal to graded levels of ANF activity in the diet. However, having once established such threshold levels, the availability of sensitive and reproducible in vitro techniques may be employed in a predictive fashion to evaluate the acceptibility of a given legume. It is even conceivable that at some future time, with the input of all of the relevant data, it night be possible to arrive at this answer using computerized technology.

Technology and Plant Breeding

Many studies have been made with respect to the effectiveness of heat treatment for the inactivation of ANFs, and the importance of such factors as the time/temperature relationship, moisture content, particle size, etc. is well recognized. Excessive heat treatment must of course be avoided in order to minimize damage to the nutritional value of the protein and other heat-labile nutrients. In an attempt to reach a compromise between these two opposing effects, many processed legume seeds may retain low, but possibly significant, levels of ANF. It becomes important from an analytical point of view to be able to detect and quantitate these residual levels of ANF. In the case of protease inhibitors the low levels of antiproteolytic activity may in fact include non-protein inhibitors such as tannins and phytates which are not destroyed by heat.

It should be recognized that the results obtained under laboratory conditions may not always be directly applicable to commercial processing conditions. Unfortunately, the latter may be subject to proprietary considerations, in which case the results may not be available to other researchers.

Comparitively little research has been conducted involving the non-thermal inactivation of ANF. The elimination or reduction of ANF may in some instances be achieved by various forms of chemical treatment or physical fractionation. Such approaches have proven effective in reducing the tannin content of faba beans, for example treatment of faba beans with polyvinylpyrrolidone or removal of the seed coat by dehulling. Vitrually unexplored is the possible use of enzymes for the detoxification of ANFs. Fermentation by mircroorganisms which produce "ANFases" should be investigated. The discovery of such enzymes would open up the possibility of using immobilized enzymes in the form of a continuous flow reactor for the inactivation of ANFs.

A few ANFs have been eliminated or at least reduced by plant breeding, for example the tannins in peas and white-flowered faba beans, alkaloids in lupins, and

trypsin inhibitor in soybeans. But, in general, efforts by the plant breeder to remove ANFs have received low priority since the main objectives of the plant breeder are improved yield and disease resistance. These properties could be adversely affected if ANFs were to be eliminated since they serve to protect the plant against predation by insects, bacteria, and fungi. Furthermore, the elimination of ANF could necessitate the increased application of pesticides resulting in a further pollution of the environment. Nevertheless, if plant breeders are to be encouraged in their efforts to eliminate ANFs, there must be close interaction with the nutritionists, who should establish priorities as to which ANF should be removed, and with the chemist, who must provide than with simple and sensitive methods for screening large populations of plant cultivars.

One point that may perhaps be overlooked is the fact that the elimination of ANFs by any technology that does not denature protein, such as heat treatment, may not be fully effective in optimizing the nutritive value of legumes. There is ample evidence to indicate that the proteins of many legumes must be denatured if they are to be effectively digested by enzymes in the digestive tract of the animal. Thus, even if technologies are evolved which eliminate ANFs without heat treatment, or if the plant breeder succeeds in producing non-ANF-containing legumes, the application of heat may still be necessary. Heat treatment alone, however, will not serve to eliminate those ANFs which are relatively heat stable such as the tannins, phytates, alkaloids, and vicine/convicine. In such cases, the plant breeder will still have an important role to play.

Analysis and isolation.

Protease inhibitor activity. As pointed out above, one of the most vexing problems of ANF research is the lack of standardization in the analytical methods used to measure their in vitro activity. This is particularly true in the case of protease inhibitors where so many different methods and units are employed that it becomes very difficult to compare the results of one laboratory with another. Most of the current methods for measuring trypsin inhibitor activity involves the use of bovine trypsin regardless of the fact that the legume under study may be used in the diet of a completely unrelated animal species. Since it has been shown that the proteases of different animals exhibit varying degrees of inhibition by legume inhibitors, the predictive value of using bovine trypsin could give misleading results unless correlated with the biological effects observed in the target animal.

Another problem arises in connection with the fact that seed components other than proteinacous inhibitors can inhibit proteases, for example, the tannins in peas and faba beans. Since these are relatively heat stable, their presence in heat-processed legumes may lead to an over-estimation of the "true" protease inhibitor content. This problem can perhaps be addressed by the development of immunological techniques for the detection and quantitation of specific protease inhibitors, or by the use of affinity chromatography for the specific absorption and subsequent election of protein protease inhibitors.

