EFFECTS OF BIOPROCESSED ANTINUTRITIONAL FACTORS ON BEAN PROTEIN QUALITY-with special emphasis on *Phaseolus vulgaris* L.

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EFFECTS OF BIOPROCESSED ANTINUTRITIONAL FACTORS ON BEAN PROTEIN QUALITY-with special emphasis on *Phaseolus vulgaris* L.

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Legumes, e.g. beans and peas, can contain antinutritional factors. Some varieties of faba beans (*Vicia faba*), soya beans (*Glycine max*) and white kidney beans (*Phaseolus vulgaris*) can contain in their raw state antinutritional factors such as tannins, trypsin inhibitors and lectins respectively which negatively effect the protein digestibility by nonruminants e.g. pigs. Also the storage protein is not easily digested by nonruminants. The main aim of the present study was to find a reasonable alternative for the technological elimination of antinutritional factors and cleavage of storage proteins in beans to increase the protein digestibility in pigs. Germination experiments with soya- and white kidney beans resulted in a decrease of the amount of trypsin inhibitors and lectins respectively but the amount of tannins remained constant during germination of faba beans. When these three germinated legume varieties were used for *in vitro* protein digestibility experiments with pepsin and pancreatin results indicated an increased protein digestibility in all the three legumes. Further research on white kidney beans resulted in the isolation of an enzyme extract from germinated beans, capable of cleaving lectins and phaseolin, a storage protein. After investigations it appeared that also specific proteases and glycosidases from microbial organisms, other plants and animals were able to cleave lectin and/or phaseolin into smaller fragments. Germination increased the deviation of the amino acid composition of faba-, soya- and white kidney beans from what is ideally needed for feeding of pigs. Feeding germinated white kidney beans to pigs as compared to ungerminated beans improved the digestion of the bean protein by a decrease of the antinutritional factors and an increase in true ileal bean protein digestibility.
PhD Thesis, Department of Animal Nutrition, Wageningen Agricultural University, Haagsteeg 4, 6708 PM Wageningen, The Netherlands.

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STELLINGEN

1. Het resultaat van enzymatische *in vitro* incubatie op de proteolyse van lectinen is bij aan- of afwezigheid van een zout medium verschillend.

   Dit proefschrift

2. De proteolyse van lectinen en phaseoline in voerbonen *(Phaseolus vulgaris)* tijdens kieming is aan elkaar gerelateerd.

   Dit proefschrift

3. Wateropname tijdens kieming beinvloedt de *in vitro* eiwitverteerbaarheid van sojabonen (*Glycine max*), veldbonen (*Vicia faba*) en voerbonen (*Phaseolus vulgaris*).

   Dit proefschrift

4. Kieming is het meest optimale proces voor de proteolyse van lectinen en phaseoline in voerbonen (*Phaseolus vulgaris).*

   Dit proefschrift

5. Micro-organismen zijn geschikt voor de productie van enzymen, die lectinen en phaseoline proteolyseren en glycolyseren.

   Dit proefschrift

6. Kieming bij licht verbetert de ileale eiwit verteerbaarheid van gekiemde voerbonen (*Phaseolus vulgaris*) in varkens maar verlaagt daarbij de eiwitkwaliteit.

   Dit proefschrift

7. Het zoeken naar het allerkleinste deeltje in de fysica duurt al een eeuwigheid.

8. Grensverleggend pre-embryonaal onderzoek in de geneeskunde heeft haar grenzen meer dan overschreden.

9. Hedendaagse TV-programma's (vanaf 20.30 uur) verlagen zich steeds vaker tot het propageren van rijkdom, genot en geweld.

10. Leerlingen op de middelbare school zijn een zeer goede graadmeter voor het signaleren van veranderende opvattingen over morele normen en waarden in onze samenleving.

11. Het opnemen van kindercrèches in bejaardentehuizen is voor de eersten een volwassen beslissing en voor de laatsten een bevrijding.

12. House-muziek dient binnenskamers te blijven.

13. De roep naar stilte en rust in deze tijd is een verademing voor de Geest.

14. Een toenemend gebruik van Nederlandse woorden in het Limburgse dialect is eigenlijk ongehoord.

15. De overeenkomst tussen het kiemen van een voerboon en het spelen op een schuiftrombone is het vele waterverbruik; een verschil is dat bij de laatste de maximale lengte per definitie vast ligt of staat (?)

16. Er is Hoop.

Frans Savelkoul
Effects of bioprocessed antinutritional factors on bean protein quality - with special emphasis on *Phaseolus vulgaris* L.
Wageningen, 18 oktober 1994.

Voor Louise en Wiesje,
mijn familie en schoonfamilie

Opgedragen aan de parochiegemeenschap St. Victor en Gezellen te Wamel.

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Susteren, augustus 1994

Frans

CONTENTS

INTRODUCTION

General Introduction

Legumes have been used since thousands of years for consumption by animals and humans (FAO, 1982). Several legume crops such as lentils, lupins, peas and beans are nowadays still used for food production (Wiseman and Cole, 1988). From the latter legumes a great scala of bean varieties exists all over the world.

Three species of bean varieties, faba beans (*Vicia faba*), kidney beans (*Phaseolus vulgaris*) and soya beans (*Glycine max*), are widely used in animal feed industry as well as human food industry. However, consumption of these protein-rich beans in their raw state by monogastrics can cause digestion problems (Liener, 1989). The problems often arise after consumption of faba-, kidney- and soya beans containing antinutritional factors, such as respectively tannins, lectins and trypsin inhibitors but also poorly digestible storage proteins (Savelkoul, 1992). Apart from this, Sgarbieri and Whitaker (1982) stated that toxic proteins, antinutritional factors, amino acid composition of proteins, availability of amino acids and the digestibility of proteins limit the nutritive value of beans. To increase the protein quality, legume seeds were often technologically processed.

In this thesis results are published of alternative methods through which antinutritional factors can be inactivated and storage proteins made better digestible. These methods are based on biotechnological principles such as enzyme-incubation, germination and incubation with micro-organisms.

Effects of biotechnologically inactivated antinutritional factors and storage proteins in and from faba-, kidney- and soya beans were measured by *in vitro* and *in vivo* experiments. Results have been presented in this thesis.

History

Faba beans (*Vicia faba*) were already eaten in the ancient Rome and some beans were also added to grain flour for bread baking (Lowgren, 1988). These beans can have a high content of condensed tannins (polyphenols). Because of their complexing properties with proteins, condensed tannins can also react as non-specific trypsin inhibitors and as amylase inhibitors (Deshpande et al, 1986).

Kidney beans (*Phaseolus vulgaris*), have been found in pre-inca tombs in Peru and in Mexico dated from 4000 BC (FAO, 1982). Kidney beans have a lower protein content then faba beans. In addition kidney beans contain high amounts of lectins and phaseolin. The latter is a storage protein that is poorly digestible for nonruminants e.g. pigs. Moreover, certain varieties may contain considerable amounts of amylase inhibitors and trypsin inhibitors (Van der Poel, 1990; Wilson and Laskowski, 1973).

Soya beans (*Glycine max*), the first legume to be described, were already used in China in 2800 BC (FAO, 1982). They have been studied thoroughly because of their high protein and lysine content, but also because they contain considerable amounts of Kunitz and Bowman-Birk type trypsin inhibitors (Wilson et al, 1988). When compared to faba and kidney beans, soya beans do have the highest protein content.

The use of technological processes like thermal treatments has proven to be very effective for degrading lectins (Van der Poel, 1990), trypsin inhibitor activity (Rackis et al, 1986), and tannins (Van der Poel et al., 1991). Asp and Björck (1989) mentioned problems caused

by Maillard reactions during overheating or poor amino acid availability as a result of technological treatments.

Biotechnological processes are also believed to have potential through which antinutritional factors may be removed. Up till now breeding has led to the best results with regard to decrease antinutritional factors on a large scale. The specific role of antinutritional factors can also be as a defense mechanism against predators (Ryan, 1973), weather conditions and diseases. Therefore it is important to assess also the possibilities of breeding against antinutritional factors. It has been shown that the bean yield can be affected by changes in antinutritional factors obtained by breeding (Bond and Smith, 1989).

Research

In order to develop alternative methods, compared to technological processes, for inactivating antinutritional factors and storage proteins a literature study was done. In this study the effect of germination, by the work of enzymes, on the content and activity of amylase inhibitors, lectins, tannins and trypsin inhibitors in legumes has been reviewed (Chapter I).

Firstly the results of the review led to the decision to concentrate the attention of further experiments on three legume species, faba-, kidney- and soya beans which can have a high content of tannins, lectins and trypsin inhibitors respectively. Secondly that germination could function as an alternative process for the improvement of protein quality of legumes, compared to technological processes. A series of experiments was performed to study the enzymatic action. This was done by enzymatic protein hydrolysis of flour of germinated faba and soya beans, using a pepsin-pancreatin enzyme system. SDS-PAGE was used to study the changes in protein pattern of these legumes during germination. In addition, the effect of germination on the content of condensed tannins in flour from germinated faba beans and trypsin inhibitor activity (TIA) in flour from germinated soya beans was observed (Chapter II).

The effect of germination on white kidney beans (*Phaseolus vulgaris*, var. Processor) was also investigated further. Therefore, whole bean extracts were studied during a germination period up to seven days for their lectin pattern and phaseolin pattern, lectin content, binding capacities of functional lectins towards brush border membranes and trypsin inhibitor content. In addition the *in vitro* enzymatic hydrolysis by pepsin and pancreatin of the protein from flours of (un)germinated white kidney beans was performed. Results of this germination experiments led to the decision to concentrate the attention in further experiments on white kidney beans. Thereby enzymes responsible for the degradation of lectins, phaseolin and trypsin inhibitors in white kidney beans during germination were further investigated (Chapter III).

In addition, protease activity was measured in white kidney beans germinated up to seven days. Also the enzyme-extract responsible for this protease activity was isolated. Phaseolin and lectins, which cause a poor digestibility and antinutritional effects in nonruminants respectively, were isolated and purified from the same line of raw white kidney beans. Furtherone lectins and phaseolin were studied for their susceptibility to degradation by the

isolated enzyme-extract (Chapter IV).

In order to produce large quantities of enzymes capable of modifying antinutritional factors and storage proteins in legume seeds, microbial enzymes were also screened. The antinutrional factors lectins and trypsin inhibitors and the poorly digestible storage protein phaseolin, from white kidney beans (*Phaseolus vulgaris* L) were bioprocessed by incubating them with proteases and glycosidases from different origins (Chapter V).

The experiments of germination, of enzyme-incubation and of incubation with micro-organisms (Chapter III, IV and V) were compared. The results indicated that flour of germinated white kidney beans would be the best alternative, compared to technologically treated products. The flour of germinated beans could therefore be used in animal feeding experiments for observing effects of inactivated antinutritional factors on the apparent ileal digestibility in pigs. Also the effect of germination on the nutritive value of legume seeds in terms of protein digestibility (*in vitro*) and amino acid composition was measured. The results indicated a decrease of the content of essential amino acids during germination (Chapter VI).

Finally after these experiments effects of germination of white kidney beans on apparent ileal digestibility was investigated in *in vivo* experiments with pigs. Thereby four diets were formulated. The first diet, the control diet, contained no beans. The other three diets contained respectively 7.5% raw beans, 7.5% raw beans treated with pancreatin and 7.5% beans, germinated for seven days. Results indicated that the latter diet had the highest apparent ileal digestibility, compared to the other diets (Chapter VII).

In the general discussion the aim was to make overall conclusions from the results obtained from the several experiments. The first main conclusion was that flour of germinated beans is the best alternative product, compared to technologically treated beans, for the use in animal feedstuffs. The second main conclusion was that the considerable inactivation of antinutritional factors and storage protein overruled the negative effect of the decrease of the content of essential amino acids during germination thereby leading to an increase of the apparent ileal protein digestibility of the diet, containing germinated beans, in pigs.

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CHAPTER 1

The presence and inactivation of trypsin-inhibitors, tannins, lectins, and amylase-inhibitors in legume seeds during germination. A review.

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Abstract

During the germination of legume seeds, enzymes become active in order to degrade starch, storage-protein and proteinaceous antinutritional factors. The degradation of storage-protein is necessary to make peptides and amino acids available in order to stimulate seed growth and early plant growth. Proteinaceous antinutritional factors such as amylase inhibitors, lectins and trypsin inhibitors are present in legume seeds and protect them against predators. However, during germination, they degrade to a lower level by the action of several enzymes. The effect of germination on the content and activity of amylase inhibitors, lectins, tannins and trypsin inhibitors is discussed.

Introduction

A variety of legume seeds is used in human as well as animal nutrition as suitable sources of amino acids and energy. These seeds usually contain large amounts of stored materials which can be used as precursors for synthetic processes during germination. Examples of stored materials are starch, protein and phosphorus. It has been recognized for a long time that legume seeds contain mechanisms against predators such as micro-organisms, insects, birds and animals (Ryan, 1973). Such protection mechanisms are present in the form of antinutritional factors. To this group of factors belong amylase inhibitors, lectins and trypsin inhibitors (Gupta, 1987; Liener, 1989). In the last decade much research has been done, aimed at improving the nutritive value of protein-rich feedstuffs such as *Glycine max*, *Phaseolus vulgaris* and *Vicia faba* with regard to animal nutrition. A limitation for the use of these three protein-rich products in the raw state in animal feeding is the presence of antinutritional factors which have a negative influence on the protein digestibility in monogastrics, including humans (Liener, 1989). Therefore much research has been devoted to the inactivation of antinutritional factors by plant breeding (Bond and Smith, 1989) and technology (Van der Poel, 1989). A new approach, not yet fully explored would be the inactivation of nonproteinaceous antinutritional factors such as tannins and proteinaceous antinutritional factors such as amylase-inhibitors, lectins and trypsin-inhibitors by enzymes developed during germination. This paper reviews the mode of action of enzymes and enzyme systems which are responsible for the inactivation of these four antinutritional factors during germination of *Glycine max*, *Phaseolus vulgaris* and *Vicia faba*.

Proteolysis during germination

Germination is the first stage of growth of a plant during which the primary root and stem break through the seed coat. Environmental factors act as external conditions for germination of seeds; water intake: for weakening the seedcoat, metabolism and seed growth, air supply: as oxygen intake necessary for the respiration and temperature: an important factor for metabolism.

It is believed that during germination proteases are responsible for the inactivation of proteinaceous antinutritional factors like lectins, amylase- and trypsin inhibitors. Therefore it is of interest to review rate and type of proteolysis in germinating seeds.

According to Mikola (1983) during germination of seeds there are three stages of proteolysis which can be distinguished.

During the initial hydrolysis (first stage) amino acids are liberated and subsequently used for the synthesis of enzymes responsible for the conversion of reserve substances to forms suitable for transport.
This is followed by stage 2, the bulk hydrolysis during which reserve proteins are hydrolysed into amino acids for the growing seed.
Finally during the third stage, which happens during the senescence of storage tissue, the cellular proteins and enzymes are broken down into amino acids which are subsequently used by the seedling before the onset of autotrophic growth.

In legume seeds several enzymes that take part in the hydrolysis of storage proteins are located in different parts of the cell (Mikkonen, 1986). Most of the carboxypeptidase activity in *Phaseolus vulgaris* (var. kidney bean) is associated with the protein bodies (Mikkonen et al., 1986). Naphtylamidases (neutral aminopeptidases) are localized in the cytoplasm of resting kidney beans (Mikkonen et al., 1986). The activity of naphtylamidases seems to decrease during germination in kidney beans (Feller, 1979). Alkaline peptidases, similar to those which have been characterized in barley (Sopanen and Mikola, 1975; Sopanen, 1976), have very high activities in resting kidney beans and are localized in the cytoplasm (Mikkonen and Mikola 1986; Mikkonen et al., 1986). The first step of protein breakdown is probably carried out by carboxypeptidases and acid proteases inside the protein bodies but in resting seed however the activities of these enzymes are low (Mikkonen et al., 1986). This effect can also be explained by the presence of an inhibitor in the resting bean seeds (Nielsen and Liener, 1984) or by the lack of water. In the following some aspects are discussed with respect to important inhibitors and effects of germination on their content and activity.

Trypsin inhibitors

Some of the antinutritional factors inhibit the activity of proteolytic enzymes in the digestion tract of animals. In legume seeds protease inhibitors are widely distributed and differ in specificity and in potency of inhibition, which depends on the origin of the target enzyme (Birk, 1989).
Inactivation of trypsin in the gut by trypsin inhibitors from soybeans induces the intestinal mucosa to release cholecystokinin (CCK). This hormone stimulates pancreas acinar cells to produce more trypsin, chymotrypsin, elastase and amylase. When this negative feedback continues, an important loss of S-containing amino acids is created. This leads to a depression in growth, pancreatic hypertrophy/hyperplasie and carcinogenic effects (Liener, 1989). Trypsin inhibitors from other legumes depress growth and cause pancreatic hypertrophy in much the same way as the soybean inhibitors (Liener and Kakade, 1980).

Two types of trypsin inhibitors exist, Bowman-Birk- and Kunitz inhibitors. Most of both types of inhibitors inhibit trypsin and many of them inhibit chymotrypsin (Birk, 1989). The Kunitz soybean trypsin inhibitor is the first plant protease inhibitor which was isolated and characterized (Kunitz, 1947). This inhibitor has a molecular weight of about 21,000 and includes two disulphide bridges (Birk, 1987). Standard trypsin inhibitor methods do not differentiate the trypsin inhibitors into Bowman-Birk and Kunitz inhibitors (Kakade, 1974; Roozen and de Groot, 1988). However Brandon et al. (1988) developed an enzyme-linked immuno-assay (ELISA) for the Kunitz trypsin inhibitor of soybeans by using monoclonal

antibodies derived from mice. The assay is specific for isoforms of Kunitz soybean trypsin inhibitor of type Ti^a and Ti^b, discriminating against molecules denatured by heat or alkalinity and shows no cross-reactivity with the Bowman-Birk trypsin inhibitor.

The Bowman-Birk inhibitor from soya beans serves as the prototype for a family of inhibitors and has a molecular weight of about 8,000 with a high content of cystine, forming seven disulfide bridges.

From *Phaseolus vulgaris* (var. kintoki bean) at least five different trypsin inhibitors were isolated (Tsukamoto et al., 1983). Two distinct sites for trypsin and chymotrypsin were discovered in both inhibitors 2 and 4. Inhibitor 5 inhibited only trypsin. Jacob et al. (1986) isolated a trypsin-chymotrypsin inhibitor from *Phaseolus vulgaris* (var. kidney bean) with a molecular weight of approximately 13,000.

During germination the trypsin inhibitor activity (TIA) decreased in horsegram (*Dolichos biflorus*), mothbean (*Phaseolus aconitifolius*) (Subbulakshmi et al., 1976) and jack bean (*Canavalia ensiformis* L. DC) (Babar et al., 1988). The trypsin inhibitor activity in French bean decreased during 10 days of germination by 30% (Nielsen & Liener, 1988). However Chang & Harrold (1988) concluded that the TIA in Navy beans (*Phaseolus vulgaris*) did not significantly decrease after 6 days of germination (table 1.). They observed an increase in TIA in Pinto beans (*Phaseolus vulgaris*) after 6 days of germination by more than 39 %.

Table 1. The degradation of trypsin-inhibitor activity (TIA) during germination of legumes.

Legume	TIA ungerminated (TIU/g beanflour) (mg/100 g DM)	Germination Time (days)	TIA-degradation (%)	
Dolichos biflorus (Horse gram)	50200	3	16	a)
(*Phaseolus aconitifolius*) (Mothbean)	4300	3	40	a)
Canavalia ensiformis (Jack bean)	12	1.5	31	b)
Phaseolus vulgaris (Navy bean)	17	6	3	c)
Glycine max (Soybean)	-	13	100	d)

a) Subbulakshmi et al., 1976; method of Kakade et al., 1969
b) Babar et al., 1988; method of Erlanger et al., 1961
c) Chang and Harrold, (1987); method of Kakade et al., 1974
d) Results were reflected as percentages Bowman-Birk inhibitor by Tan-Wilson et al., 1982; method of Tan-Wilson et al., 1982

The content of the Bowman-Birk inhibitor in the cotyledons of soybean (*Glycine max* [L.] Merrill cv. Fiskeby) seeds decreased till the 13th day. After that day no Bowman-Birk inhibitor could be detected anymore but Kunitz proteinase inhibitor was still present (Tan-Wilson et al., 1982). During germination and seedling growth of soybeans (*Glycine max*) Hartl et al. (1986) observed new forms of the Kunitz soybean trypsin inhibitor in the cotyledons distinct from that of the resting seed. They concluded that these forms arise from the Kunitz soybean trypsin inhibitor present in the ungerminated seed by limited specific proteolysis. Wilson et al. (1988) discovered that at least three proteases (K1, K2 and K3) attacked the native Kunitz soybean trypsin inhibitor of soya beans (*Glycine max* L. Merrill cv Amsoy 71). Protease K1, was a cysteine protease that peaked in activity at day 4 of seedling growth with a maximal activity at pH 4. The activity of protease K2 peaked later in seedling growth, at day 8 with a maximal activity at pH 4. Protease K3 was active at pH 8 and increased in activity from day 10 till day 14 of seedling growth.

Tannins

Inhibition of digestive proteolytic enzymes is not restricted to protein protease inhibitors, such as trypsin-inhibitors, also nonprotein protease inhibitors are known. This was demonstrated by Roozen and de Groot (1987, 1988) who introduced a method for the analysis of residual trypsin inhibitor activity in soya flour. They concluded that total trypsin-inhibitor activity could only partly be ascribed to proteins. The non-specific inhibition activity was thought to result from tannin, a polyphenol. Deshpande (1986) had made that same conclusion by observing that polyphenols could react with proteins and enzymes so they could also act as trypsin-inhibitor and amylase-inhibitor.

The presence of tannins in food legumes is widespread (Reddy et al., 1985; Rao and Deosthale, 1987; Mangan, 1988).
Marquardt et al. (1977) observed that a water extract from faba bean hulls, when incorporated into a chick diet, depressed the rate of growth, efficiency of feed utilization and liver size.
Tannins are water-soluble compounds with a molecular weight in the range of 500-3000 and they can be divided into two groups, the hydrolysable and condensed tannins (Goldstein and Swain, 1963; Gupta and Haslam, 1979, Marquardt et al., 1977). Hydrolysable tannins can be split into sugars and recognizable phenolic carboxylic acids. Condensed tannins cannot be split in this way (Marquardt, 1989). Condensed tannins are the most widespread and typical of the plant tannins. They consist of oligomers of the flavan-3-ols (the catechins) and related flavanol residues which typically produce anthocyanidins on acid degradation (Mangan, 1988). The condensed tannins cause a decrease in the digestibility of protein and carbohydrate as a result of the formation of insoluble enzyme-resistant complexes with tannins (Reddy et al., 1985).
Deshpande (1986) commented that in faba beans the condensed tannins were present in high concentration and interact with dietary proteins. The resulting tannin-protein complexes are believed to be responsible for growth depression, low protein digestibility and increased fecal nitrogen. Tannins have also been reported to cause damage to the intestinal tract, to be toxic after absorbtion from the gut, an to interfere with the absorption of iron. Finally tannins have been claimed to have a carcinogenic effect (Liener, 1989).

Tannins are located primarily in the seed coats of cereals and legumes. Mechanical dehulling almost quantitatively removes condensed tannins (Rao and Deosthale, 1982; Deshpande, 1986). Marquardt et al. (1977) isolated, purified, identified and characterized condensed tannins from faba beans (*Vicia faba* L. var. minor).

Bressani and Elias (1980) concluded that there was a decrease in tannin content during germination of legume seeds and Price et al. (1979) made the same observation that also in developing and maturing sorghum grain. Sawathar et al. (1981) observed that the polyphenol content in horse gram (*Dolichos biflorus*) decreased from 1.6% to 1.1% after 2 days of germination. In moth bean (*Phaseolus aconitifolius*) the polyphenol content was reduced from 1.3% to 1.0% after 1 day of germination. After 36 hours of germination however there was a significant increase from 1.0% to 1.7%.

Rao and Deosthale (1982) investigated the tannin content of different varieties of pigeonpea (*Cajanus cajan*), chickpea (*Cicer arietinum*), blackgram (*Phaseolus mungo*) and greengram (*Phaseolus aureus*) (table 2). They suggested that enzymatic degradation was responsible for the loss of tannin during germination.

Tabel 2. The change in tannin content during germination of legumes (Rao and Deosthale, 1982).

Legume	Tannin content* ungerminated (mg/100 g DM)	Germination Time (days)	Tannin degradation (%)
Cajanus cajan (Pigeonpea)	1141	2	60
Cicer arietinum (Chickpea)	165	2	60
Phaseolus mungo (Blackgram)	836	2	50
Phaseolus aureus (Greengram)	612	2	50

* Tannin content estimated by the Vanillin-HCl method

Rahma et al. (1987) observed that after 1 day of germination in faba beans (*Vicia faba*) a reduction of about 23% in tannin content was measured. During further germination this reduction decreased to about 9%. An explanation for these peculiar results can be that tannins become oxidized during germination. Thereby the molecular structure is changed and therefore can no longer be detected by the Folin-Denis reagent which was used. Babbar et al. (1988) concluded that the level of polyphenols in jack bean (*Canavalia ensiformis* L. DC) after 1.5 day of germination was decreased by 35%.

In both plant and animal cells the enzyme polyphenol-oxidase (PPO) is present and has the ability to catalyze the hydroxylation of various monophenols and the aerobic oxidation of diphenols (Boyer, 1977). Rao and Deosthale (1987) observed PPO-activity in seeds of the legumes: chickpea (*Cicer arietinum*), pigeon pea (*Cajanus cajan*), greengram (*Phaseolus aureus*) and black gram (*Phaseolus mungo*) which were germinated for 0, 12 and 24 hours. While PPO-activity increased during germination, tannin content of the legumes decreased. However no significant inverse relationship was observed between the level of PPO-activity and the tannin content of the germinated legumes.

Lectins

Lectins are glycoproteins which bind to specific sugars or proteins (Roberts and Goldstein, 1984). This reaction can be shown in vitro by the agglutination of red blood cells. Agglutination is the interaction of a lectin with cells and is used to reveal the presence of a lectin in a biological source (Lis and Sharon, 1986).

Lis and Sharon (1981) reviewed the purification of lectins by affinity chromatography. Koehler et al. (1986) differentiated the lectin activity in twenty-four cultivars of dry beans (*Phaseolus vulgaris* L.).

The feeding of lectins derived from particularly *Phaseolus vulgaris* beans resulted in biological effects such as: 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 (Liener, 1986; Pusztai, 1987). Lectin molecules consist of one or more subunits. When the number of units in such a complex decreases, a dimunition of its agglutinating ability is observed (Kik et al., 1989).

Legume lectins are initially synthesized as single polypeptide chains of a molecular weight of about 30,000. These chains, after removal of a 20-residue hydrophobic leader amino acid sequence, may be cleaved into a larger ß- and a smaller α-subunit, with the possible loss of a few amino acids. In many legumes the α- and ß- subunit associate into dimers of about 50,000 and tetramers of about 100,000 and 120,000 . *Phaseolus vulgaris* (var. Processor) contain two basic types of lectin subunits of about 30,000 molecular weight, designated E (erythro) and L (leuco) (Strosberg et al., 1986).

Vicia faba contains mannose/glucose-binding lectins, these are tetrameric glycoproteins composed of two light (α) and two heavy
(ß) chains. *Glycine max* contains N-acetylgalactosamine/galactose-binding lectins, which are tetrameric glycoproteins composed of equal amounts of two slightly different subunits, each of which contains an N-terminal alanine. *Phaseolus vulgaris* (red kidney) contains also N-acetylgalactosamine/galactose binding lectins but they differ from soya bean lectins in that they comprise five isolectins, which are glycoproteins and each of which consists of four subunits held together by noncovalent bonds (Goldstein and Poretz, 1986).

Valdebouze et al. (1980) observed that all the haemagglutinating activity in ungerminated pea (*Pisum sativum*), soybean (*Glycine max*) and faba bean (*Vicia faba*) was caused by the lectins present in the cotyledons. The haemagglutinating activity (HA) in soybean (*Glycine max*), pinto bean (*Phaseolus vulgaris*), mung bean (*Vigna radiata*), faba beans *Vicia faba*,

horse gram (*Dolichos biflorus*) decreased during germination. However the hemagglutinating activity in mothbean (*Phaseolus aconitifolius*), germinated for three days, did not decrease (Chen et al., 1977; Rahma et al., 1987 and Subbulakshmi et al., 1976) (Table 3).

Table 3. The reduction in hemagglutinating activity (HA) during germination of legumes.

Legumes	HA ungerminated (HA/g beanflour)	Germination Time (days)	HA-reduction (%)	
Dolichos biflorus (Horse gram)	2600	3	77	a)
Phaseolus aconitifolius (Moth bean)	0	3	0	a)
Glycine max (Soybean)	12800	4	96	b)
Vigna radiata (Mung bean)	1600	4	100	b)
Phaseolus vulgaris (Pinto bean)	12800	4	100	b)
Vicia faba (Faba bean)	3200	6	89	c)

a) Subbulakshmi et al., 1976; method of Liener and (1953)
b) Chen et al., 1977; method Prigent and Bourillon (1976)
c) Rahma et al., 1987; method of Liener and Hill (1953)

During 6 days of germination the hemagglutinating activity decreased in *Phaseolus vulgaris* (cv. Pinto) by 30 % (Chang & Harrold, 1988). Nielsen & Liener (1988) observed that during the 9 days of germination the hemagglutinating activity in French beans (*Phaseolus vulgaris*) decreased by 90 %. Bansal et al. (1988) concluded that complete elimination of hemagglutinin in chickpea (Cicer arietinum L.) was achieved after 8 days of germination.

Boylan and Sussex (1987) purified an endopeptidase (a cysteine endopeptidase) involved with the storage-protein degradation in *Phaseolus vulgaris* L. cotyledons. The isolate endopeptidase partly hydrolysed the bean-seed lectin, phytohemagglutinin. Thereby extensively degrading the 36000-Da subunit. However the, 34000-Da subunit remained intact.

Amylase inhibitors

Amylase inhibitors interfere with the starch digestion in animals by inhibiting pancreatic

amylase.

Jaffe et al. (1973) partially purified kidney bean (*Phaseolus vulgaris*) inhibitor which acted on pancreatic and salivary amylases.

Marshall and Lauda (1975) observed that an inhibitor of α-amylase, which they called phaseolamin, was specific for animal α-amylases, with no activity towards the corresponding plant, bacterial and fungal enzymes, or any other hydrolytic enzyme tested. Phaseolamin appeared to be a protein with a molecular weight in the range 45,000 to 50,000, and a specific α-amylase inhibitor rather than a proteolytic enzyme. Marshall and Lauda (1975) concluded that the function of phaseolamin most likely was to serve as a protective agent against insects and other predators by inhibiting their digestive amylases.

Powers and Whitaker (1977) purified also an α-amylase inhibitor from *Phaseolus vulgaris* with a molecular weight of 49,000, which contained four subunits. This α-amylase inhibitor had an inhibiting activity against porcine pancreatic amylase and human salivary α-amylase.

Pick and Wöber (1978) purified and partially characterized a proteinaceous α-amylase inhibitor from *Phaseolus vulgaris* (commercially white beans) with a molecular weight of 43,000.

Frels and Rupnow (1984) purified and partially characterized two α-amylase inhibitors from black bean (*Phaseolus vulgaris*). The inhibitors were designated I-1 and I-2 based on their order of elutions from a phenyl-Sepharose column. Both inhibitors were mannose containing glycoproteins, composed of subunits; active against bacterial, mold, and plant α-amylases with molecular weights of 49,000 (I-1) and 47,000 (I-2). I-2 was more resistant to heat denaturation than I-1.

Lajolo and Filho (1985) partially characterized the amylase inhibitor of black beans (*Phaseolus vulgaris*, var. Rico 23), with a molecular weight of 53,000. The amylase inhibitor happened to be active against mammalian α-amylases.

Kotaru et al. (1989), concluded that the α-amylase inhibitor in cranberry bean (*Phaseolus vulgaris*) was quite resistant to the proteolytic pepsin and trypsin digestions but relatively susceptible to chymotrypsin digestion.

Sathe et al. (1983) observed that the α-amylase inhibitor activity decreased by 67.1 % after 5 days of germination of great northern beans (*Phaseolus vulgaris* L.).

A similar observation was made by Kotaru et al. (1987) who also observed changes of α-amylase inhibitors during germination. Thereby the α-amylase inhibitor activity in kidney beans (cranberry bean) decreased after seven days of germination by 40 %.

Discussion

Feeding protein rich legume seeds to monogastric animals is hampered by the presence of antinutritional factors. Therefore

ways have to be found to eliminate such factors. Technological treatment is one possible way, but this is usually a very energy demanding and therefore expensive proces. Heat treatment may result in damage to the protein (Van der Poel, 1990). A potential alternative seems to be germination because it is beyond reasonable doubt that proteinaceous antinutritional factors like lectins, amylase- and trypsin inhibitors are partly degraded during germination. This has been observed in varieties of *Glycine max*, *Phaseolus vulgaris* and *Vicia faba* (Hartl et al, 1986; Kotaru et al., 1987; Lajolo and Filho, 1985; Sathe et al., 1983; Tan-Wilson et al., 1982; Wilson et al., 1988).

However depending on conditions the extent of degradation differs with variety of the legumes, type of antinutritional factors and enzymes which degrade proteinaceous trypsin inhibitors in beans of *Glycine max* and *Phaseolus vulgaris* during germination is abundant (Wilson, 1973; Sathe et al., 1983; Wilson et al., 1988).

Much less research has been put into the inactivation of lectins by enzymes during germination of legumes. This is somewhat surprising because various lectins have been described in detail. For instance in lectin-rich legumes like *Phaseolus vulgaris* the amino acid sequence of lectins has been thoroughly examined (Hoffman et al., 1982; Hoffman, 1984). Therefore research on the enzymatic degradation of lectins during germination in *Phaseolus vulgaris* seems very relevant.
Research on the eventual change in tannin content during germination has also been investigated in *Vicia faba* (Rahma et al., 1987), but methods for measuring tannin content are still in a developing stage. That tannins are significantly degraded by enzymes during germination of legumes has not yet been observed. Tannins can be oxidized by polyphenoloxidases, but a significant relationship between polyphenol-activity and tannin content in legumes during germination has not yet been proved (Rao and Deosthale, 1982; Rao and Deosthale, 1987).

The initial hydrolysis of reserve protein is executed by specific enzymes like carboxypeptidases and acid proteases. Lectins are in fact also reserve proteins so it may be possible that they are hydrolysed by the same above mentioned enzymes.

If one wants to make use of the effect of germination it is necessary to isolate and characterize the enzymes responsible for ANF inactivation. In this process of isolation it is important to measure specifically the activities of enzymes which can degrade lectins in *Phaseolus vulgaris.*

It is also of importance to use a substrate from *Phaseolus vulgaris* like phaseolin or lectins themselves and not hemoglobin or casein in order to have a specific reliable enzyme-substrate reaction. When using bean flour one has to be aware of the fact that a total enzyme activity is measured, instead of the activity of only one enzyme from a part of the bean.

The feeding of the germinated legumes like *Glycine max, Phaseolus vulgaris* and *Vicia faba* to nonruminants in order to improve the protein digestibility does not seem to be very attractive. Germination of legumes will most probably result in a loss of biomass of more than 1% biomass per day of germination. Research has to prove that the negative effect of loss of biomass is overruled by the positive effect of ANF degradation during germination of the above mentioned legumes.

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CHAPTER II

In vitro enzymatic hydrolysis of protein and protein pattern change of soya and faba beans during germination

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Abstract

In addition to technological processes like heat treatment germination can be an alternative process for the improvement of protein quality of legumes. This was demonstrated by enzymatic protein hydrolysis of flour of germinated faba and soya beans, using a pepsin-pancreatin enzyme system. SDS-PAGE was used to study the changes in protein pattern of these legumes during germination. In addition, the effect of germination on the content of condensed tannins in flour from germinated faba beans and trypsin inhibitor activity (TIA) in flour from germinated soya beans was studied. Germination for five days resulted in a maximum increase in enzymatic protein hydrolysis by 21.3% in flour from faba beans and by 25.7% in flour from soya beans after 12 hours of germination. Protein patterns, obtained with SDS-PAGE demonstrated a considerable protein breakdown during germination between day 2 and 3 in faba beans and between day 1 and 2 in soya beans. The tannin content in flour from faba beans decreased by 29.7% after seven days of germination, but the tannin content of the hulls of the faba beans did not change during that period of germination. The trypsin inhibitor activity (TIA) in flour from soya beans decreased by 25.5% after seven days of germination. We conclude that the increased enzymatic hydrolysis of protein in both legumes cannot be explained by a decrease of tannins or trypsin inhibitors. The possible explanation is that through degradation of proteins during germination of the legumes, the cleaved protein fragments are more susceptable for hydrolysis by pepsin-pancreatin.

Introduction

Native storage proteins in ungerminated legume seeds like phaseolin in white kidney beans (*Phaseolus vulgaris*) are quite resistant against proteolytic degradation in the digestive tract of monogastric animals like pigs and poultry (Van der Poel, 1990). Storage proteins in ungerminated legume seeds like legumin and vicillin in faba beans (*Vicia faba*) and ß-conglycinin and glycinin in soya beans (*Glycine max*) can possibly have the same effect as phaseolin in monogastric animals. True digestibility of raw faba and soya beans appeare to be relatively low (Van der Poel et al., 1991; Kratzer et al., 1990). Even when this is taken into account the apparent protein digestibility is lower than expected because of the presence of antinutritional factors such as protease inhibitors (trypsin-/chymotrypsin inhibitors), tannins and lectins (Birk, 1989; Deshpande et al., 1986; Liener, 1986). In order to improve the protein quality of legumes much research aiming at the elimination of antinutritional factors, often of technological nature, has started in recent years (Van der Poel et al., 1992; Vohra and Kratzer, 1991). Apart from technological treatments there seems scope for alternative processes. One promising area of biotechnology research aiming at the improvement of protein quality of legumes is germination (Savelkoul et al., 1992). Wassef et al. (1988) concluded that proteolysis of protein in soya bean meal after treatment with papain and trypsin was increased when the meals were defatted, prepared from germinated soya beans or digested for long periods. They also found that germination and seedling growth of soya beans were accelerated by impaction, soaking in water and growth at a temperature of 30°C. Rahma et al. (1987) studied the effect of germination on the *in vitro* protein digestibility of faba beans. Using pepsin-pancreatin as well as pepsin, pancreatin and trypsin as single enzyme systems they concluded that the *in vitro* protein digestibility increased up till 4 days of germination.

In order to investigate the effect of germination on *in vitro* enzymatic hydrolysis of bean protein an experiment was conducted in which faba beans and soya beans with a relatively high content of tannins and trypsin inhibitors, respectively, were germinated for up to 7 days. Total crude protein content, water absorption, enzymatic hydrolysis of protein and protein pattern of flour from faba and soya beans were measured. In addition the effect of germination on the content of tannins and trypsin inhibitor activity was measured in bean flour from faba beans and soya beans, respectively. Finally, during germination the tannin content of the hulls of faba beans was measured in order to compare results with the content of the tannins in the beanflour.

Materials and methods

Materials

Faba beans (*Vicia faba*, var. Pistache) were obtained from Joordens Zaadhandel BV; Neer, The Netherlands; soya beans (*Glycine max*) were obtained from Schouten-Giessen; Giessen, The Netherlands.

Germination

All bean varieties were germinated in wet sand under standard conditions (12 hours light/day and 20°C) at the former Government Seed Testing Station (RPvZ), Wageningen (Van der Burg et al., 1983). Germination was stopped by harvesting after 0, 0.5, 1, 2, 3, 4, 5, 6 and 7 days.

Preparation

After germination beans harvested by sieving, washed carefully to remove sand, dried between filter paper, weighted to measure the water intake, frozen in fluid nitrogen and homogenized in a Waring Blendor. The resulting crude bean flour was subsequently freeze-dried (FTS System & Co), ground at 1 mm in a Retsch-mill and stored at 4°C in plastic buckets.

Enzymatic hydrolysis

The determination of the protein hydrolysis of faba beans and soya beans, germinated for various lengths of time was carried out by a modified method of the enzyme-assay developed by Babinszky et al. using pepsin and pancreatin (Babinszky et al., 1990). Bean flour samples containing 200 mg protein were incubated in a 25 ml pepsin/HCl solution (0.1 mol/l HCl, pH 1.0; pepsin 0.25 g/l) in a shaking waterbath at 40°C for 15 minutes. After incubation the solution was neutralized with 5 ml $NaHCO_3$ (44 g/l). Phosphate buffer (25 ml, 0.165 M, pH 6.8) containing 0.25 g/l pancreatin, 0.25 ml/l amylase (NOVO) and 0.25 ml/l termamyl (NOVO), 76 mg/l bile salts (SIGMA) and 40 mg/l lipase (SIGMA) was added and the incubation continued (40°C, 15 minutes).

The second incubation was stopped by the addition of 2.5 ml 1.0 mol/l HCl and 1.0 ml 1.0 mol/l acetic acid and the incubated mixture filtrated over a glass filter crucible, with a layer of ashfree floc (Whatman). The ashfree floc with the undissolved sample was collected and transferred in a Kjeldahl flask. Total nitrogen (N) in the original samples and in the residue after filtration was determined by the Kjeldahl method. Protein hydrolysis was calculated from the ratio between the amount of nitrogen in the residue and in the original feed sample (Savelkoul et al., 1990; ISO 1979).

Extraction procedure for electrophoresis

Soya bean flour was extracted (1 g:10 ml) with 50 mM Na_2CO_3, pH 9.5, for 1 hour at room temperature (20°C) and centriguged for 1 hour at 40,000 g. Faba bean flour was extracted with 0.1 M NaCl (1 g:10 ml), pH 8.0, for 1 hour at room temperature (20°C) and centrifuged for 1 hour at 40,000 g.

Electrophoresis

Protein patterns of flour of faba and soya beans were analyzed according to the procedure of Laemmli (Laemmli, 1970) with the sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE). A running gel, containing 15% acrylamide, and a stacking gel, containing 4% acrylamide were used. Electrophoresis was performed using a Biorad Mini-PROTEAN II Cell at 200 V.

Protein assay

Protein content of faba- and soya beans prior to their use in SDS-PAGE was determined with the bicinchoninic acid(BCA)-method (Hill and Straka, 1988). The BCA protein assay reagent was obtained from Pierce Chemical Co (Rockford, IL).

Tannin content assay

It was felt of interest to investigate whether tannins from faba beans are in some way inactivated or decreased during germination. Tannins are located primarily in the seed coat of cereals and legumes (Deshpande et al., 1986). Therefore the seed coat of 100 faba beans, ungerminated and germinated for 4 and 7 days respectively, were removed manually. They were freeze dried and analysed for their condensed tannin content by the vanillin-H_2SO_4 method (Kuhla and Ebmeier, 1981) with catechine as a standard. Also, bean flour from *Vicia faba* germinated for up to 7 days was tested for its tannin content, using the same method.

Trypsin inhibitor activity (TIA) assay

Trypsin-sepharose 4B affinity chromatography was used to purify protein-type trypsin

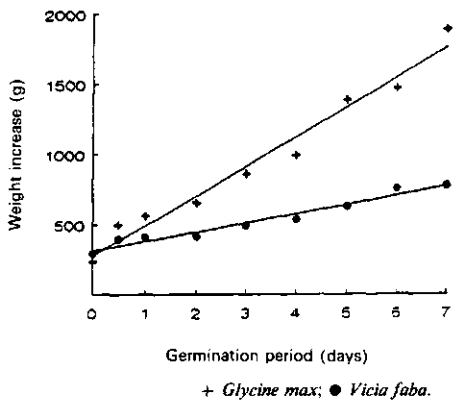
inhibitors from soya bean flour according to the method of Roozen and de Groot (Roozen and de Groot, 1989). The amounts of protein obtained by this procedure were determined by a Coomassie protein assay with Kunitz inhibitor as a standard.

Results and discussion

Water absorption of legumes during germination

The water absorption of faba beans contrasted that in soya beans considerably. Soya beans absorbed water much faster and in greater amounts than faba beans (figure 1).

Fig. 1. Water intake during germination



+ *Glycine max*; ● *Vicia faba*.

Because of their relatively thick hulls, faba beans absorb water more slowly compared to soya beans. That the water absorption in faba beans increased continuously during germination was also found by Rahma et al. (1987). Also plant development was observed to start earlier in soya beans than in faba beans respectively.

Crude protein of legumes in relation to germination

During germination the total crude protein content in faba beans, and soya beans hardly changed, and was 28.3 % ± 0.1 and 40.2% ± 1.7 respectively . This is somewhat in contrast to other findings. Lee and Karunanithy (1990) observed an increase in total crude protein content of more than 21% in *Glycine max*. But they measured crude protein in beans separate from that in ungerminated ones and after dehulling, drying at 100°C and grinding. Ndzondzi-Bokouango et al. (1989) found that flour from faba beans, germinated up till six days had a somewhat higher content of proteins and non protein nitrogen (dry matter basis) than that of non-germinated seeds.

Enzymatic hydrolysis

The effect of germination on enzymatic hydrolysis of protein in faba beans and soya beans was initially studied in an *in vitro* system using the original method of Babinszky et al. (1990). Results indicated that effects of antinutritional factors on protein digestibility remained undiscovered. The digestive enzymes pepsin, pancreatin (a mixture including trypsin and chymotrypsin) and amylase were apparently present in far too high concentrations. The high concentrations probably overruled the effects of germination (Savelkoul and Diekema, unpublished results).

By decreasing the concentration of pepsin, pancreatin and amylase the effect of germination became visible. Results of the enzymatic protein hydrolysis of faba beans during seven days of germination are shown in table 1. Germination increased protein hydrolysis and at day 5 a maximum increase in the protein hydrolysis (21.3 %) was reached. Rahma et al. (1987) observed an almost identical pattern but with a maximum at day 4 of germination. Germinating soya beans for only 12 hours resulted in a considerable increase in the protein hydrolysis (25.7%), but germination for more than 12 hours did not increase protein degradation any further (Table 1).

Table 1 *In vitro* enzymatic hydrolysis of the protein (%; mean ± S.D.) of flour from germinated faba beans and soya beans

Germination Period (days)	Faba beans	Soya beans
0	69.2^{c} ± 3.73	65.1^{b} ± 1.60
0.5	72.1^{bc} ± 1.03	81.8^{a} ± 3.40
1	77.4^{ab} ± 0.09	79.4^{a} ± 2.44
2	80.0^{a} ± 1.48	82.2^{a} ± 0.69
3	80.9^{a} ± 2.92	81.0^{a} ± 0.80
4	82.9^{a} ± 0.60	80.8^{a} ± 3.27
5	83.9^{a} ± 0.61	81.3^{a} ± 0.91
6	83.5^{a} ± 1.53	81.6^{a} ± 1.84
7	82.8^{a} ± 4.95	84.2^{a} ± 1.99

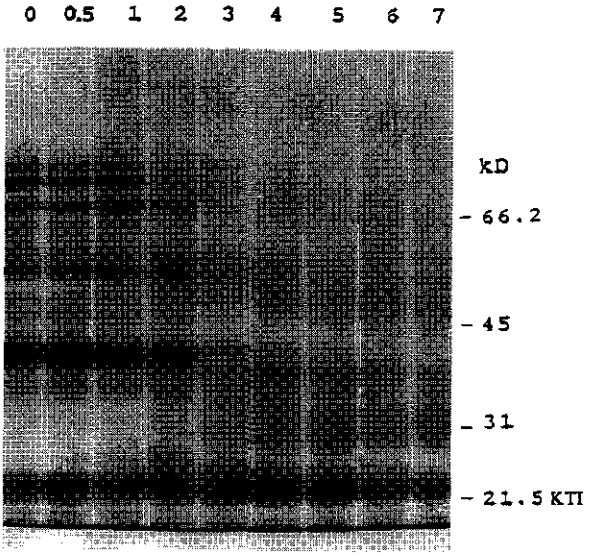
Means with different superscripts differ significantly ($P<0.05$).