It was the strong opinion of the workshop that an international committee be organized for the purpose of developing a standardized protocol for the determination of trypsin inhibitor activity. Such a methods must define in a very precise fashion as many of the variables as possible including the method of extraction, enzyme (origin and purity), substrate, time and termperature of incubation, etc. A collaborative study involving several different laboratories will be needed to evaluate the reproducibility of the recommended methods. Ideally, each laboratory participating in such a study should use the same stock of reagents, particularly the enzyme, substrate, and reference inhibitors.

Lectins. Although hemagglutinating activity, because of its relative simplicity, has been most commonly used to measure the lectin content of legumes, its relevance to the

biological effects which they induce in animals is highly questionable. This assay is complicated by the fact that lectins show a high degree of specificity towards the red blood cells of different animal species. This in itself may bear little relationship to their in vivo toxicity which results primarily from their binding to the intestinal mucosa. Thus the most relevant in vitro assay would be one which measures the binding of lectins to the gut wall of the target animal. An assay which shows considerable promise in this regard is the so-called FLIA test (functional lectin immunoassay) which is based on the ability of the lectin to bind to microtiter plates which have been coated with either a carbohydrate matrix or a gut wall brush border membrane preparation. A method such as this could be adapted to the measurement of the binding of lectins and isolectins from various legumes to the gut wall of the target animal. The predictive value of such an assay should, however, be verified by feeding the same legume to the target animal with special attention being paid to any pathological effects that may be produced on the mucosal surface of the small intestine.

Other ANFs. As far as tannins are concerned, the main problem here is which of the several analytical methods which have been proposed are most relevant to their in vivo effects. No clear cut answer has yet to emerge, indicating the need for further research in this area.

Although the importance of alkaloids as an ANF in lupins is generally recognized, their nutritional significance is difficult to evaluate because of the presence of α-1,6-galactosides and the inherent amino acid deficiency (mainly methionine) of the protein.

Antinutritive factors which affect the utilization of carbohydrate deserve more attention. In particular the role of oligosaccharides and the α-amylase inhibitors in relation to energy requirements remains to be elucidated.

In conclusion, it was evident from the papers presented in this workshop and from the ensuing discussions that much research remains to be done before recommendations can be made as to what constitutes acceptable threshold levels of ANFs that are present in legumes fed to animals of agricultural importance. A corollary to this is the need for reproducible analytical methods with proven predictive value which will enable the nutritionist, feed processor, and plant breeder, all working in close cooperation with each other, to achieve the ultimate goal of optimizing the utilization of legumes as a feed ingredient for animals.

CLOSING COMMENTS.
M.P.M. Vos.

I thank the organizers for affording me the opportunity to make some closing remarks. I would like to discuss some aspects regarding the relevance and importance of ANF research for the feed industry and animal production.
Professor Liener has already summarized in his speech the scientific aspects which were discussed during the ANF Workshop. We have learned from the different contributions and the discussions that from a scientific point of view the workshop was a success and valuable. For the point of view of the feed industry the workshop was also important because in these various contributions, the nutritional aspects and analytical methods were discussed in detail.
For the feed industry it is very important that the scientists be active in research that can be applied in practice. In the past we have learned to make progress by carrying out growth and digestibility experiments. Nowadays fundamental research is becoming more and more important for understanding why some problems related to digestion and utilization cannot be solved by traditional research. Therefore fundamental research is indispensable for making progress in increasing the nutritional value of feed. This is certainly the case for ANF-research. Only fundamental research can give us an insight into the way ANFs are acting in the animal. There is no doubt that we can only attack the ANF problem adequately when we know in which way the ANFs cause their negative effects. In this view the ANF workshop has proved to be an important meeting. To stimulate the research which can be applied in practice, the Commodity Board for Feedingstuffs in the Netherlands gives financial support for such research.
The Commodity Board for Feedingstuffs is an organization of farmers, and representatives of trade and industry with statutory powers. Within the Commodity Board there is a cooperation between farmers, trade and industry, including employers and employees. Farmers, trade and industry in the feed sector discuss developments and subjects which are of interest to all of the links in the trade and industrial chain.
An important activity of the board - aside from serving the interests of the enterprises - is promoting and guiding research. This is of importance to the feed trade and industry and often also to livestock farming by co-financing a number of projects. The aim of this "common research" is to promote the economic power of the feed industry and livestock farming. For the competitive position of the Netherlands in the EC-market in the field of production and trade of animal products, it is important to promote new technologies and developments in valudation and production of feedingstuffs.
The Commodity Board has funds to finance such research. Yearly, the board spends about 4 to 5 million Dutch guilders on these activities. These funds are financed by all enterprises in the feed trade and industry.
The Board also supports research and common activities by setting up several committees and working groups for practical and scientific advice.
In this way very close contact has been created between the practice of feed production, feeding and animal production on the one hand and scientific research on the other hand. Certainly this form of cooperation stimulates the rapid transfer of the results of scientific research to practical application.