The difference in rate of enzymatic hydrolysis by pepsin-pancreatin between faba and soya beans is probably caused by the faster water intake in soya beans through which protein cleavage in the soya beans is started earlier.

Protein pattern of faba and soya beans during germination

Although total crude protein content did not change during germination, protein pattern of the faba and soya bean flour changed considerably between day 2 and 3 of germination. During germination of the soya bean, protein bands with molecular weights between 97.4 and 31.5 kDalton, mainly ß-conglycinin- and glycinin-subunits, started to disappear between day 1 and 2 of germination (Sathe et al., 1987). Starting at day 2 of germination protein bands with molecular weights between 25 and 35 kDalton appear at the gel, as is shown in Figure 2.

Fig. 2. Electrophoretic profile of soya bean proteinpatterns during germination. Numbers on top refer to days of germination.

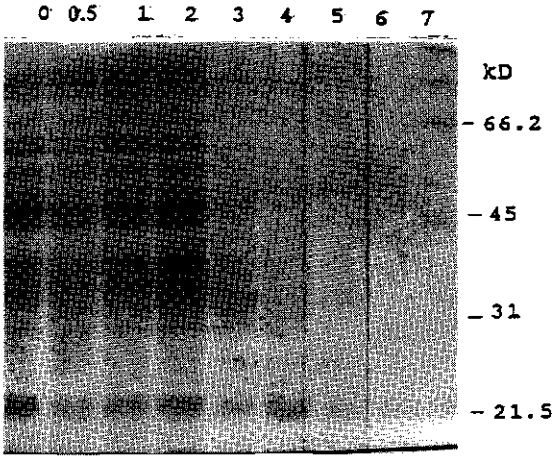


KTI = Kunitz trypsin inhibitor

This finding confirms results found by Wilson et al. (1988) who also investigated germinating soya beans.

Studying the protein pattern of flour from germinating faba beans we observed a very sharp change in protein pattern between day 2 and day 3 of germination. Protein bands with high and low molecular weights, mainly legumin- and vicillin-subunits, disappeared during this period as is shown in Figure 3 (Müntz et al., 1986).

Fig. 3. Electrophoretic profile of faba bean proteinpatterns during germination. Numbers on top refer to days of germination.



Rahma et al. (1987) observed also a disappearing of protein bands during germination of faba beans but on a more continuous basis. However they used a different variety (*Vicia faba*, var. Giza-2) than was used in our experiment (*Vicia faba*, var. Pistache).

Tannins in faba beans and trypsin inhibtors in soya beans

The content of tannins of ungerminated faba beans was 829 (± 13) catechine equivalents (mg catechine/ 100 g DM) but after seven days of germination it decreased by 29.7%., as was calculated with the Vanillin-H_2SO_4 method of Kuhla and Ebmeier [18]. Using the Pholin-Denis method Rahma et al. (1987) found that the reduction of the tannin content in faba bean flour (var. Giza 2) by more than 23% at day 4 reversed again during the following days to less than 9% after 6 days of germination. Bressani and Elias (1980) also found a decrease in tannins during germination of legume seeds. Our results showed that germination had almost no effect on the tannin content of the faba bean hulls which changed from 2.3% at day 0, to 2.4% at day 4 and to 2.0% at day 7.
The content of the trypsin inhibitor in the ungerminated soya beans was 20.4 (± 6) mg/g DM, but decreased by 25.5 % after seven days of germination as was detected by the TIA-method of Roozen and de Groot (1989). Tan-Wilson et al. (1988) investigated a Kunitz trypsin inhibitor in soya beans (var. Fiskeby) which was still detectable after 10 days of germination. Wilson et al. (1988) observed that the total amount of Kunitz trypsin inhibitor in soya beans (var. Amsoy 71) started to decline after 8 days of germination but was still present in the cotyledons after 14 days of germination.

Summary and conclusion

The large water absorption in soya beans shortly after germination, and not the degrading trypsin inhibitor activity, causes a maximum in enzymatic protein hydrolysis after 12 hours already. This would indicate that protein fragments (from germinated soya beans) are better digested than the whole protein, in its native form (in ungerminated soya beans).
Results suggest that the increasing susceptibility to proteolysis of protein by the pepsin-pancreatin enzyme-system in faba beans (*Vicia faba*, var. Pistache) during germination is not because of a reduced tannin content. Changes in the proteins of the faba beans themselves, forming complexes with the tannins which are than not measured anymore by the vanillin-H_2SO_4 method, could have led to artificial results.

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CHAPTER III

The degradation of lectins, phaseolin and trypsin inhibitors during germination of white kidney beans, Phaseolus vulgaris L.

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Abstract

White kidney beans (*Phaseolus vulgaris*), cv Processor, contain a relatively high content of phaseolin (storage protein), lectins, a special group of glycoproteins and a considerable amount of protein-type trypsin inhibitors. Protein digestion of raw 'Processor' beans in monogastrics, for example pigs, is disturbed by, the poorly digested, phaesolin, lectins, which can bind to carbohydrates in brush border membranes of the small intestinal epithelium, and trypsin inhibitors. The effect of germination of white kidney beans on lectins, phaseolin and trypsin inhibitors was studied in order to achieve a degradation of lectins, phaseolin and trypsin inhibitors and an increase of *in vitro* enzymatic hydrolysis of the protein of bean flour. Therefore, whole bean extracts were studied during a germination period up to seven days for their lectin pattern and phaseolin pattern, lectin content, binding capacities of functional lectins towards brush border membranes and trypsin inhibitor content. In addition the *in vitro* enzymatic hydrolysis by pepsin and pancreatin of the protein from flours of (un)germinated white kidney beans was studied. SDS-PAGE demonstrated a degradation of E-lectins and a disappearance of L-lectins and phaseolin during germination. Results indicated a decrease of the lectin content by 85%, a loss of binding capacities of functional lectins towards brush border membranes by 91 %, and a decrease of trypsin inhibitors by 76%, in bean flour after germination for seven days. A maximum in *in vitro* enzymatic hydrolysis of protein from bean flour was already established after germination for half a day.

Introduction

Legumes are commonly used as a protein source by all kind of animals (Wiseman and Cole, 1988). Many of these legume varieties contain in their raw state antinutritional factors which have a negative influence on the protein digestibility in monogastrics, including humans (Liener, 1989). Two of these antinutritional factors are the lectins and trypsin inhibitors. The (iso)lectins of *Phaseolus vulgaris* consist of five tetrameric subunits: E_4, E_3L_1, E_2L_2, E_1L_3 and L_4 which can be distinguished on the basis of their affinity to blood cells. E-subunits have a high affinity for erythrocytes while L-subunits have a high affinity for lymphocytes (Felsted et al., 1975; Goldstein and Poretz, 1986; Kik et al., 1989; Pusztai and Stewart, 1978; Pusztai and Watt, 1974; Roberts and Goldstein, 1984). Several trypsin/chymotrypsin inhibitors have been isolated from *Phaseolus vulgaris* beans with molecular weights in the range of 8,000-13,000 Daltons (Jacob and Pattabiraman, 1986; Tsukamoto et al., 1983; Wilson and Laskowski, 1972).

Technological methods like HTST-processes (High Temperature Short Time) are available to denaturate lectins and decrease trypsin inhibitors in kidney beans (Van der Poel, 1990; Van der Poel et al., 1990). HTST-processes are not only energy consuming, when not applied properly, they may, because of Maillard reactions, also result in a reduced protein digestion.

It has also been observed that germination has an eliminating effect on antinutritional factors and therefore a biological approach to inactivate lectins, phaseolin and to decrease trypsin inhibitors seems also to have potential (Savelkoul et al., 1992a; Savelkoul et al., 1990; Savelkoul et al., 1992b) The effect of germination on the protein pattern of amylase inhibitors, trypsin inhibitors and storage proteins in different legumes has been studied well, the protein pattern of lectins and the trypsin inhibitor activity for protein-type trypsin

inhibitors in white kidney beans during germination has not (Boylan and Sussex, 1987; Moreno et al., 1990; Müntz et al., 1985, Nielsen and Liener, 1984, Roozen and de Groot, 1992; Wilson et al., 1988). For futural *in vivo* feeding experiments with bioprocessed 'Processor' beans we decided first to study the effect of germination on changes in the protein pattern of lectins, lectin content, *in vitro* binding capacities of functional lectins and trypsin inhibitor content in white kidney beans.

Materials and methods

Extraction of bean flour for affinity chromatography

White kidney beans (*Phaseolus vulgaris* cv Processor), were germinated in wet sand, under standard conditions with 12 hours light per day at 20°C, for 0.5, 1,2,3,4,5,6 and 7 days (Van der Burg et al., 1983). After germination they were harvested, weighed, frozen in liquid nitrogen and ground in a Waring Blendor. The crude bean flour was lyophilized and milled (1 mm screen) in a Retsch-mill. Extraction was started by adding 10 g of bean flour to 100 ml 6.7 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl. This suspension was extracted overnight at 4°C by stirring and centrifuged at 39,000 g. The supernatant, referred to as the saline extract, was stored overnight at 4°C.

Lectin purification by affinity chromatography

Porcine thyroglobulin (Sigma Chem. Co.) (3 g) was coupled to cyanogen bromide activated sepharose 4B (Pharmacia) (15 g) in a coupling buffer of 0.1 M Na_2CO_3 (pH 8.0) containing 0.5 M NaCl, according to the method of Felsted *et al.* (1981). The coupled resin was added to the saline extract, mixed for 2 hours and centrifuged at 5650 x g for 5 minutes. The packed resin was suspended in 50 ml of 6.7 mM potassium phosphate (pH 7.4) containing 0.15 M NaCl. Of this suspension 25 ml was transferred to a column. First unbound material was washed away with 6.7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. This was followed by washing with a 1 mM potassium phosphate buffer (pH 7.2) in 1.0 M NaCl. The lectins were eluted with a 0.05 M glycine-HCl buffer (pH 3.0).

Bean flour extraction and estimated protein content for electrophoresis

White kidney bean flour was extracted with 0.5 M NaCl (1:10 w/v) at 4°C for 1 hour by stirring and centrifuged at 12,000 g according to the method of Chang and Harrold [2]. Protein pattern of the bean flours was then analyzed by SDS-PAGE, according to Laemmli [10]. The protein content of the beans for the use in SDS-PAGE was determined by using the BCA-method by Hill and Straka (1988).

Enzyme-linked immuno sorbent assay (ELISA)

The total amount of lectins in *Phaseolus vulgaris* was determined by the ELISA-method

of Hamer *et al.* (1989). In this method antibodies against lectins from white kidney beans (*Phaseolus vulgaris*) were raised in New Zealand white rabbits. Total IgG was isolated by affinity chromatography with purified protein G and used as anti-lectin antibodies (anti-lectin IgG). The anti-lectin antibodies were coated in microtitre plates and aspecific protein binding sites were blocked with serumalbumine. Extracts of bean flour were added to the microtitre plates and subsequently incubated with anti-lectin IgG-peroxidase antibodies. Finally the content of bound anti-lectin IgG peroxidase was quantified with a colour reaction catalysed by peroxidase.

Functional lectin immuno assay (FLIA)

A FLIA-BBM technique with a coating of porcine small intestinal brush bordermembranes (BBM) was used to detect binding capacities of functional lectins (Hamer et al., 1989). Extracts of bean flour were added to and subsequently incubated with BBM, thereby creating optimal conditions for functional lectins to bind to the carbohydrates in the brush border membranes. The content of the functional lectins was quantified further with anti-lectin IgG-peroxidase antibodies as described in the ELISA-technique above.

Trypsin inhibitor assay (TI)

Trypsin-sepharose 4B affinity chromatography was used to purify trypsin inhibitors from kidney bean flour according to the method of Roozen and de Groot (1992). Thereby bean flour samples were extracted (1:50 w/v) by stirring for two hours at room temperature in phosphate buffer containing 0.5 M NaCl (pH 8.0) and centrifugated for 15 minutes at 25,000 g. The supernatant was used for application on a trypsin-sepharose column. After washing with phosphate buffer, containing 0.5 M NaCl (pH 8.0) the column was eluated with, again, phosphate buffer, containing 0.5 M NaCl (pH 8.0) and 0.05 M acetate buffer, containing 0.5 M NaCl (pH 5.2). Trypsin inhibitors were eluted with glycine buffer, containing 0.5 M NaCl, pH 3.0.

Enzymatic hydrolysis

For *in vitro* enzymatic hydrolysis of protein from bean flour, samples from white kidney beans, germinated for 0, 0.5 an 1 to 7 days, were weighed to contain 200 mg protein and subsequently incubated for 15 minutes at 40 °C in a pepsin (0.1 mol/l HCl, pH 1.0; pepsin 0.25 g/l) and pancreatin (0.25 g/l in 0.165 M, phosphate buffer, pH 6.8) solution. Finally hydrolysed protein was calculated by the Kjeldahl method and expressed in percentages as described in Savelkoul *et al.* (1990).

Results and discussion

Protein pattern of Phaseolus vulgaris during germination

The protein content of the supernatant was analysed after extraction and centrifugation of white kidney bean flour. Each lane of the SDS-PAGE gel was loaded with 15 µl solution, containing 15 µg protein. Results of electrophoresis are shown in figure 1. A change in protein pattern appeared, between day one and day two of germination, and continued during the following days of germination. Thereby protein with high molecular weight disappeared while protein with a low molecular weight appeared, as also could be detected by Yomo and Srinivasan (1973). Three subunits of phaseolin with a molecular weight of 54,000, 49,000 and 46,000 on the SDS-PAGE gel disappeared during germination while components having a molecular weight in the range of 21,000 to 27,000 appeared. A simular change in protein pattern of phaseolin was found by Nielsen and Liener (1984) who germinated *Phaseolus vulgaris* (cv Improved Tendergreen) up to 10 days. On gel lectins are divided into E-lectins and L-lectins with molecular weights of 34,000 and 32,000 Daltons respectively. E- and L-lectins from red kidney beans (used as standards) and white kidney beans appeared to have the same molecular weight on gel. During germination E-lectins and L-lectins decreased as can be seen in Figure 1.

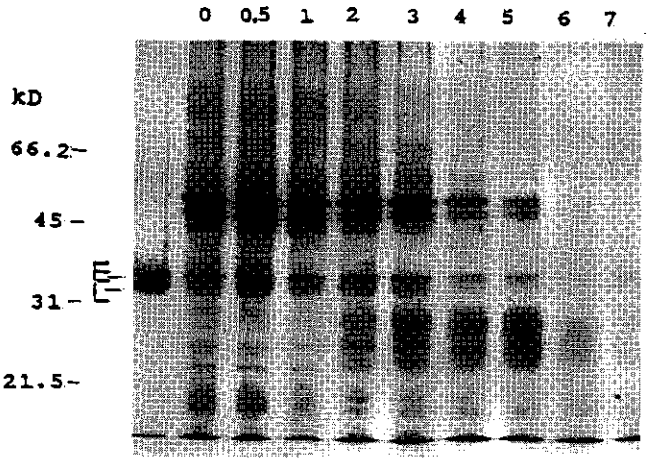

Fig. 1. Analysis of the protein pattern of (un)germinated white kidney beans (*Phaseolus vulgaris*) by SDS-PAGE. Purified E- and L- lectins from red kidney beans were used as standards (E,L). Extracts from bean flour samples represent the different proteinpattern of ungerminated (0), 0.5 day germinated (0.5), 1 day germinated (1), 2 days germinated (2), 3 days germinated (3), 4 days germinated (4), 5 days germinated (5), 6 days germinated (6) and 7 days germinated white kidney beans (7). Numbers in the left margin indicate approximate $M_r.10^3$.

The L-lectin protein band disappeared almost completely while the E-lectin protein band was still visible, after seven days of germination. The same phenomenon was detected by Yomo and Srinivasan (1973. These results contrasted with those reported by Boylan and Sussex (1987). They incubated lectins with a purified cysteine endopeptidase which cleaved phaseolin during germination of kidney beans (cv Taylor's Horticultural). In their experiments it appeared that E-lectins were degraded completely while L-lectins were not. E-lectins were first cleaved into two fragments (molecular weight 20000 and 15000 Dalton) and subsequently into fragments which were not recovered on the gel. This indicates that the purified cysteine endopeptidase was not identical with the enzyme(s) that cleaved E- and L-lectins during germination. Another explanation can be that certain substrate specificities are blocked during purification of the cysteine endopeptidase.

Lectin content and functional lectins in bean flour

Figure 2 shows the results of ELISA for total lectins.

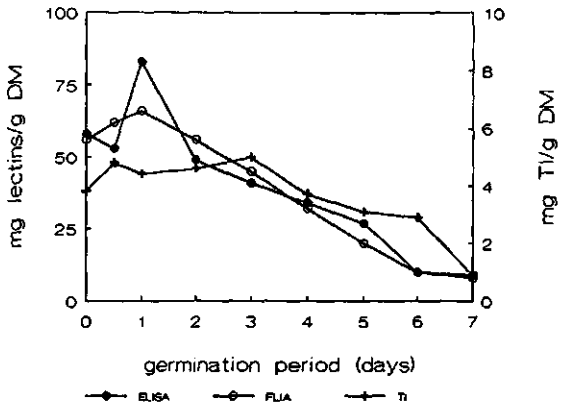

Fig. 2. ELISA-analysis of the total content of lectins, FLIA-BBM-analysis of the binding capacities of lectins and trypsin inhibitor activity (TIA), during germination of white kidney beans. The figure represents the amount of total lectins (ELISA) remaining after different germination periods, the decreasing binding capacity of functional lectins (FLIA) towards brush border membranes of the small intestine of pigs and the loss of trypsin inhibitor activity, in bean flour from (un)germinated white kidney beans.

Using ELISA to estimate the content of total lectins (E-/L) in germinated seeds, it appeared that the content of total lectin was decreased by 85% germination for seven days. However, from Figure 2 it can be seen that the content of total lectin did not decrease in a linear way. Initially it increased at day 1 of germination. Thereafter it decreased during the following days of germination with a greater decrease between day 5 and 6.
The ELISA-method does not differentiate between functional lectins which can bind to

brush border membranes and nonfunctional lectins which can not. In order to better simulate *in vivo* conditions a FLIA-experiment was executed. The results of the FLIA (Fig. 2) indicate that the functional lectins decrease during germination. Similar to the total lectins, the level of functional lectins also seem to increase during the first day of germination and their degradation is also gradual during germination.

Trypsin inhibitor content

The content of trypsin inhibitors in the ungerminated kidney beans was 3.8 mg/g DM. It first slightly increased till the third day of germination but than decreased by 76% after seven days of germination. The results are also shown in Figure 2.

Purification, electrophoretic analysis and content of lectins

The pattern of E- and L-lectins, isolated by affinity chromotography, was analysed by SDS-PAGE. The E- and L-lectins were eluted at the same time, although it seemed that they were differently cleaved during germination. After elution the purified lectins were first desalted on a PD-10 column (Pharmacia), containing Sephadex G-25 M (Fig. 3).

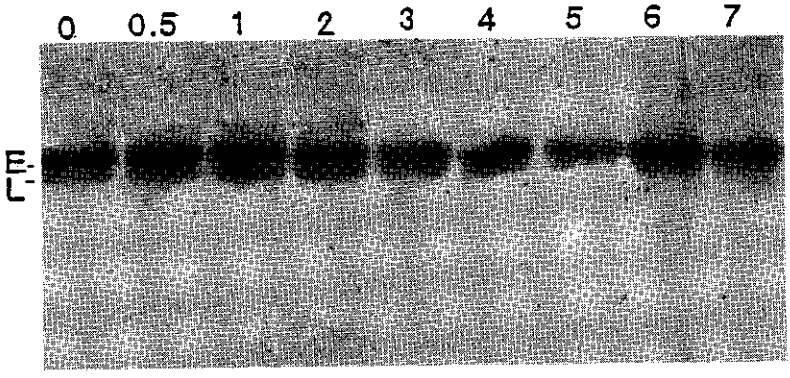

Fig. 3. SDS-PAGE of E- and L-lectins isolated from bean flour of (un)germinated white kidney beans (*Phaseolus vulgaris*). The figure represents the pattern of E-lectins (E) and L-lectins (L), isolated by affinity chromatography, of ungerminated (0), 0.5 day germinated (0.5), 1 day germinated (1), 2 days germinated (2), 3 days germinated (3), 4 days germinated (4), 5 days germinated (5), 6 days germinated (6) and 7 days germinated white kidney beans (7).

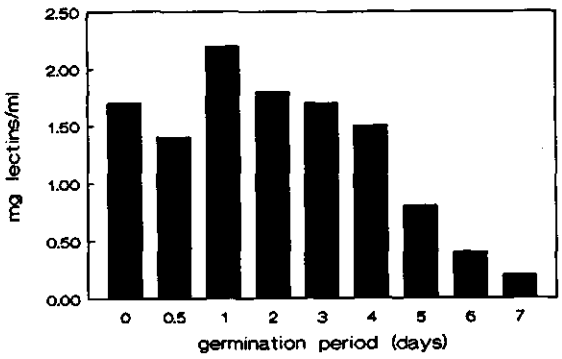

Fig. 4. Protein content of purified lectins from (un)germinated white kidney beans (*Phaseolus vulgaris*). The figure represents the amount of lectins from fractions containing the top of the peak of lectins of the elutionprofile.

Enzymatic hydrolyis

The enzymatic hydrolysis of the bean protein increased to a maximum after twelve hours of germination already. During the following days of germination no significant increase could be measured (Table 1). Reports in literature present different results of *in vitro* protein digestibility of *Phaseolus vulgaris* beans (Chang and Harrold, 1988; Nielsen and Liener, 1988). During the first 12 hours of germination the increase of weight by water intake was also considerable as can be seen in Table 1.

Table 1. *In vitro* enzymatic hydrolysis of the protein (IVEH in %; mean ± S.D.), crude protein (CP in %), dry matter (DM in%) of flour from germinated white kidney beans and weight of 1600 kidney beans during germination.

Germination period (days)	IVEH (%)	CP (%)	DM (%)	Weight (g)
0	67.2^{b} ±2.69	22.4	93.1	275.5
0.5	80.6^{a} ±2.54	22.0	92.2	413.4
1	79.8^{a} ±1.59	22.1	91.2	424.0
2	78.2^{a} ±4.06	22.7	90.6	484.8
3	75.8^{a} ±1.53	22.5	91.0	652.3
4	81.4^{a} ±4.05	23.5	89.2	1148.1
5	79.3^{a} ±5.19	23.9	89.1	1178.0
6	79.7^{a} ±1.60	26.7	88.9	1720.9
7	81.1^{a} ±0.22	25.6	88.9	1895.2

Means with different superscripts differ significantly ($P<0.05$)

Summary and conclusion

The initial increase in lectin content (ELISA), binding facilities of functional lectins (FLIA) and trypsin inhibitor content would suggest that germination starts with the release of some additional lectins and trypsin inhibitors, which are already present but cannot be detected. Trypsin inhibitors were not detected, probably because of their low molecular weights, from the protein pattern of flour from (un)germinated kidney beans on the SDS-PAGE gel which was used (Jacob and Pattabiraman, 1986).

Germinated beans were used in *in vitro* enzymatic hydrolysis experiments in order to investigate the possibility that digestive enzymes like pepsin and pancreatin can cleave not only protein in general but are also the very toxic lectin and the trypsin inhibitors (Savelkoul et al., 1990). The fact that the enzymatic protein hydrolysis reached a maximum after 12 hours of germination, cannot be caused by the hydrolysis of lectins and trypsin inhibitors. The content of lectins (ELISA) and the binding capacities of functional lectins towards brush border membranes (FLIA) increased till the first day and trypsin inhibitor content (TI) till the third day of germination. Probably water absorption in the beans shortly after germination could be an explanation for the increase in enzymatic protein hydrolysis. This effect was also observed in soya beans as previously described (Savelkoul et al., 1992b).

Our next aim will be the characterization of the enzyme(s), responsible for the cleaving of the E- and L-lectins, phaseolin and trypsin inhibitors during germination of white kidney beans.

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CHAPTER IV

Biological studies for degrading lectins and phaseolin from *Phaseolus vulgaris* L. I Effects of bean own proteases

F.H.M.G. Savelkoul, G.J. Kampert, B. Diekema and S. Tamminga

Abstract

Protease activity was measured in beans (*Phaseolus vulgaris*, var. Processor) germinated up to seven days. Between the third and the fourth day of germination protease activity increased considerably. From the fourth day on, lectins and phaseolin subunits were cleaved into smaller fragments, which could be observed from results obtained with SDS-PAGE. An enzyme extract was isolated from beans, germinated for four days. Purified phaseolin and purified lectins isolated from the same line of raw white kidney beans (*Phaseolus vulgaris* L, var. Processor), which cause a poor digestibility and antinutritional effects in nonruminants, respectively, were studied for their degradability against this enzyme-extract. After extraction and precipitation with ammonium-sulphate up to 70 % the enzyme-extract degraded purified phaseolin and purified lectins. When complete raw beans were used as a substrate, phaseolin and lectins were also degraded by the enzyme-extract. Results from FLIA-experiments (Functional Lectin ImmunoAssay) indicated that the amount of functional lectins, able to bind with brush border membranes from the pigs gut wall, was decreased by some 80% after whole raw beans had been incubated with the enzyme-extract.

Introduction

Studies on protein quality, protein digestibility and amino acid sequence of legume seeds in particular have been performed extensively during the past years. Such studies are executed because of their value as a tool for fundamental research and, in addition, to provide background information for applied research in animal nutrition (Wiseman and Cole, 1988). For example soya beans (*Glycine max*) have been studied thoroughly because of their high protein and lysine content, but also because they contain considerable amounts of Kunitz and Bowman-Birk type trypsin inhibitors (Wilson et al., 1988). Faba beans (*Vicia faba*), when compared with soya beans, have a somewhat lower protein content but can have a high content of condensed tannins (polyphenols). Because of their complexing properties with proteins, condensed tannins can also react as non-specific trypsin inhibitors and as amylase inhibitors (Deshpande et al., 1986). Compared with soya and faba beans, kidney beans (*Phaseolus vulgaris*), have a even lower protein content. In addition these beans can contain high amounts of lectins (glycoproteins) and phaseolin. The latter is a storage protein that is poorly digestible for nonruminants e.g. pigs. Moreover, certain varieties may contain considerable amounts of amylase inhibitors and trypsin inhibitors (Van der Poel, 1990; Wilson and Laskowski, 1973). The lectins from *Phaseolus vulgaris* (var. Processor) are very toxic for nonruminants, including humans (Huisman, 1990; Pusztai, 1980). It has been reported previously, that the elimination of lectins and trypsin inhibitors from *Phaseolus vulgaris* beans is no guarantee for a high digestibility of protein or lysine (Van der Poel *et al*, 1990). The possible level of inclusion of *Phaseolus vulgaris* beans in diets for monogastric animals therefore is limited.

Commonly applied methods for the inactivation of lectins and alteration of phaseolin are thermal treatments, methods by which these (glyco)proteins are denaturated by heat. Alternative methods were not available up till now. The use of proper conditions during thermal treatment is effective in reducing the negative effects of lectins but digestibility of bean proteins may still be poor. High temperature/short-term treatments (HTST) have proved to elevate the apparent ileal digestibility of bean protein and lysine in swine (Van

der Poel, 1991) but these treatments may have some negative effects on e.g. amino acid availability (Asp and Björck, 1989). Less agressive alternative methods which do not show such negative effects are not yet available or have not been developed yet. As phaseolin may play a significant role in the digestion of bean proteins, we decided therefore to investigate possibilities to degrade phaseolin and to eliminate lectins enzymatically. It is known that E- and L-lectins and phaseolin from beans are degraded during germination as could be determined with SDS-PAGE (Savelkoul, 1994). Most research has been focussed on the process of germination and the role of proteases in degrading phaseolin from kidney beans (Boylan and Sussex, 1987; Nielsen, 1988; Nielsen and Liener, 1984; Sgarbieri et al., 1982). However, the role of proteases responsible for the degradation of lectins during germination of beans has been studied to a limited extent only. Boylan and Sussex (1987), for example, purified an endopeptidase from kidney beans belonging to the group of cysteine-proteases. The protease degraded phaseolin during germination and also degraded purified lectins to some extent. Csoma and Polgar (1984) isolated a cysteine-protease from germinated *Phaseolus vulgaris* beans.
The objective of the present experiments was first to investigate germination conditions for the enzyme-extract to select maximum protease-activity. In addition the effect of an isolated partly purified enzyme-extract from germinated *Phaseolus vulgaris* beans (var. Processor) on the degradability of purified phaseolin, purified lectins and raw beans was studied.

Materials and methods

Plant materials

White kidney bean seeds (*Phaseolus vulgaris* L, var. Processor) were obtained from Bakker Brothers, Noord Scharwoude, The Netherlands. Seeds were germinated up to seven days in wet sand at 20°C under standard conditions as described by Van der Burg et al (1983). After germination, bean seeds were harvested, washed, lyophilized and milled to pass a 1 mm screen in a Retsch mill as described by Savelkoul et al (1992).

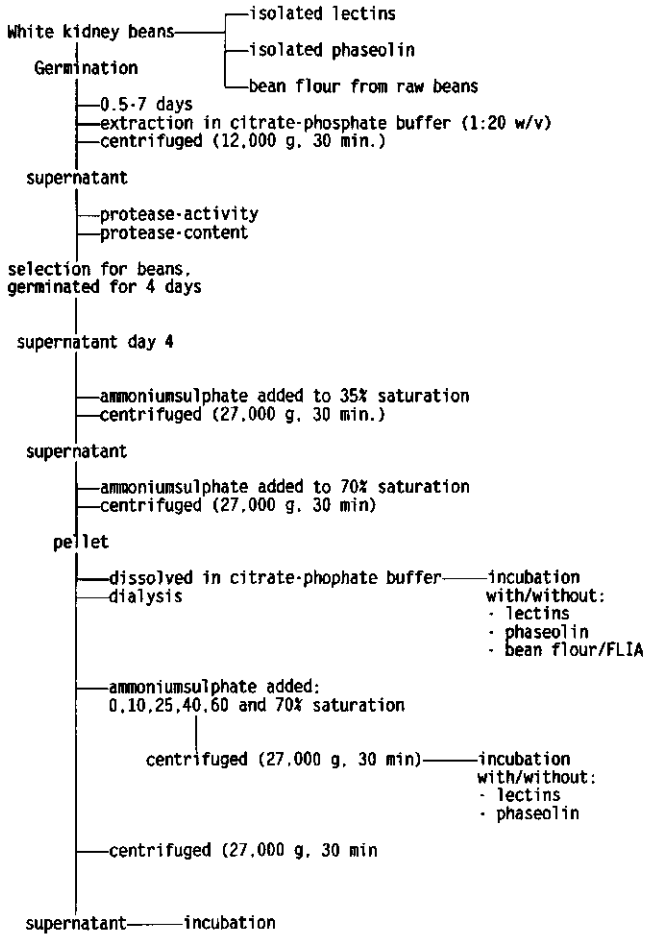
Flow chart of the isolation and incubation steps

The isolation and incubation steps for the isolation of the enzyme-extract are given in Fig 1.

Purification of lectins from raw beans

Bean flour (10 g) from raw beans was extracted overnight at 4°C in a 6.7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl (100 ml) and centrifuged at 39,000 g. From the supernatant lectins were purified by affinity chromatography for which porcine thyroglobulin (Sigma, St. Louis, USA) was coupled to cyanogen bromide activated sepharose 4B (Pharmacia, Uppsala, Sweden). The coupled resin was mixed with the supernatant for two hours and the resulting suspension was centrifuged at 5,650 g for five minutes. The supernatant was discarded, the remaining packed resin was transferred to a column (C 10/20, Pharmacia) and the lectins were eluted with 0.05 M glycine-HCl buffer (pH 3.0), containing 0.5 M NaCl, according to the method of Felsted et al (1975).

Fig. 1. Flow chart of the germination, isolation and incubation steps of the enzyme-extract.



Purification of phaseolin from raw beans

Bean flour (10 g) from raw beans was extracted (1:10 w/v) in a 0.5 M NaCl buffer containing 0.25 M ascorbic acid (pH 2.2) for one hour at room temperature and after that centrifuged at 23,000 g for 30 minutes at 4°C. Protein in the supernatant fraction was precipitated by dilution with five times its volume with water (Milli-Q Plus water quality Type 1) and centrifuged at 23,000 g for 30 minutes at 4°C, according to the method of Marquez and Lajolo (1981). The obtained pellet was redissolved in a 0.5 M NaCl solution.

Protease activity of bean flour extracts

Bean flour (1 g) from raw beans and from beans germinated for 0.5,1,2,3,4,5,6 and 7 days was extracted according to the method of Nielsen and Liener (1984) in 20 ml of a 0.025 M citrate/phosphate buffer (pH 5.7) containing 0.02% NaN_3 and 0.01 M 2-mercaptoethanol during one hour at 4°C. The extract was subsequently centrifuged at 40,000 g for 30 minutes at 4°C. The protease activity in the supernatant was measured according to the method of Twining (1984) in which 0.05 ml of a resorufin labeled casein substrate was dissolved (0.4% w/v) in H_2O (Milli-Q Plus water quality Type 1) and subsequently incubated for 30 minutes at 37°C with 0.1 ml supernatant and 0.05 ml 0.2 M Tris (pH 7.8), containing 0.02 M $CaCl_2$. The reaction was stopped with 0.48 ml 5% (w/v) TCA, after which the mixture was incubated for 10 minutes at 37°C and centrifuged at 10,000 g for 5 minutes at room temperature. Finally 0.4 ml of the supernatant was diluted with 0.6 ml 0.5 M Tris (pH 8.8), the solution mixed and the absorbance at 574 nm read against a blank at room temperature. Protein contents of the extracts were measured by a modified micro-Lowry method according to Peterson (1977).

Precipitation with ammoniumsulphate of bean flour extracts from beans germinated for four days

Bean flour (8 g) from beans germinated for four days was extracted in 160 ml of a 0.025 M citrate/phosphate buffer, containing 0.01 M DTT and 0.02% NaN_3, pH 5.6 for 1 hour at 4°C according to the method of Boylan and Sussex (1987). The extract was then centrifuged at 12,000 g for 30 minutes at 4°C and, under constant stirring over a period of 30 minutes at 4°C, ammoniumsulphate was slowly added to the supernatant (150 ml) to 35% saturation. The solution was stirred for an additional hour and then centrifuged at 27,000 g for 30 minutes at 4°C. The pellet was discarded and the supernatant was further saturated with ammoniumulfate to 70% saturation and which was subsequently centrifuged at 27,000 g for 30 minutes at 4°C. The supernatant was discarded and the pellet redissolved in 11.5 ml 0.025 M citrate/phosphate buffer (pH 5.6) containing 0.02% NaN_3 and 0.01 M DTT. From this solution 5 ml was dialysed overnight against a 0.025 M citrate/phosphate buffer containing 0.02% NaN_3 and the remaining 6.5 ml frozen. After dialysis 6.5 ml was obtained and frozen and stored at -20°C until further use.

Estimated protein content for electrophoresis

Protein content of bean flour extracts was determined by using the BCA-method (bicinchononic acid) according to Hill and Straka (1986). Protein patterns were then analyzed with SDS-PAGE, according to the method of Laemmli (1970). Protein standards (BIO-RAD Laboratories, Richmond, CA) comprising bovine serum albumin (66.2 kD), hen egg white ovalbumin (45 kD), bovine carbonic anhydrase (31 kD) and soybean trypsin inhibitor (21.5 kD) were electrophoresed under the same conditions as the bean flour extracts.

Incubation of bean flour extracts

Dialysed as well as undialysed bean flour extracts were incubated at 37°C before and after centrifugation. Samples were taken each hour during incubation. After centrifugation at 40,000 g for 30 minutes at 4°C, and calculation of the protein level of the supernatant, samples were subjected to electrophoresis.

Enzymatic hydrolysis of lectins, phaseolin and bean flour protein

Purified lectins (0.4 mg in 0.4 ml 0.025 M citrate/ phosphate buffer, containing 0.02% NaN_3 and 0.01 M DTT, pH 5.6) were incubated with 0.6 ml (un)dialysed enzym-extract. During incubation at 37 °C samples were taken after different time periods and centrifuged at 40,000 g for 30 minutes at 4°C.

Purified phaseolin (0.3 mg in 0.3 ml 0.5 M NaCl) was incubated with (un)dialysed enzyme-extract (0.2 ml). Incubated samples were treated in the same way as the purified lectins.

A mixture of purified lectins (0.2 mg in 0.2 ml 0.025 M citrate/ phosphate buffer, containing 0.02% NaN_3 and 0.01 M DTT, pH 5.6) and phaseolin (0.3 mg in 0.3 ml 0.5 M NaCl) were also incubated with (un)dialysed enzyme-extract. Incubated products were treated in the same way as the purified lectins.

Total bean flour protein (0.375 g) from ungerminated beans was also incubated with (un)dialysed enzyme-extract (15 ml). This enzyme-extract was obtained by the isolation of 80 g of bean flour, from beans germinated for four days, in 1.6 l buffer, according to the method of Boylan and Sussex (1987). During incubation at 37°C samples of 1 ml were taken after different time periods and treated in the same way as the purified lectins.

Ammoniumsulphate saturation and incubation of the dialysed sample

Ammoniumsulphate was added stepwise to 10 ml of a dialysed sample (0% ammoniumsulphate) which was obtained by the isolation of 80 g of bean flour, from beans germinated for four days, in 1.6 l buffer, mentioned above. At the stages of 0%, 10%, 25%, 40%, 60% and 70% ammoniumsulphate saturation, pH was measured and samples of 1 ml were taken. Purified lectins (0.1 mg in 0.1 ml 0.025 M citrate/phosphate buffer, containing 0.02% NaN_3 and 0.01 M DTT, pH 5.6) were incubated with 0.1 ml of the 1 ml samples (saturated with 0%-, 10%-, 25%-, 40%-, 60%- and 70% ammoniumsulphate) and incubated up to 24 hours. Thereafter the incubation products were centrifuged at 40,000 g for 30 minutes at 4°C and the supernatants subjected to electrophoresis.

Functional lectin immuno assay (FLIA)

In this method antibodies against lectins from white kidney beans (*Phaseolus vulgaris* L) were raised in New Zealand white rabbits. Total IgG was isolated by affinity chromatography with purified protein G and used as anti-lectin antibodies (anti-lectin IgG). A FLIA-BBM technique with a coating of porcine small intestinal brush border membranes (BBM) was used to detect binding capacities of functional lectins (Hamer *et al*, 1989).

Extracts of bean flour were added to and subsequently incubated with BBM, thereby creating optimal conditions for functional lectins to bind to the carbohydrates in the brush border membranes. Thereafter the whole complex was incubated with anti-lectin IgG-peroxidase antibodies. Finally the content of bound anti-lectin IgG peroxidase was quantified with a colour reaction catalysed by peroxidase.

Results

Protease activity and protein content of bean flour from germinated beans

Germination of kidney beans resulted in an increase in total protease-activity which started on the first day of germination, with a sharp increase at day four and a maximum at day five of germination whereafter it decreased (Fig 2). The total amount of extracted protein decreased during germination particularly during the first 12 hours and between day 3 and 4 of germination (Fig 2).

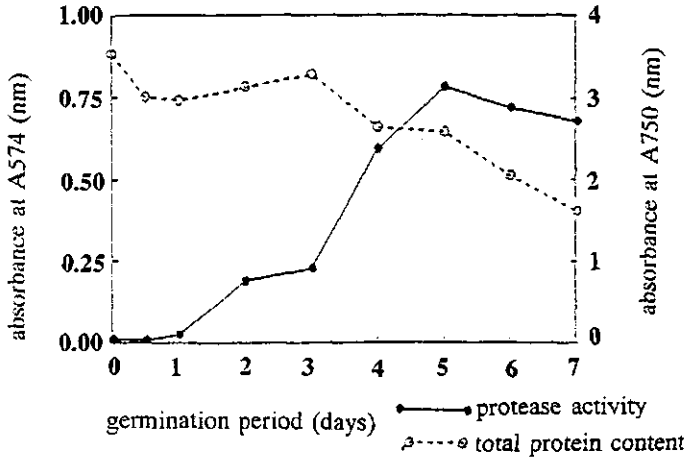


Fig 2. Changes of total protein content and protease activity in bean flour from 'Processor' beans, germinated up to seven days. Protein was extracted from 1 g bean flour in 20 ml 0.025 M citrate/phosphate buffer (pH 5.7) containing 0.02% NaN_3 and 0.01 M 2-mercaptoethanol.

In earlier research it was found that cleavage of lectins and phaseolin started between day 3 and 4 of germination (Savelkoul et al., 1994), it was therefore decided to isolate the enzyme-extract to be used in further studies from kidney beans, germinated for four days.

Incubation of flour from ungerminated beans with enzyme-extract (saturated with 70% ammoniumsulphate) and Functional Lectin Immuno-assay (FLIA)

Incubation of the flour from raw white kidney beans with the enzyme-extract resulted in degradation of phaseolin after 24 hours (Fig 3).

From the lectins however only the L-type subunits were degraded, shown by SDS-PAGE, during that period. The E-lectins were degraded only slightly. When the reaction-mixture of bean flour incubation was studied before and after 24 hours of incubation, it appeared that the amount of functional lectins was decreased by over 80% from 39.5 mg/ml to 7.6 mg functional lectins/ml reaction mixture.

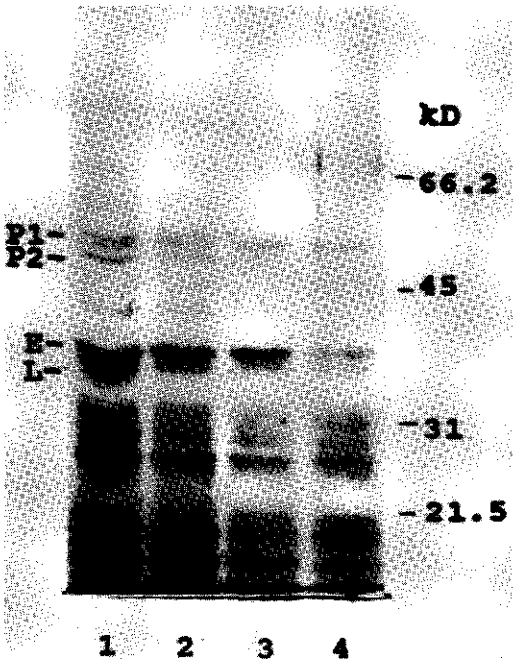


Fig 3. SDS-PAGE (15% polyacrylamde gel) analysis of the enzyme-extract during incubation, after extraction and ammoniumsulphate precipitation. Lane 1 shows the protein pattern of the enzyme-extract before incubation, lane 2,3 and 4 show the protein pattern of the enzyme-extract after 1, 4 and 8 hour(s), respectively of incubation (E = E-Lectin, L = L-Lectin, P1,P2 = Phaseolin).

Incubation of the enzyme-extract, after ammoniumsulphate precipitation (70% saturation) and dialysis

Incubation of the enzyme-extract, saturated with 70% ammoniumsulphate, during 8 hours resulted in a degradation of phaseolin and E- and L-lectins. Thereby L-lectins were degraded faster than E-lectins as can be seen in Fig 4. The previously saturated (70% ammoniumsulphate) enzyme-extract was also incubated after dialysis. During the incubation period of 8 hours it appeared that, in contrast with the saturated enzyme-extract, the E-lectins were cleaved more than the L-lectins. Phaseolin was also cleaved during the incubation period of 8 hours as is shown in Fig 5.

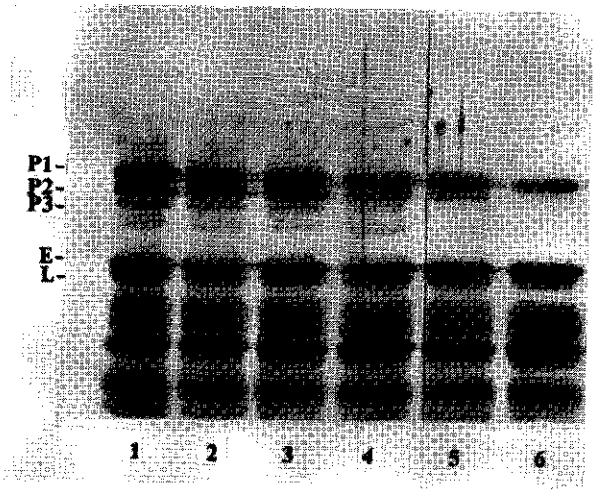


Fig 4. SDS-PAGE (15% polyacrylamide gel) analysis of the incubation of bean flour from raw beans with enzyme-extract, saturated with 70% ammoniumsulphate. Lane 1 shows the protein pattern bean flour before incubation, lane 2,3,4,5 and 6 after 1,2,4,6 and 24 hours of incubation (E = E-Lectin, L = L-Lectin, P1,P2,P3 = Phaseolin).

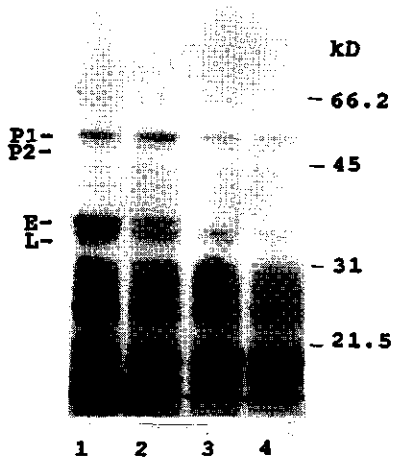


Fig 5. SDS-PAGE (15% polyacrylamide gel) analysis of the enzyme-extract during incubation, after extraction, ammoniumsulphate precipitation and dialysis. Lane 1 shows the protein pattern of the enzyme-extract before incubation, lane 2,3 and 4 show the protein pattern of the enzyme-extract after 1, 4 and 8 hour(s), respectively, of incubation (E = E-Lectin, L = L-Lectin, P1,P2 = Phaseolin).
Purified phaseolin and purified lectins were used as a substrate mix and incubated with the dialysed enzyme-extract. During an incubation period of 8 hours they were both degraded considerably as can be seen on the protein pattern of the gel in Fig 6.

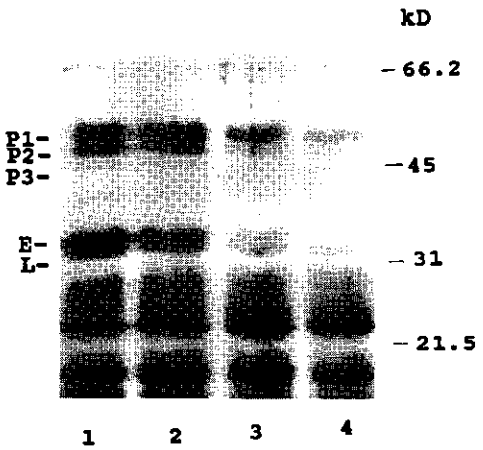


Fig 6. SDS-PAGE (15% polyacrylamide gel) analysis of a substrate- mix of purified lectins and phaseolin incubated with an enzyme-extract during, which has been extracted, precipitated with ammoniumsulphate and dialysed. Lane 1 shows the protein pattern of the enzyme-extract before incubation, lane 2,3 and 4 show the protein pattern of the enzyme-extract after 1, 4 and 8 hour(s), respectively, of incubation (E = E-Lectin, L = L-Lectin, P1,P2,P3 = Phaseolin).

Saturation of the dialysed enzyme-extract with ammoniumsulphate and incubation of the enzyme-extract

Saturation with ammoniumsulphate, up to 70%, resulted in a considerable pH change from pH 5.35 (0% saturation) to pH 4.95 (70 % saturation) as can be seen in Table 1.