In the following I would like to discuss some points as to why research into ANF is so important for the feed industry.
Until recently soya was the main protein source for pigs and poultry. When toasted, it is good protein source for fattening pigs and poultry. However, when incorporated in diets for young piglets and veal calves at substantial levels, the weight gain and feed conversion efficiency are negatively influenced. Therefore for these animals, besides milk protein, other protein sources have to be used in the diets. In the future this will become even more important. Moreover, a number of other protein sources also cause negative effects when incorporated into the diets.
The Dutch feed industry is stimulating research focussed on understanding why there are negative effects and how these can be reduced.
For different reasons it is also important to be less dependent on soya. It is never advisable to be dependent on one raw material.
Crop failure or speculations can raise the price of such a raw material to the point where it is no longer acceptable in economic terms.
In this regard I would refer to the marked increase in the price of soya in 1974 and in the middle of 1988.
Moreover, the increased cost of soya will lead to an increase in the prices of other protein-rich raw materials.
Concerning the supply of protein, the European policy is directed to becoming less dependent on imports from the world-market.
For this as well as other reasons the EC is stimulating production of protein rich crops which grow well under European climatic conditions. Main crops for this purpose are the legume seeds and rapeseed. As already discussed in detail during this workshop these seeds contain ANFs limiting their use in feed for monogastric animals.
To give an idea about the amounts of feed produced in Europe and the use of protein rich seeds in these diets, I will give some figures.

About 96.5 million tons of compound feed has been produced in the entire EC in 1987. The use of soya in compound feed is about 18 million tons totally.
Comparing this to the present production of peas and beans in the EC, which is about 4.2 million tons, the conclusion can be drawn that this production is indeed increasing; however, it can never be a satisfactory substitute for soya. Moreover, soyabean meal contains about twice as much protein as the legumes, which means that double the quantity of legumes would be required to give an equivalent level of protein. Nevertheless, it had already been proved that by increasing the production of legumes, the dependence on soya can be decreased. This is, as mentioned, a positive item.

From these data it is evident that there is a need for the use of protein sources other than soya. To accomplish this, the EC is stimulating the grow of these crops with subsidies. However this will be done only temporarily. In the meantime, therefore, we have to solve the ANF problem. This will also create possibilities for the use of these seeds in the future. The importance for industry to accomplish this is demonstrated by the fact that in Europe this kind of research is subsidized by the Eureka organization, by the EC itself, and, in the Netherlands, by the Commodity Board for Feeding-stuffs.

Because of the importance of ANF research for the European feed industry and of course also for other parts of the world, I would make an appeal for scientists active in ANF research to work together in cooperative programmes. We believe that this is the best way to make progress. Organizing a Workshop on ANF like this will be an important tool for maintaining contacts and updating knowledge. I would also make an appeal to pay special attention to research that is of practical importance. Some of these aspects are:

- Optimization of analytical methods for ANFs. For the industry it is very important to have adequate analytical methods for ANFs. These are the tools necessary to characterize the quality of the ANF-containing feed ingredients.
- Research into threshold levels of ANFs. For optimization of the diets it is necessary to have data at which maximal levels of the ANF may be tolerated in the seed.
- Research on the way ANFs are acting in the animal. This information is important for understanding why ANFs are harmful to the animal, and this information also provided the basis for research on threshold levels.
- Research into the possibilities for reducing the ANF activity in the seeds. For the short term there is a need for optimizing existing methods or the development of new techniques. I would direct your attention to the possibilities of biotechnological methods, for instance the development of techniques for the enzymatic inactivation of the ANF, the so called ANF-ases.
  For the long term, plant breeding may be important for producing seeds containing low levels of ANFs.

In conclusion, therefore, I would emphasize that ANF research is important for the feed industry. I have the feeling that in this workshop results have been discussed which are important for the feed industry. I hope that this Workshop will have a follow up, I wish all the best to the scientists in these activities.

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