Table 1. pH values at different concentration of ammoniumsulphate (AS) added to the dialysed enzyme-extract

AS-saturation (%)	0	10	25	40	50	60	70
pH	5.35	5.14	5.11	5.07	5.01	4.96	4.95

Incubation of the enzyme-extract, saturated with 0, 10, 25, 40, 60 and 70% ammoniumsulphate, resulted in a protein pattern which changed when the percentage of ammoniumsulphate was increased. When the dialysed enzym-extract was incubated, E-lectins were cleaved more than L-lectins. As the percentage of ammoniumsulphate increased to 25 %, E- and L-lectins were cleaved. In the range of 40% to 70% ammoniumsulphate saturation, L-lectins were better cleaved than E-lectins. At a percentage of 70% the amount of cleaved E-lectins, in contrast with L-lectins, was minor as can be be seen in Fig 7.

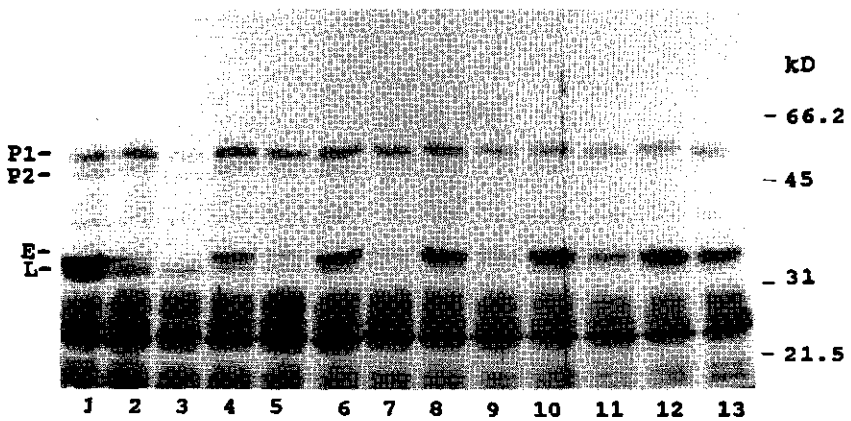


Fig 7. SDS-PAGE (15% polyacrylamide gel) analysis of the incubation of the dialysed enzyme-extract resaturated with different percentages of ammoniumsulphate (AS). Lane 1, 2 and 3 show the protein pattern of the enzyme-extract before incubation and after 4 and 24 hours of incubation respectively, lane 4 and 5 show the protein pattern of the enzyme-extract after 4 and 24 hours of incubation by 10% saturation, lane 6 and 7 after 25%, lane 8 and 9 after 40%, lane 10 and 11 after 60% and lane 12 and 13 after 70% saturation of AS (E = E-Lectin, L = L-Lectin, P1,P2 = Phaseolin).

Incubation of the enzyme-extract, after ammoniumsulphate precipitation, dialysis and centrifugation

After ammoniumsulphate precipitation and dialysis the enzyme-extract was centrifuged to obtain a clear supernatant. This supernatant was incubated for a period of 3 hours. During incubation the lectins were already cleaved after one hour of incubation but phaseolin was not cleaved any further (Fig 8).

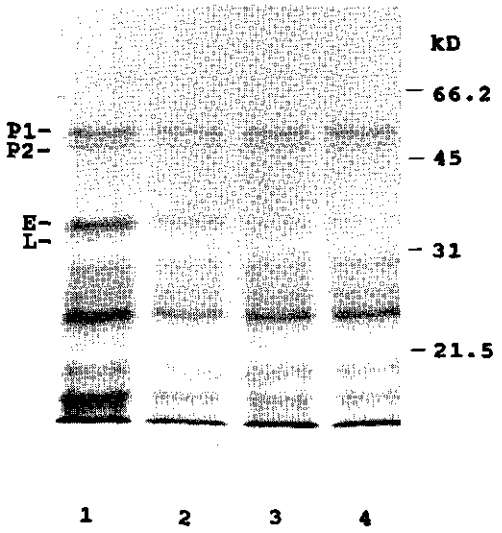


Fig 8. SDS-PAGE (15% polyacrylamide gel) analysis of the enzyme-extract during incubation, after extraction, ammoniumsulphate precipitation, dialysis and centrifugation. Lane 1 shows the protein pattern of the enzyme-extract before incubation, lane 2,3 and 4 show the protein pattern of the enzyme-extract after 1, 2 and 3 hour(s), respectively, of incubation (E = E-Lectin, L = L-Lectin, P1,P2 = Phaseolin).

The pellet, which was obtained after centrifugation, was mixed with the supernatant and incubated again. The results however were not different from the results of the previous supernatant incubation. Also the pellet was examined during incubation by mixing it first with 0.025 M citrate/phosphate buffer, containing 0.02% NaN_3 and 0.01 M DTT, pH 5.6. No breakdown of lectins, nor phaseolin could be observed, although there was still protease-activity present.

Discussion

In the first twelve hours during germination and furthermore from day three of germination the total amount of protein, extracted from the germinated beans, decreased considerably. In earlier research (Savelkoul et al., 1994) using the same batch, it was observed that protein hydrolysis increased considerable after twelve hours and after three days of germination when the incubation was executed with pepsin and pancreatin. Probably some fragments of proteins, which can be poorly hydrolysed in raw beans are cleaved during germination and than can be better hydrolysed. Boylan and Sussex (1987) observed a more gradual decrease of cotyledon protein content during germination but they used the dye-binding method of Bradford for measuring the protein content.

Results from germination experiments indicated an increase in protease-activity up to day 5 of germination. After that period protease-activity seems to decrease. In our experiments on purification of the enzyme-extract we used casein-resorufin as a substrate and 0.025 M citrate/phosphate buffer, containing 0.02% NaN_3 and 0.01 M DTT, pH 5.6 as enzyme-buffer. This buffer was used to isolate an enzyme-extract containing a maximum amount of cysteine-proteases. Casein-resorufin was used to have a nonspecific protein substrate to avoid the missing of specific cysteine-proteases. Our results are in line with those of Yomo and Srinivasan (1973) who concluded that the protein nitrogen level decreased till the eleventh day of germination and observed an increase of protease-activity till the seventh day of germination, followed by a decrease till the fourteenth day. Nielsen and Liener (1984) observed an increase of protease-activity till the nineth day of germination.

The purification of the enzyme-extract resulted in a loss of yield of 63% after precipitation with ammoniumsulphate with a concomitant increase of specific enzyme-activity of 50%. Boylan and Sussex (1987) and Csoma and Polgar (1984) also observed a similar considerable loss of yield after ammoniumsulphate precipitation.

Countrary to our results a cysteine-protease, which was isolated by Boylan and Sussex (1987), was able to cleave E-lectins completely instead of L-lectins which were not cleaved at all. Probably this discrepancy is caused by the absence of ammoniumsulphate, because the enzyme-extract can both break down E-lectins and L-lectins when 25% ammoniumsulphate in the medium is used. The resaturation experiments of the dialysed enzyme-extract indicated that the effect of ammoniumsulphate is reversible. The pH change is probably due to the saturation with ammoniumsulphate. Conformational changes of the lectins or enzyme-extract or both could be responsible for the changing cleavage pattern of the lectins.

Earlier research did show that germination had an effect on protein digestibility as could be measured with *in vitro* techniques (Liener and Thompson, 1980; Nielsen and Liener, 1984; Savelkoul et al., 1992). By *in vitro* experiments we wanted to examine the possible use of enzyme-extracts to degrade proteinaceous bean constituents. An enzyme-extract of 'Processor' beans germinated for four days contains degraded phaseolin- and lectin fragments. Incubation of this extract at 37°C resulted in a further breakdown of lectins and phaseolin. When using total bean flour as the substrate, phaseolin was degraded completely and lectins only partly. This would suggest that either the extracted enzyme was not yet sufficiently purified or that some factor inhibiting full degradation of lectins is present in the crude bean flour extract. Boylan and Sussex (1987) isolated an enzyme which was able to cleave purified phaseolin and E-lectins, but not L-lectins. Nielsen and Liener (1984) observed the proteolytic breakdown of phaseolin during germination. Proteolytic activity, responsible for this was also observed by Yomo and Srinivasan (1973).

When bean flour from raw white kidney beans is used as a substrate, as was done in our results, it appears that proteases cannot work with maximum efficiency, probably through denaturation during purification steps or inhibiting factors in the bean flour.

After centrifugation of the dialysed enzyme-extract lectins were cleaved within one hour by the enzyme-extract but phaseolin was not cleaved at all. Results of fast cleavage of phaseolin by enzymes or enzyme-extracts were reported earlier (Boylan and Sussex, 1987; Nielsen, 1988; Nielsen and Liener, 1984; Savelkoul et al., 1992). In the present experiment it was found that if the resulting pellet, obtained after centrifugation, was mixed with the supernatant again and incubated, no cleavage of lectins or phaseolin could be obtained. It may be possible that during centrifugation a co-factor is damaged or the enzyme(s) are modified.

The fact that some 80% of the functional lectins were eliminated by the bean flour incubation may have practical significance. This should be investigated further in experiments with animals to control the results obtained *in vitro* in terms of effecting the *in vivo* protein digestibility of the beans

From the results of the present experiment, it can be concluded that there seems to be scope for alternative bioprocesses to eliminate lectins and phaseolin such as the incubation of feed ingredients with enzymes. Although the use of isolated plant enzymes can be succesfull for eliminating lectins and degrading phaseolin from kidney beans, for isolating large quantities microbial enzymes are probably more practical.

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CHAPTER V

Biological studies for degrading lectins and phaseolin from *Phaseolus vulgaris* L. II Effects of bioprocessing

F.H.M.G. Savelkoul, T.W.J. Verwaaijen, M.G. Houdijk and B.A. Williams

Abstract

The antinutrional factors lectins and trypsin inhibitors and the poorly digestible storage protein phaseolin, from white kidney beans (*Phaseolus vulgaris* L.) were bioprocessed by incubating them with proteases and glycosidases from different origins. Proteases obtained from anaerobic microbes from sheep rumen were tested and appeared to degrade phaseolin and E-lectins in raw beans. Aerobic microbial proteases such as proteinase-K from Tritirachium album limber, animal enzymes; pyroglutamate aminopeptidase from calf-liver and the plant enzyme; papain, were also able to degrade these proteins partly. A N-glycosidase F from an aerobic micro-organism, Flavobacterium meningosepticum, was able to degrade purified lectins (glycoproteins) by deglycosylating the oligosaccharide component, containing mannose and N-acetyl-D-glucosamine.

Introduction

Feeding raw kidney beans (*Phaseolus vulgaris*, var. Processor) to monogastric animals results in a disturbed protein digestion, probably due to the presence of the very toxic lectins and the poorly digestible storage-protein, phaseolin in the beans (Van der Poel et al., 1991). Methods for eliminating lectin toxicity and increasing the digestibility of phaseolin involve usually application of physical processing techniques like HTST (High Temperature Short Time). One example of HTST-techniques is steam processing, used by Van der Poel et al. (1990) for studying the thermal inactivation of lectins and trypsin inhibitors, in which they also assessed the effects of these processes on protein quality. Recently biotechnological methods to decrease the toxicity of lectins and to improve the poor apparent digestibility of phaseolin for nonruminants (Savelkoul et al., 1992a; 1994) have been developed. In earlier investigations it was found that germination improved *in vitro* protein digestibility (Savelkoul et al., 1992b; Savelkoul et al., 1992c). Thereby faba beans (*Vicia faba*), soya beans (*Glycine max*) but also white kidney beans (*Phaseolus vulgaris*) were incubated with porcine pepsin in combination with porcine pancreatin to determine the protein digestibility. The increase in *in vitro* protein digestion due to germination was explained by the development of proteolytic enzymes capable of degrading both lectins and phaseolin (Savelkoul *et al.*, 1992b). When reviewing the degradation of bean proteins, especially phaseolin, Nielsen (1988) concluded that several enzymes including bean proteases, insect gut proteases and mammalian digestive proteases were able to break down phaseolin. In order to investigate further the possibilities of lectin- and phaseolin degradation in white kidney beans by bioprocesses, it was decided to test (an)aerobic micro-organisms, microbial enzymes, animal and plant enzymes.

Materials and methods

Plant materials

White kidney bean seeds (*Phaseolus vulgaris* L, var Processor) were obtained from Bakker Brothers, Noord Scharwoude, The Netherlands. Seeds were washed, lyophilized and milled at 1 mm in a Retsch mill and stored in plastic buckets.

Extraction of bean flour from ungerminated beans

Bean flour (1 g), from raw beans and beans germinated for 4 days, was extracted according to the method of Nielsen and Liener (1984) in 20 ml of a 0.025 M citrate/phosphate buffer (pH 5.7) containing 0.02% NaN_3 and 0.01 M 2-mercaptoethanol during one hour at 4°C. The extract was subsequently centrifuged at 40,000 g for 30 minutes at 4°C.

Protein assay

Protein content of kidney beans prior to their use in SDS-PAGE was determined with the bicinchoninic acid(BCA)-method of Hill and Straka (1986).

Electrophoresis

Protein patterns of extracts of kidney bean flour were analyzed according to the procedure of Laemmli (1970) with the sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE). A running gel, containing 15% acrylamide, and a stacking gel, containing 4% acrylamide were used. Electrophoresis was performed using a Biorad mini-protean II cell at 200 V.

Incubation of lectins with N-glycosidase F and O-glycosidase

Lectins (glycoproteins) from white kidney beans, purified according to the method of Felsted et al. (1975), were used for the incubation with N-glycosidase F (Boehringer mannheim, EC 3.2.2.18) which was isolated from culture filtrate of *Flavobacterium meningosepticum* (Plummer et al., 1984), and O-glycosidase (endo-a-N-acetylgalactosaminidase, Boehringer mannheim, EC 3.2.1.97) which was isolated from culture filtrate of Diplococcus pneumoniae (Unemoto et al., 1977). It was found by Felsted et al. (1981) that lectins from kidney beans contained maltose and N-acetyl-D-glucosamine as oligosaccharide components. N-glycosidase F, with a molecular weight of 35 kD, cleaves the N-glycan linkage of glycoproteins between asparagine and the carbohydrate chain. O-glycosidase with a moleculat weight of 160 kD cleaves O-glycans from glycopeptides/proteins. To start the reaction 10 µg of lectins in a 1% SDS-buffer were incubated with 2 µl N-glycosidase-F (1 mU) in 88 µl sodium phosphate buffer (20 mM, pH 7.2, containing 10 mM NaN_3, 50 mM EDTA and 0.5% v/v Triton X-100) at 37°C. Samples were taken after different periods of time. The reaction of O-glycosidase was started by incubating 10 µg (10µl) of purified lectins with 2 µl (1 mU) O-glycosidase in 100 ul 20 mM sodiumcacodylate buffer pH 6 for 15 minutes at 37°C. The reaction was stopped by the addition 100 µl of 0.8 M sodium borate buffer of pH 9.1 to the reaction mixture. Samples were taken after different periods of time.

Incubation of bean flour protein with N-glycosidase F

In order to observe a breakdown of lectins in the flour of ungerminated kidney beans by N-glycosidase F, 10 mg of bean flour was incubated with 100 µl N-glycosidase F in 5 ml sodium phosphate buffer (20 mM, pH 7.2, containing 10 mM NaN_3, 50 mM EDTA and 0.5% v/v Triton X-100) at 37°C. Samples were taken after different periods of time.

Incubation of bean flour with bacterial enzymes

Lectins and phaseolin (1 mg/ml), purified according to the method of Felsted et al. (1975) and Marquez and Lajolo (1981) respectively, were incubated with seven commercially available enzymes (100:1); endoproteinase glu-C (Boehringer mannheim, EC 3.4.21.19) from *Staphylococcus aureus V8*, leucine aminopeptidase (cytosol) (SIGMA, EC 3.4.11.1) from porcine kidney, papain (Sigma, EC 3.4.22.2) from *Carica papaya*, prolidase (Sigma, EC 3.4.13.9) from porcine kidney, proteinase-K (Boehringer mannheim, EC 3.4.21.14) from *Tritirachium album*, pyroglutamate aminopeptidase (Sigma, EC 3.4.19.3) from calf liver and thermolysine (Boehringer mannheim, EC 3.4.24.4) from *Bacillus thermoproteolyticus*.

For endoproteinase glu-C the incubation buffer contained 50 mM Tris-HCl, with 2 mg/ml SDS (pH 7.8). Leucine aminopeptidase had to be pre-incubated in 40 mM Tris-HCl containing 1.4 mM $MnCl_2$ (pH 8.5) as described by the manufacturer, before it could be used for incubation in the same buffer. For papain an incubation buffer was used which contained 0.01 M NaCl and 10 mM EDTA (pH 6.5). Prolidase was used in a medium containing 2.7 M $(NH_4)_2SO_4$, pH 8.0. The incubation buffer of proteinase-K contained 1 mM $CaCl_2$ in 50 mM Tris-HCl (pH 8.0) and that of pyroglutamate aminopeptidase contained 10 mM EDTA, 5 mM DTT and 5% glycerol (v/v) in 100 mM sodiumphosphate (pH 8.0) respectively. Thermolysine was incubated in a buffer of 50 mM Tris-HCl, containing 1 mM $CaCL_2$ (pH 8.0).

Basic medium for inocculation of the bacteria

The basic medium which was used contained a mineral solution and medium buffer (1:1). The mineral solution (100x concentrated) consisted of 200 g NH_4Cl, 10 g $(NH_4)_2SO_4$, 75 g $MgCl_26H_2O$ and 20 ml solution of trace elements. This latter solution was made per litre demineralized water by dissolving successively 50 g EDTA, 22 g $ZnSO_4.7H_2O$, 5.54 g $CaCl_2.2H_2O$, 4.99 g $FeSO_4.7H_2O$, 1.5 g $Na_2MaO_4.2H_2O$, 1.57 g $CuSO_4.5H_2O$, 1.61 g $CoCl_2.6H_2O$ and 5.06 g $MnCl_2.4H_2O$. After preparation each solution pH was brought to 6.8. The mediumbuffer (pH 7.0, 100 x concentrated) per litre mineralized water consisted of 155 g K_2PO_4 and 85 g $NaH_2PO_4.H_2O$.

Bean flour (10 g) from raw beans was extracted overnight at 4°C in a 6.7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl (100 ml) and centrifuged at 39,000 g. The supernatant (5 ml) was used as a substrate for the micro-organisms and added to the basic medium (50 ml) in a stoppered vessel. After adding micro-organisms the medium was incubated at 30°C for several hours in a shaker. Samples were taken after different periods of time.

Incubation of bean flour with pure strains of bacteria

Four different types of micro-organisms with proteolytic activities (*Bacilllus aureus*, *Bacillus subtilis*, *Aeromonas hydrophila* and *Candida tropicalis*) were used.
For the incubation with *Bacillus cereus* and *Bacillus subtilis* strain, 18 ml of basic medium was used to which 2 ml of substrate (bean flour extract) was added. The basic medium for *Aeromonas hydrophila* (subsp. proteolitica) was the same but now it contained 0.6 g of NaCl. Also a yeast strain, *Candida tropicalis*, was used for which 16 ml of the basic medium was used. To this basic medium also 2 ml of substrate (bean flour extract), and 2 ml of yeast extract was added. In a subsequent experiment with the same yeast strain 2 ml of basic medium was replaced by 2 ml of glucose (2 g/l).

Incubation of bean flour with mixed bacterial population

Two mixed bacterial populations were used, one obtained from the river Rhine and one obtained from the rumen of sheep.
A Rhinewater sample of 0.1 ml was added to the 18 ml basic medium with 2 ml of substrate (bean flour extract) and incubated for 2 days at 30°C. From this pre-accumulation a sample was taken and added again to the basic medium with the substrate. The solution was incubated at 30°C and samples were taken after different time periods.

Rumen fluid, obtained from sheep which had been fasted for 12 hours before collection time, were used as a source of microbial enzymes. The rumen fluid was first strained through a cheese-cloth according to the method of Theodorou et al. (1987). Both purified lectins (0.5 mg) and bean flour from ungerminated beans were incubated with 0.5 ml of the strained rumen fluid. During incubation at 37°C samples were taken after different periods of time and centrifuged at 4°C, for 10 minutes at 12,000 g in a Eppendorf-centrifuge. Thereafter the supernatants were subjected to electrophoresis (SDS-PAGE).

Results

Incubation of lectins with N-glycosidase F and O-glycosidase

The incubation of lectins with N-glycosidase F from *Flavobacterium meningosepticum* resulted in a deglycosylation of the oligosaccharide component in lectins who were denaturated before incubation. Thereby a decrease of the molecular weight of the E-lectin and L-lectin protein bands on SDS-PAGE-gel was observed (Figure 1). A complete disappearance of lectins was observed after 5 hours of incubation when native lectins were used as substrate (Figure 2). Before disappearing completely fragments of high molecular weight appear on SDS-PAGE gels.

Incubation of bean flour with N-glycosidase F

The incubation of bean flour from ungerminated kidney beans with N-glycosidase in order to stimulate a degradation of the lectins did not result in a breakdown of the lectins or a

cleavage of the oligosaccharide component.

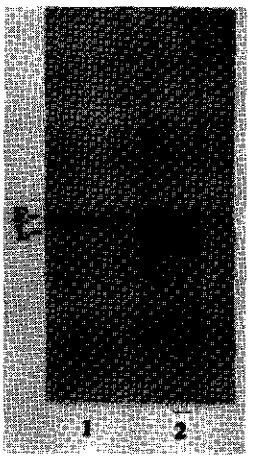

Fig. 1. SDS-PAGE (15% acrylamide) analysis of the protein pattern of denaturated lectins incubated with N glycosidase F. Lane 1 represents denaturated lectins before incubation. Lane 2 represents denaturated lectins after 24 hours of incubation with N-glycosidase F at 37°C (E = E-Lectin, L = L-Lectin).

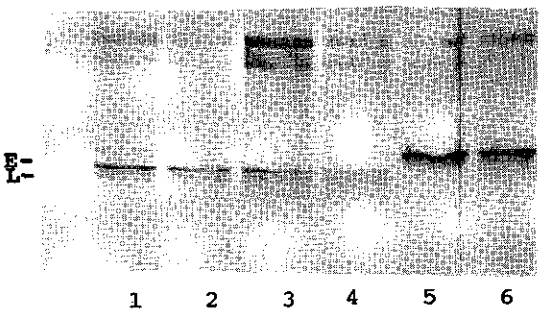

Fig. 2. SDS-PAGE (15% acrylamide) analysis of the protein pattern of incubated lectins incubated with N glycosidase F. Lane 1 represents native lectins before incubation. Lane 2 represents denaturated lectins after 1 hour of incubation with N-glycosidase F at 37°C. Lane 3, 4 5 and 6 represent the protein pattern after 2, 3, 4 and 5 hours of incubation (E = E-lectin, L = L-lectin).

Incubation of bean flour with bacterial enzymes

In subsequent research it was investigated wether commercially available proteolytic enzymes from different organisms could degrade lectins and phaseolin from raw white kidney beans. Incubation experiments showed that three enzymes degraded purified lectins: papain, proteinase-K and pyroglutamate aminopeptidase. Results from incubation experiments with flour from raw beans indicate that papain degrades both lectins and phaseolin. On the other hand proteinase-K degrades only phaseolin, moreover it was shown that pyroglutamate aminopeptidase did not degrade lectins nor phaseolin (Table 1).

Table 1. Effects of proteolytic enzymes and bacteria on the degradation of phaseolin and lectins

Enzymes	Phaseolin	Lectins		pH-optimum
		E	L	
Leucine aminopeptidase	-	-	-	8.5
Pepsin	-	-	-	1.0
Pancreatin	-	+/-	+/-	6.8
Pyroglutamate aminopeptidase	+/-	+/-	+/-	8.0
Enzyme-extract white kidney bean*	+++	+++	+++	5.7
Papain	++	+++	+++	6.5
Endoproteinase glu-C	-	-	-	7.8
Prolidase	-	-	-	8.0
Proteinase K	+	+/-	+/-	8.0
Thermolysine	-	-	-	8.0
Aeromonas hydrophyla	+	+	+	7
Bacillus cereus	-	-	-	7
Bacillus subtilus	++	++	++	7
Candida tropicalis	_	_	_	7
Rumen fluid	+++	+++	+++	6.8
Rhine water micro-oranisms	+++	+++	+++	7

* Data obtained from Savelkoul et al. (1992a)

Incubation of bean flour with bacteria

From the four tested strains of micro-organisms *Aeromonas hydrophila* and *Bacillus subtilis* grew well on bean flour extract from raw kidney beans as a substrate (Fig. 3). *Bacillus cereus* and *Candida tropicalis* were only partly able to use the substrate, with a minor growth compared to *Aeromonas hydrophila* and *Bacillus subtilis* (Table 1).

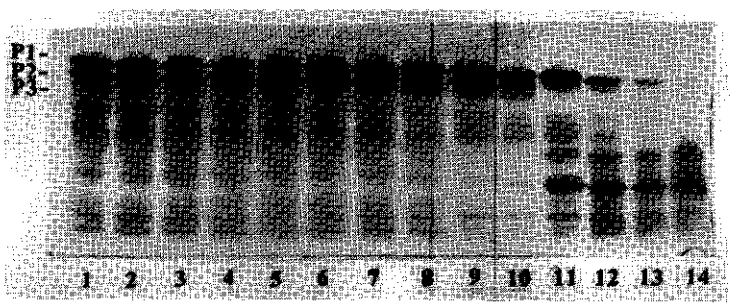

Fig. 3. SDS-PAGE (15% acrylamide) analysis of the protein pattern of bean flour from raw beans incubated with Aeromonas hydrophila, subspecies proteolytica. Lane 1 represents the protein pattern of the bean flour before incubation. Lane 2 after 10 minutes, Lane 3 after 45 minutes, Lane 4 after 75 minutes, Lane 5 after 2 hours, Lane 6 after 3 hours, Lane 7 after 21 hours, Lane 8 after 23 hours, Lane 9 after 25 hours, Lane 10 after 44 hours, Lane 11 after 50 hours, Lane 12 after 69, Lane 13 after 75 hours and Lane 14 after 141 hours of incubation (P1,P2,P3 = Phaseolin).

Incubation of bean flour with mixed bacterial populations

The complex of micro-organisms from the water sample from the river Rhine could use bean flour extract and did break down phaseolin and lectins fast (Fig. 4). Isolation of micro-organisms indicated the presence of a Bacillus subtilis strain (Savelkoul and Verwaaijen, unpublished results).

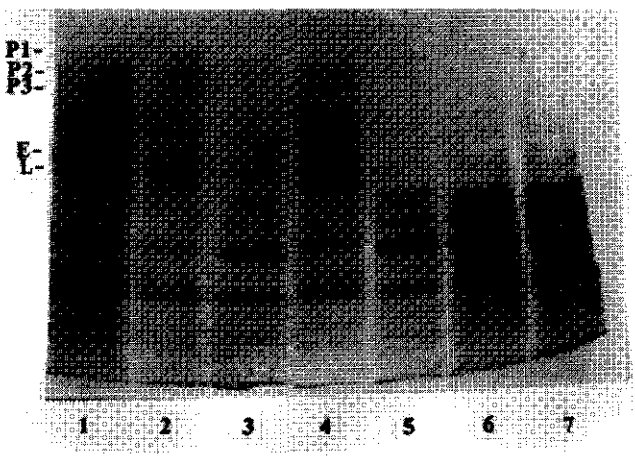

Fig. 4. SDS-PAGE (15% acrylamide) analysis of the protein pattern of bean flour from raw beans incubated with water sample of the river Rhine. Lane 1 represents the protein pattern from bean flour of raw beans before incubation. Lane 2 after 9 hours, Lane 3 after 12 hours, Lane 4 after 13 hours, Lane 5 after 14 hours, Lane 6 after 15 hours and Lane 7 after 16 hours of incubation (E = E-Lectin, L = L-Lectin, P1,P2,P3 = Phaseolin).

Incubation of flour from raw kidney beans with rumen fluid from sheep resulted in a total breakdown of phaseolin after six hours of incubation (Fig. 5).

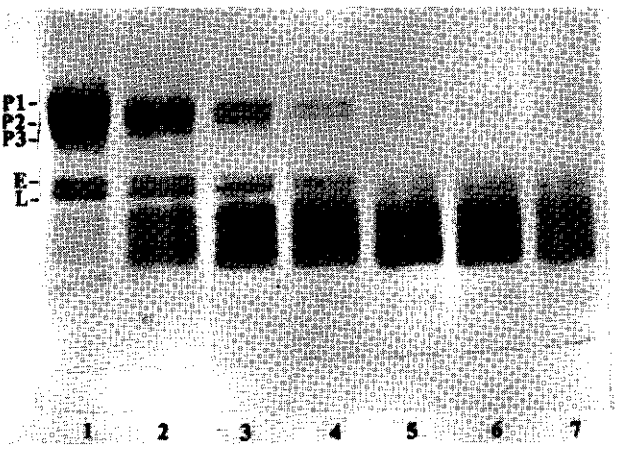

Fig. 5. SDS-PAGE (15% acrylamide) analysis of the protein pattern of bean flour during incubation of rumen fluid from sheeps. Lane 1 represents the protein pattern before incubation with rumen fluid. Lane 2,3,4,5,6 and 7 show the protein pattern after 1,2,3,4,5 and 6 hours of incubation respectively (E = E-Lectin, L = L-Lectin, P1,P2,P3 = Phaseolin).

Fragments of phaseolin were already visible after one hour of incubation. From lectins only E-lectins were degraded, L-lectins were still visible after 6 hours of incubation. At that time protein bands of phaseolin and E-lectins were not detectable anymore. But the cleavage of E-lectins did result in fragmentation products which were still detectable on SDS-PAGE-gel and blot (Savelkoul and Kampert, unpublished results).

Discussion and conclusions

The results in this paper show that bioprocesses have potential for the degradation of phaseolin and lectins from white kidney beans. Enzymes capable to degrade lectins and phaseolin seem to be widespread in nature. Proteinase-K from *Tritirachium album*, can degrade lectins and phaseolin completely or partly. But also enzymes from animals, such as pyroglutamate aminopeptidase from calf liver operate in the same way with regard to the degradation of lectins and phaseolin. The best results however were obtained with enzymes from plant origin, such as an enzyme-extract from germinated seeds and papain from Carica papaya and with mixed populations of bacteria from rumen fluid and the Rhine water micro-organisms. The latter is not too surprising because such mixed populations posses a wide variety of hydrolytic enzymes apparently with a broad spectrum of activities. A disadvantage of mixed populations of bacteria is that degradation is most likely not restricted to hydrolysis but that further degradation occurs. This limits there applicalibility in practice. Furthermore isolation and purification of most specific enzymes from such microbial sources is however possible.

Proteases seem to work better on lectins in bean flour than the glycosidases; N-glycosidase F and O-glycosidase, from which only N-glycosidase F could react on purified lectins. During the cleavage of lectins by N-glycosidase F, fragments of high molecular weight appear on SDS-PAGE gels. The change in protein structure, due to the loss of the oligosaccharide component, is probably the reason for these results. The deglycosylation proces for asparagine-linked glycans has been described also by Tarentino et al. (1985). The incubation of lectins with O-glycosidase did not result in a degradation of the lectins or a cleavage of the oligosaccharide component. N-glycosidase F was not able to break down lectins in the bean flour. Probably the presence of inhibiting factors like proteins or other sugars like raffinose, stachyose and verbascose, causing competitive inhibition, in the bean flour, have lead to this latter result.

Some plant proteases, like papain and the enzyme-extract which was isolated from germinated beans, were able to break down lectins and phaseolin simultaneously. It is interesting to know that papain is by far the most active commercially available proteolytic enzymes. Except that like the enzyme-extract from white kidney beans it is also of plant origin, it has a pH optimum of 6.5, close to that of the enzyme extract (5.7), while all the other commercial enzymes used have a much higher or much lower pH optimum.

A practical consequence of the finding that rumen microbes can but digestion enzymes like pepsin and pancreatin cannot degrade lectins is a difference in susceptibility towards lectins between ruminant animals like cattle and sheep and monogastric animals like pigs and poultry.

Before bioprocesses can be used as a mean to improve digestibility animal experimentation is necessary. Common digestive enzymes like pepsin and pancreatin did

not degrade lectins in (un)germinated kidney beans (Savelkoul et al. 1994). Studies of the protein/nutrient digestibility in nonruminants with bioprocessed kidney bean flour have to prove the results found in the above mentioned *in vitro* experiments. A practical application of bioprocessing as demonstrated here could be in the feed industry. However, considering the large quantities of enzymes needed, to break down phaseolin and lectins from kidney bean flour in feed industry, microbial enzymes seem to be the best alternative for upgrading white kidney bean protein in feed stuffs.

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CHAPTER VI

The effect of the duration of germination on the amino acid composition of legume seeds

F.H.M.G. Savelkoul, S. Tamminga, M.W.A. Verstegen and G. Post

Abstract

Seeds of the legumes *Glycine max*, *Phaseolus vulgaris* and *Vicia faba* were germinated during 12 hours up to 7 days. Germinated seeds were harvested after 0.5, 1, 2, 3, 4, 5, 6 and 7 days and their chemical composition, including amino acids, determined. After 7 days the proportion of amino acid N in total N decreased with 24, 20 and 7% for *Glycine max*, *Phaseolus vulgaris* and *Vicia faba* respectively. Reduction in essential amino acid N was more pronounced than in non-essential amino acid N, respectively 65 and 91% for *Glycine max*, 67 and 99% for *Phaseolus vulgaris* and 94 and 92% for *Vicia faba*. Within the essential amino acids the deviation from an assumed "ideal" amino acid profile increased when germination progressed. It is concluded that germination for more than 2 to 3 days is not a promising method of improving protein quality of legume seeds.

Introduction

Raw legume seeds often contain high levels of so-called Antinutritional Factors (ANF). ANF's are compounds with a negative influence on the digestion process of monogastric farm animals like pigs and poultry, usually resulting in a poor performance. Soy beans (*Glycine max.*) contain trypsin inhibitors, proteinous compounds which bind and inactivate the digestive enzyme trypsine, excreted by the pancreas (Birk, 1987, 1989). In addition to ANF's soya beans contain the storage proteins glycinin and ß-conglycinin, which have a poor digestibility. Raw white kidney beans (*Phaseolus vulgaris*) also contain components which can disturb protein digestion in monogastric farm animals. Two of these components are the antinutritional factors trypsin inhibitors (TI) and lectins (Pusztai, 1987; Liener, 1989). Lectins can cause damage to the intestinal wall (Kik et al., 1989), resulting in an impaired digestion. The third component is phaseolin, the main storage protein in raw white kidney beans. This storage protein is also poorly digested in monogastric animals (Liener and Thompson, 1980; Van der Poel 1990). In faba beans (*Vicia faba* var. Pistache), levels of lectins and trypsin inhibitors were relatively low compared to soya (*Glycine max.*) and white kidney beans (*Phaseolus vulgaris*, var. Processor). This was also found in earlier research (Savelkoul et al, 1992[4]). In certain varieties of faba beans significant amounts of tannins are present. They form complexes with protein, which may result in a negative effect on protein digestion (Jansman, 1993). The storage proteins in faba beans are legumin and vicillin. They are also poorly digestible. Apart from lectins, trypsin inhibitors, tannins and poorly digested storage protein, faba beans contain vicine and convicine. These compounds are linked with the occurence of favism in humans and their inclusion in animal diets significantly reduces biological value of protein (Griffith and Ramsey, 1993).

Lectins and trypsin inhibitors can be inactivated by means of process technology. With high temperature/short-term processes (HTST) it appeared possible to increase the apparent ileal digestibility of bean protein (Van der Poel, 1990), but sometimes this led to negative effects on amino acid availability (Asp and Björck, 1989). Studies were undertaken on alternative, and less agressive methods like enzymatic methods. These studies resulted in the conclusion that germination could be used as a model for the enzymatic approach aiming at the decrease of phaseolin and trypsin inhibitors and to eliminate lectins by means of the activity of enzymes. Research on the effect of germination on lectins, trypsin inhibitors and phaseolin showed a decrease in all three components (Savelkoul *et al*,

1992b). During germination of legume seeds the ANF's were largely disappearing. This was the reason to start research on possibilities to isolate and further characterize enzymes which are held responsible for the degradation and/or inactivation of ANF's (Savelkoul et al., 1992[c]).

Little is known on the effect of germination on amino acid profiles. During germination of seeds, proteins are initially hydrolyzed and the liberated amino acids used as precursors for the synthesis of enzymes which are required for the conversion of reserve substances to forms suitable to be transported. In later stages of development the purpose of hydrolysis is mainly to provide precursors for the onset of autotrophic growth (Savelkoul, 1992[a]). Germination is a magnitude of processes. Therefore the possibility exists that amino acids are used for other purposes than as precursors for protein synthesis. Consequently the amino acid profile of protein in germinating seeds, and as a consequence its biological value for monogastric farm animals, may change during germination.

The aim of this research was therefore to investigate the effect of germination on the amino acid composition in soy beans, kidney beans and faba beans.

Materials and methods

Plant materials

White kidney bean seeds (*Phaseolus vulgaris* L. var. Processor) were obtained from Bakker Brothers, Noord Scharwoude, faba beans (*Vicia faba*, var. Pistache) from Joordens Zaadhandel BV, Neer, and soy beans (*Glycine max.*) from Schouten-Giessen, Giessen.

Germination

Germination was done with a batch of approximately 2,5 kg of each variety of beans. Germination took place up to seven days in wet sand under standard conditions (12 hrs light/day and at 20°C) as described by Van der Burg et al. (1983). Before germination and after 0.5, 1, 2, 3, 4, 5, 6 and 7 days of germination, bean seeds were harvested. After harvesting the beans were washed, lyophilized and ground at 1 mm in a Retsch mill as described by Savelkoul et al. (1992[c]).

Chemical analysis

The lyophilized and ground samples were analysed for dry matter, organic matter, crude protein and crude fibre according to ISO procedures (ISO, 1979). Amino acids, except methionine and cystine, were determined after acid hydrolysis with 6N HCl for 22 hours at 100°C. Methionine and cystine were determined as methionine sulfone and cysteic acid after oxidation with performic acid. The oxidized samples were hydrolyzed in the same way as the samples that were not oxidized. The amino acid composition of the hydrolysate was determined using an automatic amino acid analyser (Biotronic LC 5001).

Results and discussion

After seven days of germination, crude fibre content had increased from 1.1 to 2.3 and ash

content from 6.8 to 7.7 suggesting the loss of some non fibrous organic material. This finding confirms observations made by Sathe et al., (1983) and Lee and Karunanithy (1990), who reported the loss of water soluble components such as water soluble carbohydrates during germination.

The results of the effects of germination on the amino acid composition are presented in tables 1, 2 and 3 for soy beans, kidney beans and faba beans respectively. Amino acid compositions of the seeds before germination are in good agreement with those reported in the Dutch Feed Table (CVB, 1991).

Table 1. Amino acid content (g/16 g N) in germinated soya beans

	Germination period (days)								
Amino acid	0	0.5	1	2	3	4	5	6	7
Cys[a]	1.45	1.40	1.45	1.41	1.36	1.39	1.03	1.12	0.74
Asp	12.1	11.5	12.4	12.7	16.4	18.9	22.3	27.7	28.0
Met[a]	1.48	1.35	1.40	1.40	1.17	1.32	0.52	0.98	0.89
Tre	4.28	4.02	4.01	3.73	3.86	3.59	2.80	3.23	2.84
Ser	5.81	5.66	5.83	5.49	5.47	5.07	4.04	4.21	3.47
Glu	20.0	19.1	18.8	18.5	17.9	14.2	10.7	9.6	8.2
Pro	5.09	5.07	5.43	4.96	4.79	4.01	3.94	3.11	2.69
Gly	4.35	4.29	4.36	3.99	3.89	3.59	2.94	2.79	2.84
Ala	4.23	4.31	4.39	4.24	4.20	3.94	3.28	3.56	3.15
Val	4.95	4.93	5.09	4.89	4.88	5.07	4.29	4.76	4.32
Ile	4.77	4.73	4.81	4.24	4.20	3.94	3.28	3.56	3.15
Leu	7.33	6.44	7.51	7.43	7.19	6.84	5.81	5.71	5.06
Tyr	3.25	3.16	3.34	3.27	3.16	2.57	2.42	2.39	2.24
Phe	4.77	4.88	5.08	4.77	4.59	4.15	3.81	4.16	3.80
Lys	6.13	6.02	6.09	5.88	5.18	4.74	3.94	4.14	3.62
His	2.31	14.8	2.39	2.18	2.53	2.34	2.30	2.77	2.54
Arg	7.33	7.01	7.41	6.49	5.93	5.66	4.31	4.16	3.39
AAN/TN	84.7	82.1	85.1	80.5	80.4	76.1	66.9	71.4	65.1
EAAN/TN	45.9	44.4	46.3	42.9	41.1	38.9	32.1	34.1	29.9
EAAN/AAN	54.1	54.0	54.5	53.3	51.2	51.1	48.0	47.7	45.9
SAAN/TN	1.9	1.8	1.9	1.8	1.7	1.8	1.0	1.4	1.1
MET/SAAN	45.3	43.9	43.9	44.6	41.1	43.5	29.1	41.5	49.4
LYS/AAN	8.7	8.8	8.6	8.8	7.7	7.5	7.1	6.9	6.7

AAN: N in amino acids;
TN: Total N;
EAAN: N in essential amino acids;
SAAN: N in S containing amino acids;
MET: Methionine;
LYS: Lysine.
[a] Results from performic acid-oxidised samples

The main part (60-90%) of proteins in legume seeds is present as globulins, with relatively smaller amounts present as albumins (0-20%) or glutelins (0-30%). In mature legume seeds little or no endosperm is found and storage protein is almost exclusively present in the cotyledones. On the basis of sedimentation characteristics, several different storage globulins can be distinguished. Globulins sedimenting as 11S or 7S molecular species, are widely distributed. Different globulin storage proteins have different amino acid compositions. For example the 11S legumin of *Phaseolus vulgaris* has a much higher amount of sulphur-containing amino acids than the 7S glycoprotein II, its major storage protein (Boulter & Derbyshire, 1976).

Table 2. Amino acid content (g/16 gN) in germinated kidney beans

	Germination period (days)								
Amino acid	0	0.5	1	2	3	4	5	6	7
Cys[a]	0.79	0.96	0.95	1.04	1.01	0.91	0.79	0.73	0.69
Asp	12.5	12.1	11.9	12.2	13.2	15.1	16.1	17.7	20.5
Met[a]	1.12	1.00	1.03	0.80	0.89	1.13	0.79	0.86	0.97
Tre	4.58	4.60	4.92	4.32	4.33	4.17	3.85	3.84	3.82
Ser	6.49	6.44	6.98	6.60	6.72	6.48	5.69	5.00	5.27
Glu	17.6	17.3	17.8	17.3	16.3	13.6	11.3	9.27	8.61
Pro	3.58	3.39	3.68	3.48	3.48	3.18	3.36	2.45	2.50
Gly	4.12	4.01	3.92	3.68	3.65	3.26	3.05	2.85	2.78
Ala	4.41	4.10	4.09	3.92	3.89	3.68	3.54	3.28	3.57
Val	5.95	5.23	5.58	5.32	5.47	5.46	5.05	5.10	5.27
Ile	5.20	4.60	4.79	4.56	4.46	4.81	4.37	4.50	4.51
Leu	8.32	7.86	8.22	7.72	7.94	7.66	7.13	7.02	7.01
Tyr	3.49	3.26	3.39	2.96	3.08	2.84	2.90	2.35	2.78
Phe	5.37	5.02	5.33	5.12	4.94	5.08	4.52	4.27	4.23
Lys	6.61	6.44	6.65	5.68	5.87	5.15	4.83	3.94	3.82
His	2.95	2.76	2.64	2.48	2.67	2.62	2.41	2.19	3.23
Arg	6.49	5.73	6.11	5.40	5.23	4.66	4.22	3.84	3.78
AAN/TN	84.6	79.9	82.6	77.1	77.7	74.1	69.1	64.7	68.4
EAAN/TN	48.0	44.3	46.3	41.8	42.2	40.2	36.9	34.4	36.2
EAAN/AAN	56.7	55.5	56.0	54.2	54.4	54.2	53.4	53.2	53.0
SAAN/TN	1.2	1.3	1.3	1.2	1.3	1.3	1.0	1.0	1.1
MET/SAAN	53.5	45.8	46.8	38.4	41.7	50.2	44.8	48.9	53.3
LYS/AAN	9.4	9.7	9.6	8.8	9.1	8.3	8.4	7.3	6.7

AAN: N in amino acids;
TN: Total N;
EAAN: N in essential amino acids;
SAAN: N in S containing amino acids;
MET: Methionine;
LYS: Lysine.
[a] Results from performic acid-oxidised samples

Total protein in soya bean seeds (*Glycine max.*) consists of 85% to 95% of globulins and for 5% to 15% of albumins. The main storage proteins in soya beans are called glycinin and ß-conglycinin, respectively. Earlier research on the effects of germination of soya beans (Savelkoul et al., 1992[c]), revealed that the enzymatic hydrolysis by digestive enzymes like pepsin and trypsin, reached its maximum value already after 12 hours of germination. Electrophoretic studies showed that protein bands with molecular weights of 97.4 and 31.5 kDalton, ß-conglycinin and glycinin subunits respectively, started to disappear between 1 and 2 days of germination. Conversion into smaller subunits of between 25 and 35 kDa appeared completed between 2 and 3 days.

Table 3. Amino acid content (g/16 g N) in germinated faba beans

	Germination period (days)								
Amino acid	0	0.5	1	2	3	4	5	6	7
Cys[a]	1.09	1.06	0.97	1.07	1.13	0.96	0.93	0.94	0.89
Asp	11.6	11.5	11.0	11.6	12.6	12.8	13.8	14.9	15.2
Met[a]	5.81	5.58	5.69	5.86	5.83	5.96	6.12	5.81	5.76
Tre	3.70	3.65	3.60	3.66	3.54	3.18	3.31	3.23	3.18
Ser	5.21	5.31	5.20	5.13	5.33	5.20	5.20	5.17	4.96
Glu	17.2	17.2	15.5	16.6	17.6	15.7	14.7	14.0	13.7
Pro	4.55	4.32	4.03	4.23	4.24	4.07	3.94	3.91	3.69
Gly	4.52	4.45	4.30	4.33	4.10	3.91	3.77	3.72	3.56
Ala	4.52	4.15	4.10	4.06	3.87	3.87	3.81	3.78	3.75
Val	5.25	4.81	4.90	4.70	4.87	4.90	4.83	4.81	4.83
Ile	4.69	4.42	4.26	4.40	4.50	4.40	4.24	4.17	4.07
Leu	8.09	7.54	7.59	7.60	7.58	7.41	7.28	7.30	7.28
Tyr	3.17	2.96	3.10	3.06	2.85	2.88	2.91	2.91	2.89
Phe	4.42	4.42	4.23	4.20	4.24	4.17	4.24	4.20	3.98
Lys	6.63	6.54	6.66	6.79	6.82	7.08	7.45	7.17	6.55
His	2.74	2.59	2.50	2.53	2.45	2.42	2.48	2.42	2.42
Arg	6.60	6.54	6.66	6.79	6.82	7.08	7.45	7.17	6.55
AAN/TN	81.4	79.4	77.5	79.2	80.1	78.9	79.8	78.8	76.0
EAAN/TN	45.2	43.7	43.9	44.3	44.3	44.6	45.8	44.7	42.5
EAAN/AAN	55.6	55.1	56.6	56.0	55.3	56.6	57.4	56.7	55.9
SAAN/TN	1.1	1.1	1.0	1.1	1.2	1.0	1.0	1.0	1.0
MET/SAAN	30.2	30.0	32.3	30.9	29.4	33.7	34.8	33.4	34.6
LYS/AAN	9.8	9.9	10.3	10.3	10.2	10.8	11.2	10.9	10.3

AAN: N in amino acids;
TN: Total N;
EAAN: N in essential amino acids;
SAAN: N in S containing amino acids;
MET: Methionine;
LYS: Lysine.
[a] Results from performic acid-oxidised samples

Storage proteins in *Phaseolus vulgaris* beans consist for 50 to 70% of the major storage protein phaseolin, a glycoprotein with three N-linked oligosaccharide side chains (Sturm et al, 1987). The compact molecular structure, the three dimensional structure and the presence of an oligosaccharide part are believed to be the main reasons for the high stability of native phaseolin and its resistance against digestive enzymes (Nielsen, 1982; Semino et al., 1985). Phaseolus beans showed maximum enzymatic hydrolysis after 4 days of germination (Savelkoul et al., 1992[c]), with the most dramatic increase during the first 12 hours. After 2 to 3 days of germination, protein bands with molecular weights of 45 and 66 kDa had disappeared. Subunits of between 20 and 30 kDa remained present till day 5. Similar observations were made in Great Northern Beans (*Phaseolus vulgaris* L.) by Sathe et al. (1988). Large proteins with molecular weights of 100, 60 and 35 kDa disappeared and were replaced by smaller subunits of 20-27 kDa.

The total bean seed protein of faba beans (*Vicia faba*) consists of albumins (approximately 15%), wich are metabolic proteins, and globulins (approximately 85%), the storage proteins (Müntz. et al., 1986). Comparable with glycinin an ß-conglycinin in soya, the globulin can be further divided into legumin (70%) and vicillin (30%). In faba beans a pattern slightly differing from that in soya beans was observed. Enzymatic hydrolysis by digestive enzymes reached its maximum after 3 days. Protein bands of legumin and vicilin subunits, with molecular weights of 45 and 97.4 kDa disappeared quite rapidly on days 3 and 4 (Savelkoul et al., 1992[c]).

The results in tables 1, 2 and 3 show that in all legume seeds, after germination for 7 days, part of the N in amino acids is lost. The losses amount to 24, 20 and 7% for soy beans, kidney beans and faba beans respectively. Major changes in AAN/TN started to occur between days 1 and 2. After 4 to 5 days of germination the sulphur containing amino acids methionine and cysteine started to become degraded more rapidly than average. After 7 days of germination soy beans and kidney beans showed losses in essential amino acid that were considerably greater than in non-essential amino acids. Within the essential amino acids, particularly the amounts of lysin and the sulphur containing amino acids methionin and cystine were decreased. Within the non-essential amino acids a sharp decrease was observed for glutamic acid, which was partly compensated by an increase in aspartic acid. Most changes started to occur after 2 to 3 days of germination.

Sathe *et al* (1983) studied the effect of germination on proteins, soluble amino acids and antinutritonal factors in Great Northen beans (*Phaseolus vulgaris L.*). Soluble amino acids, extractable with 0.1N HCl for 1 hr, increased considerably during germination, roughly in the opposite direction as the degradation in our experiment. During germination, a progressive decrease occurred in the major storage proteins, associated with a substantial change in soluble amino acids. After 5 days of germination soluble amino acids had increased 3.45 fold compared to the situation before germination had started. This would suggest that solubilisation precedes degradation.

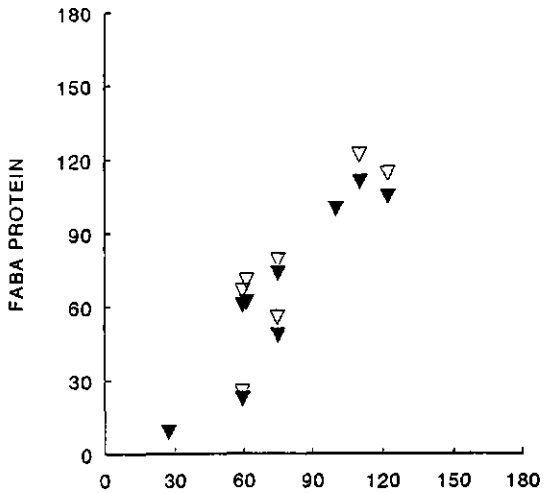
Chang and Harrold (1988) observed only minor differences in the content of amino acids (g amino acids per 100 g protein) as result of germination of pinto and pindak beans (*Phaseolus vulgaris*) in the dark at 25°C between paper towel up tp 9 days. The beans were daily harvested and lyophilized. Lee and Karunanithy (1990) also observed dramatic changes in chemical composition of phaseolus beans (*Phaseolus radiatus L* and *Phaseolus angularis L*) and soy beans (*Glycine max L* and *Glycine hispida L*), after germination. Fat and (crude) protein content went up by 20 to 25%, whereas ash content decreased with almost 50%. They explained the decreases as leakage of soluble carbohydrates and minerals

into the soak water. Sathe et al. (1983) also observed the loss of water soluble carbohydrates. Lee and Karunanithy (1990) found that together with the increase in (crude) protein, the amino acid content also increased, on average even more than the crude protein content. Expressed per 16 gN, total amino acid content ranged between 57 and 83%, which is considerably lower than the usually observed 95-100% in legume seeds (CVB, 1991). The data of Lee and Karunanithy (1990) were recalculated and individual amino acids expressed as percentage of total amino acids. Changes in amino acid pattern resembled more of less our observations, be it that the changes were less dramatic. Germination in the dark and the shorter period may be responsible for the latter.

In animal nutrition the real value of a plant protein is determined by the ease with which it can be digested and by its capacity to supply amino acids in a ratio close to what is needed for maintaining the animal or for the production of animal protein in meat, milk or eggs. The amino acids in a feed can thus be compared with the ratio in which they are ideally needed. In the concept of "ideal protein", the required amino acid composition is often expressed as the percentage in which an essential amino acid (Thr, Val, Meth+Cys, Ile, Leu, Phe+Tyr, Lys and Try) is needed in comparison with Lysine. For each 100 g of Lys, the amino acids Thr, Val, Meth+Cys, Ile, Leu, Phe+Tyr and Try are needed in quantities of 75, 75, 59, 61, 110, 122 and 19 g (Fuller et al., 1989).

The amino acid composition of soy beans, phaseolus beans and faba beans before and after germination was therefore compared with the "ideal" composition (Fuller et al., 1989). The results are shown in figures 1, 2 and 3.

Fig. 1. The effect of germination on protein quality in *Vicia faba* beans.



The results show that the amino acid composition of legune seeds before germination does deviate somewhat from the ideal composition, but not extremely. Germination does however considerably increase the deviation, particularly for soy protein and phaseolus protein. Most striking deviations from ideal were observed for Val (160%) and Phe (177%) in soy protein; for Val (185%), Phe (187%) and Ile (193%) in phaseolus protein and for Thr (65%) and Meth+Cys (38%) in faba protein.

Fig. 2. The effect of germination on protein quality in *Glycine max* beans.

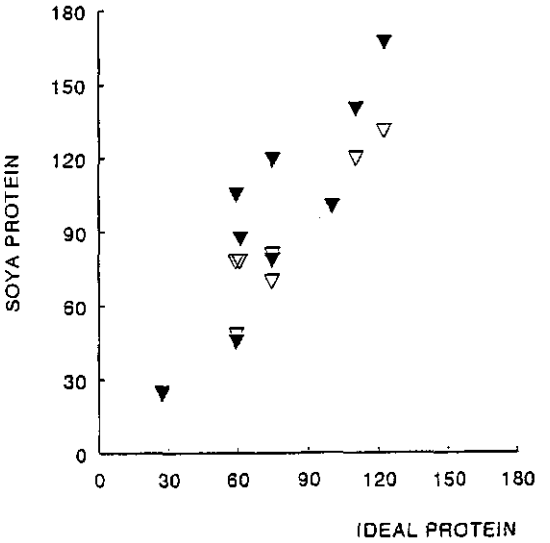
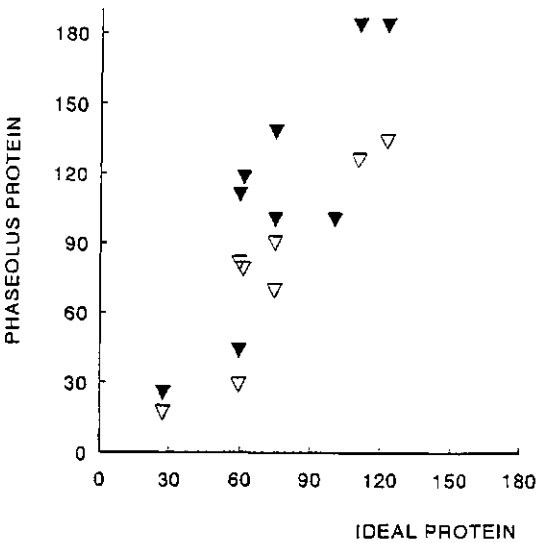


Fig. 3. The effect of germination on protein quality in *Phaseolus vulgaris* beans.



The results indicate a significant reduction in protein quality due to germination. Few results on the effect of germination of legume seeds on the nutritive value in terms of animal preformance are available. Contrary to what they observed after cooking, Savage and Thompson (1993) observed an increased true digestibility of protein from chick peas after germination, but no increase in biological value or net protein utilization (NPU).

From these and other results it can be concluded that germination of legume seeds starts initially with unfolding, making the subunits less resistant towards digestive enzymes (Savelkoul et al., 1992[c]). This is followed by a next stage in which larger subunits of between 50 and 100 kDa are degraded to smaller subunits of between 20 and 39 kDa (Sathe et al., (1983). In the next stage amino acid conversion and degradation takes place (Sathe et al., 1983). Independant of this ANF are inactivated (Savelkoul et al., 1992[b]; 1992[c]). To what extent all phases are completed depends on the germination conditions such as temperature, light or dark, length of the germination period and the presence or absence of anti-microbial agents.

Under the conditions chosen in this experiment it seems not advisable to germinate for longer than 2 or 3 days, without affecting the protein quality. This will not be long enough to eliminate ANF's so, other methods such as process technology or isolated enzymes are required additionally.

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Chapter VII

Nutritional evaluation of biological treated white kidney beans (*Phaseolus vulgaris L.*) in pigs - ileal protein and amino acid digestibility

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Abstract

The effect of feeding young growing pigs a semi-synthetic diet containing 7.5% beans, either germinated (diet GB), pancreatin treated (diet PTB), or untreated (raw beans; diet RB) on protein and amino acid digestibilities at the terminal ileum was studied. Nine castrated male pigs (13 kg liveweight) fitted with a poste-valve T-caecum cannula and two blood catheters were used. The ^{15}N-isotope dilution method was used to determine the amount of endogenous protein passing the terminal ileum and the true ileal protein digestibility. Ileal crude protein losses of 51.9, 27.4 and 51.1 g kg^{-1} dry matter intake (DMI) were found after feeding the RB,GB or PTB diet, respectively. The total amount of AA passing the terminal ileum were 48.6, 21.4 and 42.2 g kg^{-1} DMI for the diets RB, GB and PTB, respectively. The apparent ileal crude protein of the RB, GB and PTB diets were 74, 87 and 75% and for the AA digestibilities 76, 89 and 78%, respectively. The true ileal protein digestibility was 88, 93 and 93% for the RB, GB and PTB diet respectively. It can be concluded that germination of the white kidney beans clearly improves the digestion of the protein by a) decrease in the content of bean antinutritional factors and b) increase in the true ileal bean protein digestibility.

Introduction

Phaseolus vulgaris beans are a good source for protein, mineral and energy supply to monogastrics. Application of *Phaseolus vulgaris* in the diet of monogastric animals is limited. Due to a number of factors which affect protein digestibility and animal growth. According to van der Poel (1990a), reduced ileal protein digestibility with feeding raw *Phaseolus vulg.* depends on two main factors: a) the inherent protein resistance to proteolysis and b) interferences with various contained antinutritive factors (ANF's).

As described by van der Poel (1990b) several approaches may be considered to reduce and/or eliminate the ANF activity to improve the protein utilization of *Phaseolus vulgaris* in diets for monogastric animals. Beside removing the ANF's genetically, heat processing is an effective method for decreasing lectin and protease inhibitor activity (van der Poel, 1990b). Recent research on the effect of germination on ANF activity and the content of phaseolin, the main storage protein in *Phaseolus vulgaris*, showed important decreases in these components (Savelkoul *et al.*, 1994). Effects of germination of legume seeds on *in vitro* protein digestibility in nonruminants have been published only limited (Rahma *et al.* 1987; Mostafa *et al.* 1987) Investigations carried out by Savelkoul *et al.*, 1994, demonstrate improved in vitro protein digestibility of *Phaseolus vulgaris* after germination.

In the present study effects of germination on apparent ileal digestibility of nitrogen and amino acids *in vitro* in young growing pigs were investigated to elucidate the results found in vitro. It was also tested if pancreatine treatment of the bean flour can reduce the ANF activity and consequently improve the *in vivo* digestibility of bean nitrogen and amino acid.

Materials and methods

Animals

Eleven crossbred Dutch Landrace x Dutch Yorkshire castrated male pigs with an age of about 10 weeks and of mean (±SE) bodyweight 12.2 (±0.4) were used. The animals were fitted with a post-valve T-caecum cannula (PVTC cannula) as described by van Leeuwen *et al.* (1991) which allow a quantitative collection of ileal digesta. Two silicone catheters had been implanted previously, one into the external jugular vein and the other one into the arteria carotis to measure endogenous ^{15}N losses with the ^{15}N-isotope dilution technique. The animals were kept in individual metabolism crates at 24±2 °C. The humidity was kept at 50-70% as described in Schulze et al. (1994).

Diets

Four diets were formulated, a control diet (Diet D) containing no beans and three test diets containing 75g differently treated of Phaseolus vulgaris beans per kg diet. The composition of the basal diet is given in Table 1.

Table 1. Ingredient composition (g kg^{-1} air dry weight) of the basal diet

Ingredient	Composition
Casein	180
Maize starch	470
Dextrose	150
Wheat bran	100
Soya oil	25
Premix[1]	10
Minerals[2]	60
DL-methionine	1
L-cystine	2
L-threonine	1
Chromic oxide	1

[1] The vitamin/mineral mix provided the following per kilogram of feed: 9,000 IU of vitamin A; 1,800 IU of vitamin D_3; 40 mg of vitamin E; 1.36 mg of menadione as dimethyl-pyrimidinol bisulfite; 5 mg of riboflavin; 40 µg of cobalamine; 30 mg of niacin; 15 mg of d-pantothenic acid; 120 mg of choline chloride; 50 mg of ascorbic acid; 2 mg of thiamin; 3 mg of pyridoxine; .1 mg of d-biotin; 1 mg of folic acid; .38 mg of K (KI); .525 mg of Co ($CoSO_4$); .06 mg of Se (Na_2SeO_3); 80 mg of Fe ($FeSO_4$); 254 mg of Cu ($CuSO_4$); 44 mg of Mn (MnO_2); 72.8 mg of Zn ($ZnSO_4$); 40 mg of tylosin.

[2] Contributed the following per kg of feed: $CaCO_3$, 14.5 g, Monocalciumphosphate, 20.5 g; NaCl, 5 g; $KHCO_3$, 16.5 g; $NaHCO_3$, 2 g; MgO, 1.5 g.

The diets A, B and C were prepared by substituting 75g/kg of the basal diet for 75g/kg of one of the biologically treated of Phaseolus vulgaris.

Diet A: 92.5% basal diet + 7.5% raw beans
Diet B: 92.5% basal diet + 7.5% beans germinated for 7 days
Diet C: 92.5% basal diet + 7.5% pancreatine treated raw beans
Diet D: 100% basal diet

Chromic oxide was added to each of the diets as a digestibility marker at a level of 1g/kg.

Phaseolus vulgaris treatments

White kidney beans (*Phaseolus vulgaris*, var. processor) were provided by Fr. Bakker Brothers, Noord-Scharwoude, the Netherlands. The beans were subjected to three different treatments in the experimental diets: A) raw, B) germinated and C) treated by pancreatine.
Raw: The raw white kidney beans were ground (1mm mesh screen) before they were included in the experimental diet.
Germination: The seeds were germinated up to seven days in wet sand at 20◦C under standard conditions as described by Van der Burg *et al.* (1983). After germination bean seeds were harvested, washed, frozen in fluid N, lyophilized and ground through a 1mm mesh in a Retsch mill as described by Savelkoul *et al.* (1992a).
Pancreatine treatment: After grinding the seeds through a 1mm mesh screen in a Retsch mill the bean flour was incubated for 12 hours with 6% porcine pancreatin (Merck) in a aquaus solution at room temperature. Then the bean pulp was lyophilized and thereafter ground again in a Retsch mill (1mm mesh screen).
The chemical composition of the experimental diets and the levels of various ANF's of the differently treated beans and the basal diet are given in Table 2.

Experimental procedure

Nine crossbred castrated male pigs, 10 week old with an initial mean (± SE) liveweight of 12.2 (± 0.4) kg were used. They were fitted with a post-valve T-caecum (PVTC) cannula and two indwelling catheters, one into the external jugular vein (for taking blood samples) and the other one into the carotid artery (for the infusion of ^{15}N-Leucine solution). The pigs were individually housed in smooth-walled metabolism cages in a temperature controlled room (23 to 26°C; 50 to 70% relative humidity).

Throughout the 9 day experimental period, the pigs were fed 92.5% of a basal semi-synthetic diet with casein as the sole protein source, supplemented with 7.5% biologically treated *Phaseolus vulgaris* beans or raw beans. Biological treatment of the beans contained a) 7 day germination or b) 12 hours pancreatin incubation (6% pancreatin). The level of food intake given to the animals during the experiment provided about two times their maintenance requirement for energy. Chromic oxide (1 g kg^{-1}) was included in the diet as an indigestible marker. The diet was mixed with water (1:2, w/v) immediately prior to feeding and fresh water was available for 30 min after each meal.

^{15}N-leucine was continuously infused (5.04 mg kg^{-1} liveweight day^{-1}) during the 9 day experimental period. Ileal digesta were collected continuously for 12 hours on days 7,

8, and 9 of the experimental period.

Table 2. Dry matter, protein and amino acid content (g kg^{-1} air dry basis) of the various experimental diets.

	Basal diet supplemented with various treated beans[1]			Basal diet
	Untreated	Germinated	Pancreatin	
Dry matter	904.2	906.6	910.0	905.9
Protein	182.5	187.5	188.8	180.6
Indispensable Amino Acids, g kg^{-1} dry matter				
Arginine	8.2	8.1	7.5	7.3
Histidine	5.8	6.0	6.3	5.7
Isoleucine	10.7	10.8	11.0	10.6
Leucine	18.8	18.8	19.1	18.7
Lysine	15.4	15.0	15.2	15.3
Methionine	6.1	6.2	5.8	6.1
Cysteine	3.7	3.2	3.4	3.4
Phenylalanine	10.4	10.2	10.6	9.7
Tyrosine	10.0	9.9	9.9	9.7
Threonine	10.0	10.0	10.0	9.7
Valine	13.8	13.8	14.0	13.8
Dispensable Amino Acids, g kg^{-1} dry matter				
Alanine	7.3	7.1	7.3	6.7
Aspartic acid	16.2	18.0	16.0	14.8
Glutamic acid	44.4	43.5	44.0	44.7
Glycine	5.1	4.9	5.3	4.5
Proline	20.0	19.5	19.9	20.3
Serine	12.6	12.2	12.3	11.9

[1] *Phaseolus vulgaris var. Processor*

The digesta were immediately frozen at -20°C. Prior to chemical analyses the digesta were freeze-dried, ground (< 1-mm) and pooled per animal. Blood samples were taken three

times a day, at 09.00, 15.00, and 21.00, respectively. Immediately after sampling, the blood was centrifuged. The supernatant was pooled each day for each animal and stored at -20°C. Prior to chemical analysis, pooled plasma samples were further treated according to the procedure described by Schulze et al. (1994).

Total nitrogen (N) and dry matter (DM) were determined in the diet and freeze-dried ileal digesta following AOAC (1984) procedures. Chromium was determined in the diet and ileal digesta by the method of Bosch et al. (1989). Amino acids were determined in the diet and ileal digesta, with the exception of methionine and cystine after hydrolysis with 6 M HCl for 22 hours at 100°C according to the method of Slump (1969). Cystine and methionine were determined following oxidation with performic acid prior to acid hydrolysis (Moore, 1963). The various biologically treated beans and the basal diet were analyzed for the contents of total lectin using an ELISA-method (Hendriks et al., 1987) and the amount of functional lectin using a functional lectin immuno assay (FLIA) according to the principles of Hamer et al. (1989). The content of trypsin inhibitors (TIA) of the various treated beans and of the basal diet was determined according to the method described by Van Oort et al. (1989). The ^{15}N-enrichment in total N of ileal digesta, diet and TCA-soluble plasma was analyzed by mass spectrometry. All analyses were performed in duplicate.

Ileal digestibility of DM, crude protein (N x 6.25) and amino acids (AA) was calculated using the content of chromium in the diet relative to chromium in ileal digesta. The contribution of endogenous protein (N x 6.25) to total ileal protein was calculated from the ratio of ^{15}N-enrichment excess in ileal digesta total N to that in the TCA-soluble plasma according to the equation given by Schulze et al. (1994). The true ileal protein digestibility was calculated from the apparent ileal protein digestibilities and from the recovery of endogenous protein in ileal digesta.

Data analysis

Daily flow rates of DM, N, and AA at the terminal ileum and flow rates of amino acids (AA's) per animal relative to the ingestion of 1kg food dry matter were calculated according to the following equation (units are g/kg DM):

$$\text{Flow rates} = \text{nutrient concentration in ileal digesta} \times \frac{\text{diet chromium}}{\text{ileal chromium}}$$

The apparent digestibility coefficients (app.DC) were determined using the following equation (units are g/kg DM):

$$\text{app. DC (\%)} = \frac{\text{dietary nutrient - ileal nutrient flow}}{\text{dietary nutrient}} \times \frac{100}{1}$$

Nitrogen and amino acid digestibility coefficients of the different treated beans included in the experimental diets were calculated using the substitution method.

Based on the daily N and DM digestibility coefficients, the effects of collection day, animal within treatment and treatment were analysed by analysis of variance (SAS-GLM Procedure, SAS, 1990) according to the following model:

$$Y_{ij} = \mu + T_i + A_k xT_i + D_j + D_j xT_i + e_{ijk}$$

in which Y_{ij}=dependent variable; μ=overall mean; T_i=treatment (i=1,2,3,4); D_j=day of collection (j=1,2,3); $A_k(T_i)$=animal$_k$ in treatment$_i$ (k=1,2,3) and e_{ijk}=residual error. The effect of treatment was tested against animals within treatment. The day effect was tested against the residual error.
The treatment effect of the individual and total amino acid digestibility coefficients of the diets were analysed according to the following model:

$$Y_i = \mu + T_i + e_i$$

in which Y_i=dependent variable, μ=overall mean, T_i=treatment (i=1,2,3,4) and e_i=residual error.

When significant effects for both models were obtained, differences between the treatment means were compared by the Tukey's test (SAS, 1990).

Results

The pigs remained healthy. Animals readily consumed the experimental diets. Only in treatment PBT feed consumption was less. Feed rests of diet C were dried and weighed back. On the final day of the experiment the mean (±SE) bodyweight of the pigs was 13.6 (±0.8) kg. Adequate homogenous samples of ileal digesta were collected from all animals during the collection periods. For one animal on diet RB, in the second and third collection period, however not enough sample was collected. Further calculations of flow rates and digestibility coefficients were carried out using the first digesta collection of this animal.

The content of lectins and TIA in white kidney beans during germination (GB) with the 'Processor' variety of white kidney beans were high for lectins (total and functional) and TIA. Level decreased considerably after seven days of germination by 95%, 95% and 82%, respectively. However, incubating the beans with porcine pancreatine enzymes reduced the total amount of lectins (ELISA) and the amount of functional lectins (FLIA) and the TIA in the beans by only 8%, 17.5% and 50%, respectively. ANF level in the basal diet were very low TIA (Table 3). The ANF contents of the different diets were markedly influenced by the ANF contents of the different included treated beans.

The inclusion of 7.5% of the different treated beans increased the crude fibre contents of the diets RB, GB, PBT from 10.1% of diet C to 15.2%, 20.5% and 14.5%, respectively, the contents of nitrogen, crude fat and ash were only slightly influenced (Table 2).
The inclusion of raw and germinated beans in the diets reduced the passage of DM, protein, and AA at the terminal ileum compared to the diets supplemented with untreated- or pancreatin treated beans (Table 4). The mean ileal apparent AA and N digestibility coefficients for the pigs fed the different diets are shown in Table 5.

Table 3. Content of antinutritive factors in various biologically treated beans and in the basal diet.

	Various treated beans[1]			Basal diet
	Untreated	Germinated	Pancreatin	
Trypsin inhibitor activity[2]	8.49	1.52	4.26	0.17
Lectins (ELISA)[3]	116	5.70	107	n.d.[5]
Lectins (FLIA)[4]	74	3.70	61	n.d.

[1] *Phaseolus vulgaris var. Processor*
[2] Trypsin inhibitor activity in mg trypsin inhibited per gram of sample
[3] Total amount of lectins in mg per g sample
[4] Amount of functional lectins in mg per g sample
[5] Non detectable.

The mean ileal apparent digestibility of N and AA's were significantly decreased ($P<.05$) by adding raw and pancreatine treated beans compared with the basal diet. No significant different influences on mean ileal apparent N and AA digestibility were found when adding germinated beans related to the basal diet.

From the data of the diets (Table 2) and the content of dry matter, protein and amino acids (Table 4), the apparent ileal N and AA digestibility of the experimental diets were calculated (Table 5).

The mean apparent ileal AA digestibility showed higher coefficients than calculated for the mean apparent ileal N digestibility.
Apparent ileal digestibility of N and AA of the beans were very much increased when the beans were germinated.

The loss of endogenous protein is even higher when pancreatin treated beans are used in the diets when they are compared with diets in which raw beans are inclused. The diet containing pancreatin treated beans cause also the highest endogenic protein losses (Table 6). Also for the bean protein itself true ileal protein digestibility in pancreatin treated beans is higher than in germinated beans (Table 7).

Table 4. Mean content of dry matter, protein and amino acids (AA) (g kg^{-1} dry matter intake) at the terminal ileum in pigs fed different experimental diets.

	Basal diet supplemented with various treated beans[1]			Basal diet	
	Untreated	Germinate	Pancreatin		SEM
Number of pigs	3	3	3	2	
Dry matter	203.9[a]	149.2[ac]	209.9[a]	132.1[bc]	38.29
Protein	51.94[a]	27.38[b]	51.13[a]	27.88[b]	7.313
Total AA	48.56[a]	21.36[b]	42.17[ac]	22.17[bc]	8.510
Content of Indispensable AA					
Arginine	2.00[a]	0.90[b]	1.70[ac]	1.01[bc]	0.283
Histidine	1.35[a]	0.66[b]	1.37[a]	0.68[ab]	0.279
Isoleucine	2.38[a]	0.99[b]	2.04[a]	0.98[b]	0.383
Leucine	3.34[a]	1.44[b]	3.06[a]	1.48[b]	0.532
Lysine	2.88[a]	1.07[b]	2.04[ab]	1.11[ab]	0.733
Methionine	0.48[a]	0.25[b]	0.43[ac]	0.28[bc]	0.072
Cysteine	1.10[a]	0.55[b]	0.98[ab]	0.60[ab]	0.234
Phenylalanine	2.09[a]	0.85[b]	1.87[a]	0.88[b]	0.302
Tyrosine	1.61[a]	0.68[b]	1.44[a]	0.75[b]	0.247
Threonine	3.06[a]	1.54[b]	2.90[a]	1.70[ab]	0.583
Valine	2.96[a]	1.35[b]	2.70[a]	1.40[b]	0.477
Content of Dispensable AA					
Alanine	2.47[a]	1.26[b]	2.28[ac]	1.34[bc]	0.404
Aspartic acid	4.77[a]	2.01[b]	4.24[a]	2.06[b]	0.797
Glutamic acid	8.27[a]	3.44[b]	6.56[ab]	3.40[b]	1.621
Glycine	2.84[a]	1.47[b]	2.58[ab]	1.50[ab]	0.599
Proline	3.15[a]	1.29[a]	2.77[a]	1.34[a]	0.895
Serine	3.81[a]	1.62[a]	3.24[ac]	1.68[bc]	0.678

[1] *Phaseolus vulgaris var. Processor*

[a,b,c] Means in the same row followed by different letter differ at $P < 0.05$.

Table 5. Mean apparent ileal dry matter, protein and amino acid (AA) digestibility (%) in growing pigs fed different experimental diets.

	Basal diet supplemented with various treated beans[1]			Basal diet	
	Untreated	Germinate	Pancreatin		SEM
Number of pigs	3	3	3	2	
Dry matter	79.6^{a}	85.1^{ac}	79.0^{a}	86.8^{bc}	3.83
Protein	74.3^{a}	86.8^{b}	75.3^{a}	86.0^{b}	3.61
Total AA	75.5^{a}	88.6^{b}	78.2^{ac}	87.6^{bc}	4.32
Indispensable AA					
Arginine	75.6^{a}	88.8^{b}	77.3^{ac}	86.2^{bc}	3.56
Histidine	76.5^{a}	88.9^{b}	78.1^{ac}	88.1^{bc}	4.80
Isoleucine	77.8^{a}	90.9^{b}	81.5^{a}	90.8^{b}	3.54
Leucine	82.2^{a}	92.3^{b}	84.0^{a}	92.1^{b}	2.81
Lysine	81.3^{a}	92.8^{b}	86.6^{ab}	92.7^{ab}	4.75
Methionine	92.2^{a}	96.0^{b}	92.7^{ac}	95.5^{bc}	1.15
Cysteine	69.9^{a}	82.9^{a}	71.3^{a}	82.3^{a}	6.57
Phenylalanine	79.9^{a}	91.7^{b}	82.3^{a}	90.9^{b}	2.95
Tyrosine	83.8^{a}	93.2^{b}	85.5^{a}	92.3^{b}	2.48
Threonine	69.2^{a}	84.6^{bc}	71.0^{a}	82.5^{ac}	5.85
Valine	78.6^{a}	90.2^{b}	80.7^{a}	89.9^{b}	3.45
Dispensable AA					
Alanine	66.1^{ac}	82.2^{b}	68.6^{ac}	80.0^{bc}	5.56
Aspartic acid	70.5^{a}	88.8^{b}	73.6^{a}	86.1^{b}	4.96
Glutamic acid	81.4^{a}	92.1^{bc}	85.1^{ac}	92.4^{bc}	3.67
Glycine	44.1^{a}	69.7^{a}	51.1^{a}	66.9^{a}	11.75
Proline	84.3^{a}	93.4^{a}	86.1^{a}	93.4^{a}	4.48
Serine	69.8^{a}	86.8^{b}	73.7^{ac}	85.9^{bc}	5.39

[1] *Phaseolus vulgaris var. Processor*

[a,b,c] Means in the same row followed by different letter differ at $P < 0.05$.

Table 6. Mean true[1] protein digestibility and endogenous ileal protein losses in pigs fed different casein based experimental diets.

	Basal diet supplemented with various treated beans[2]			SEM
	Untreated	Germinated	Pancreatin	
True protein digestibility	88.1[a]	93.2[b]	93.4[b]	1.43
Endogenous protein				
g per kg DM intake	27.8[a]	13.3[b]	37.5[c]	4.34
% of total ileal protein	53.4[a]	48.4[a]	73.5[b]	4.54
g per 100 g protein intake	13.8[a]	6.4[b]	18.1[c]	2.13

[1] Directly determined real protein digestibility using the ^{15}N-isotope dilution method.
[2] *Phaseolus vulgaris var. Processor*
[a,b,c] Means in the same row followed by different letter differ at $P < 0.05$.

Table 7. The apparent and true ileal protein digestibilities of the various treated beans[1].

	Phaseolus vulgaris var. Processor		
	Untreated	Germinated	Pancreatin treated
Apparent	-52.6	93.1	-6.9
True	-19.5	53.4	57.6

[1] Mean apparent and true ileal protein digestibilities of the various treated bean were determined with the difference method using the mean digestibility values given in Table 4 and 5 and assuming a true ileal protein digestibility of the basal diet of 98%.

Discussion

Different legume sources will have different digestibility. Also the level of inclusion in the diet, the contents of ANF's, the methods of storage and processing will influence their digestibility (Gupta, 1987). Low digestibilities in Phaseolus vulgaris beans can be partly due to the resistance of native bean storage proteins toward hydrolysis by digestive proteolytic enzymes like pepsin and pancreatin (Liener & Thompson, 1980; Savelkoul et al., 1992a). Consequently, upgrading the digestibility of protein value of Phaseolus vulgaris beans by technological and biological treatments of the beans should result in almost complete degradation of lectins and phaseolin.
According to van der Poel et al. (1990b), the upgrading of beans by processing is possible by thermal treatments which are effective to decrease the levels of lectins (Antunes &

Sgarbieri, 1980) and the activity of protease inhibitors (Rackis et al., 1986). In addition, high-temperature steaming for a short time distinctly improved the ileal digestibility of N by conformational changes of the storage protein, which was not observed with (prolonged) steaming at 102°C (van der Poel et al, 1990a). Technological treatments however may have some drawbacks in terms of requirements of fuel, labour, maintenance of equipment and the possible hazard of overheating (Savelkoul et al., 1992b). Germination, an alternative method, considerably increased the sensitivity of the beans toward proteolysis. Differences in the amino acid pattern after germination appear to result mainly from degradation of phaseolin (Savelkoul et al., 1992a). Germination also decreased the ANF contents in the beans (Table 2). These results are in very good aggreement with a reported decrease of the lectin content by 85%, a loss of binding capacity of functional lectins towards brush border membranes by 91% and a decrease of TIA by 76%, in beans after germination of seven days, by Savelkoul et al. (1994). According to Savelkoul et al. (1992b) the reason for the decreased lectin contents seemed to be the development of enzymes capable of degrading lectins during germination.

Savelkoul et al. (1994) showed an improvement of the *in vitro* N digestibility when compared to raw beans. However, as discussed by van der Poel et al. (1991a) in vitro enzymatic procedures gives levels of N digestibility which differ from results *in vivo because* measurements do not include endogenous N losses. Therefore in vitro data can only be used as an indication and the effect of bean germination on the digestibility needs to be evaluated by an in vivo experiment.

The apparent ileal N and AA digestibility show a clear increase when the different treated beans were included in the diets. There are no significant differences ($P>.05$) between the diets B and D and the diets A and C. Results show that pancreatin only partly reduced TIA. Lectins were hardly effected. Germination for seven days however reduced both TIA and lectins to minimal levels also apparent digestibility rates of the beans were improved during germination. Romero & Ryan (1978) and Santoro et al. (1989) observed that adhesion of phaseolin to the gut wall, extended the exposure time of the protein to the gut enzymes, resulting in partially degradation of phaseolin and render it susceptible to further proteolysis by trypsin and chymotrypsin. This may be an explanation for our results for diet C. The negative apparent digestibility coefficients of the beans A and C may be related to a) poor digestion of the bean protein, b) an increased endogenous excretion and c) there may also be an effect of the included bean on the digestibility of the basal diet. Toasted beans with about the same contents of ANF's when included in a diet comparable to diet C of our experiment were used by van der Poel et al. (1991b). The very low apparent digestibility of N of bean C used in our experiment agrees very well with the results found by van der Poel et al. (1991b). The poor N and AA digestibility of raw beans (bean A) observed in our investigations seems to be relevant when compared to the results of the treated beans. This is supported by van der Poel et al. (1990c) who also observed a negative apparent faecal N digestibility of raw beans in pigs.

The decreased lectin content, loss of binding capacity of functional lectins towards brush border membranes, TIA and the assumed increase of sensitivity of the phaseolin toward proteolysis in beans after germination resulted in a substantial improvement of the apparent ileal N and AA digestibility. This result agrees data of apparent ileal N digestibility of short-term processed phaseolus vulgaris beans at higher temperatures given by van der Poel et al. (1991a).

Germination of *Phaseolus* beans resulted in a considerable loss of amino acids, particularly glutamic acid, lysine and the S-containing amino acids. This loss is still apparent when

Phaseolus beans were included in a pig diet at a rate of 75g/kg and providing so 10% of the total protein. The loss of amino acids is however more than compensated by the increased ileal protein digestion.

The results of the negative apparent ileal protein digestibility of pancreatin treated beans are in accordance with those found by Huisman (1992) and Van der Poel (1991b) who used however toasted beans in their experiments. The true ileal protein digestibilty in pigs from pancreatin treated beans is even higher than from germinated beans. An explanation for this could be that pancreatin contains trypsin and chymotrypsin which can bind to trypsin- and chymotrypsin inhibitors thereby blocking the inhibitor activity. Endogenic protein losses in pigs are higher when pancreatin treated beans are used instead of germinated beans. In this pancreatin treated beans lectins and phaseolin are still present and therefore partly responsible for the high losses of endogenous protein. The beans were pretreated with pancreatin (12 hours at room temperature), this could simulate a germinationperiod of 12 hours in which we observed an increased content of trypsin inhibitors (Savelkoul et al., 1994) but also an increased susceptibility for hydrolysis of protein (Savelkoul et al, 1992a). A higher trypsin inhibitor activity could have led to the excretion of more endogenous protein in the small intestine..

In conclusion, the results of the present investigation show that mainly trypsin inhibitors can be eleminated by pancreatin treatment. However, these biological treatment of the Phaseolus vulgaris bean provided no beneficial effect on apparent ileal N and AA digestibility. Germination of the beans resulted in an improvement of the apparent ileal N and of the AA digestibility most likely resulting from the degradation of lectins, trypsin inhibitors and phaseolin. Further research will have to indicate to what degree the different treatments influenced endogenous losses on the one and true digestibility on the other.

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GENERAL DISCUSSION

Introduction

Legume seeds can be important sources of protein in feeds for nonruminants. Raw seeds, from various legume species have limited use as such in the diet of nonruminants because of their poor apparent digestibility. Components in the raw seed, such as antinutritional factors (Liener, 1989) but also storage proteins, are responsible for this (Van der Poel, 1990). In the past several procedures to remove antinutritional factors in legume seeds have been developed. These procedures mostly concern technological processes. In recent years alternative approaches have been followed such as biotechnological processes through which antinutritional factors can be removed and/or storage proteins can be made more digestible. Methods have been explored and results are promising. One of these processes is germination of legumes during which storage proteins are hydrolysed and antinutritional factors are decreased both by the work of enzymes (Chapter I).

In this thesis germination was used as a model to study enzymatic degradation of antinutritional factors and storage proteins in legume seeds. Thereby both the enzymes and the substrate (antinutritional factor or storage protein) were studied under the most ideal conditions for the enzyme-substrate reaction, namely in the bean itself. Natural conditions are guaranteed during the process of germination. This in contrast to the situation when an isolated substrate, such as lectins, phaseolin or flour from raw beans, is incubated with the isolated enzyme-extract, proteases or glycosidases.

In the experiments three combinations were used for testing the effect of bean enzymes from germinating beans on the substrate; (a) germinated beans, (b) raw beans mixed with purified enzymes and (c) purified substrate mixed with purified enzymes. The advantage of using germinated beans is that reaction products are still present in the beans. Thereby the germination process can be prolonged to such a time that reaction products, for instance from proteinaceous antinutrional factors, have become harmless for animals. The germinated beans then contain "processed antinutrional factors" which no longer interfere with the animal's digestion process and decrease the feeding value of the germinated bean product. In fact the germination might even have increased the feeding value.

Common procedures

The use of technological processes like thermal treatments has proven to be very efficient for degrading lectins (Van der Poel, 1990), trypsin inhibitor activity (Rackis et al., 1986), and tannins (Bressani and Elias, 1988; Van der Poel et al., 1991). Some reports, showed the occurence of Maillard reactions during overheating or poor amino acid availability as a result of technological treatments (Asp and Björck, 1989).

Alternatives for technological processes as means for reducing antinutritional factors are by plant breeding and/or by enzyme technology. Up till now plant breeding is the field through which the best results have been achieved on a large scale. Decreases in lectins (Osborne and Bliss, 1985) and/or trypsin inhibitor activity (Hymowitz, 1986) have been reported. In practice, eliminating antinutritional factors through breeding may work out negatively because antinutritional factors play an important role as defense mechanism against predators (Ryan, 1973), weather conditions and diseases. This may be detrimental because

Bond and Smith (1989) reported that the bean yield can be negatively affected by reduction of antinutritional factors obtained by breeding.

Alternative but less destructive treatments for eliminating antinutritional factors and degrading storage proteins could be the application to the feed of enzymes derived from germinated legumes or microbes. From our own studies (Chapter III and IV) we concluded that antinutritional factors and storage proteins in legumes are degraded during germination by the work of enzymes.

Legume species, germination and antinutritional factors

It was decided to investigate the potential value of enzymes for the elimination of antinutritional factors and the improvement of the poor digestion of storage proteins firstly through the germination of legumes.

Three legume species were chosen for a series of experiments in which the biological degradation of different antinutritional factors during germination was studied (Chapter II and III). Faba beans (*Vicia faba*, var. Pistache) were selected because of their high content of condensed tannins. Soya beans (*Glycine max*) were used for their considerable amount of trypsin inhibitors and white kidney beans (*Phaseolus vulgaris*, var. Processor) for their high content of lectins (Chapter III). First results from experiments showed that lectins in white kidney beans (*Phaseolus vulgaris*), trypsin inhibitors in soya beans (*Glycine max*) and tannins in faba beans (*Vicia faba*) were degraded during germination (Chapter II and III). Tannin content in the hulls remained constant during germination, but digestibility of protein in the dehulled seeds was increased considerably in beans untill the fifth day and tannin content in the bean flour from beans germinated for seven days was also decreased. This indicates that the bean protein during germination changes in such a way that it decreases the tannin content after the milling procedure of the beans.

The first aim of the investigations presented in this thesis was to establish a model for the mode of action of the enzymes on antinutritional factors and storage proteins in legume seeds during germination. Therefore specific enzymes, antinutritional factors and storage proteins were isolated and purified. Purification and isolation of these products is a time consuming proces, also after each purification step a considerable part of the material was lost. This means that only a small amount of the products was obtained at the end (Chapter IV). For future animal feeding experiments higher amounts of the storage proteins, antinutritional factors and enzymes are needed. Microbes can easily be grown in large quantities and under controlled conditions. For these reasons research was started to produce large quantities of the desired enzymes from microbes (Chapter V). A number of microbial proteolytic enzymes were also screened for their potential value in modifying antinutritional factors and storage proteins in legume seeds. To obtain an ideal controlled substrate-digestion of antinutritional factors and storage protein by enzymes, germinated beans were used in a feeding experiment. Finally the effect of germination on the nutritive value of legume seeds in terms of amino acid composition and protein digestibility (*in vitro* and *in vivo*) was studied (Chapter VI and VII).

Proteolysis of (glyco)proteins during germination is carried out by proteases which can be distinguished as endopeptidases and exopeptidases. The endopeptidases are acting in the interior of the polypeptide chain and are responsible for the early stages of protein

breakdown. The exopeptidases only act at the terminal ends of the polypeptide chain and take over from the endopeptidases at an intermediate stage and then complete the release of free amino acids (Barrett, 1986).

Cysteine proteases act as endopeptidase and have been shown to hydrolyse the storage proteins of resting seeds (Ryan and Walker-Simmons, 1981; Mikkonen, 1990). When leupeptin, a cysteine protease inhibitor, was added to the endosperm of the castor bean, germination and seedling development was considerably inhibited (Alpi and Beevers, 1981). Cysteine endopeptidase are also responsible for the breakdown of glycinin and Kunitz trypsin inhibitor in soya beans and phaseolin and lectins in *Phaseolus vulgaris* beans were also cleaved by a cystein endopeptidase (Boylan and Sussex, 1987; Nielsen and Liener, 1984) which confirm our results (Chapter IV). In mung beans (*Vigna radiata*) the Bowman-Birk inhibitor seems not to be cleaved by cystein protease but by protease F, probably a serine protease (Wilson and Tan-Wilson, 1987).

In vitro protein hydrolysis of faba, soya and white kidney beans

Once the effect of germination on the apparent disappearance of antinutritional factors had been established, the next step was to see if this apparent disappearance had also resulted in the undoing of the factor associated with a decrease in feeding value. For that purpose the potential protein value was studied by the *in vitro* method of Babinsky et al. (1990). Initial results indicated that there was no difference in *in vitro* protein digestibility between germinated and ungerminated beans. This was probably due to the high concentration of pepsin, pancreatin and amylase (Chapter II). When the concentration of added enzymes was lowered, germination effects became visible. Germination influenced rate of digestion by the added enzymes. Thereby soya and white kidney bean protein digestibility appeared to have their maximum in protein digestibility already after twelve hours of germination. In faba beans the maximum increase was not reached until after five days of germination. This would indicate that soaking is enough to establish maximum increase in protein digestibility in soya and white kidney beans but not in faba beans. In literature many experiments are reported on the effect of germination on protein digestibility. Results in literature show a wide variaty because of differences in germination conditions, type and concentration of enzymes and length of the incubation period. For example in moth beans (*Phaseolus aconitifolius*) protein digestibility, by a pepsin hydrolysis, after 12 hours of germination increased only by 5% (Satwadhar et al., 1981). The protein hydrolysis was increased by 13.4% in white kidney beans after a combined hydrolysis of pepsin and pancreatin (Chapter III). After 36 hours of germination the protein digestibility of moth bean increased further to 82% (Satwadhar et al., 1981). Subbulakshmi et al. (1976) reported an *in vitro* protein digestibility of 94% in moth beans after 72 hours of germination. They used a combination of pepsin and pancreatin in their method.

Mostafa et al. (1987) measured the *in vitro* protein hydrolysis by pepsin and trypsin of soya beans. The first 3 days of germination protein digestibility by a pepsin hydrolysis resulted in an increase by more than 25% to 87%. During the same germination period, the protein digestibility with trypsin only increased by 5% to a total level of 28%. After that time protein digestibility with pepsin did not increase any further but with trypsin it increased with another 9% to a level of 37%. Our own germination experiment with soya beans of a mixed batch resulted in a maximum increase of *in vitro* protein digestibility of 16.7%

after twelve hours of germination (Chapter II). Faba beans (*Vicia faba*, var. Pistache) did have a more continuous increase of *in vitro* protein digestibility during germination (Chapter II) as was also found by Rahma et al. (1987).
The mode of action of digestion after varying duration of germination was also explored by studying the degradation using electrophoresis for the separation of protein fragments resulting from hydrolysis during germination. Results from the protein pattern determined by electrophoresis of kidney and soya beans do not indicate a cleavage of storage protein after twelve hours of germination (Chapter 2 and 3). A germination time of twelve hours was not effective to reduce for lectins and trypsin inhibitors. Tannin content did not change at all during germination of faba beans. Also the storage protein in faba beans was not changed during 12 hours of germination. This indicates that changes in protein during the early stages of germination are probably restricted to conformational changes making the proteins more susceptible for digestive enzymes. Germination of faba beans for twelve hours was however not enough to increase *in vitro* protein hydrolysis.

Enzymatic hydrolysis of lectins and phaseolin

It was decided to continue research on white kidney beans (*Phaseolus vulgaris*). This decision was effected by the results from the combined research experiments. Thereby three aspects lead to this. First of all natural growth conditions for soya beans are not optimal in the northern countries of West-Europe. Secondly assays for determining condensed tannins from these faba bean varieties, which can grow well in northern Europe, are still in a developing stage. The third reason for the decision to work with kidney beans was the fact that methods like ELISA (Enzyme Linked Immuno Assay) and FLIA (Functional Lectin Immuno Assay) for lectins from kidney beans had become available for the use in research experiments (Hamer et al., 1988).

Results from germination experiments indicated that lectins and phaseolin were cleaved from day three of germination onwards as could be observed by electrophoresis (Chapter III). This information was the reason for further research on protease-activity from extracts of kidney beans, germinated for various time periods. The aim was to isolate, purify and characterize the enzyme(s) which are responsible for the cleavage of lectins and phaseolin during germination. The isolated enzyme-extract was able to cleave purified lectins and phaseolin already after the ammoniumsulphate precipitation step in the isolation procedure (Chapter IV). In complete bean flour phaseolin and lectins were also degraded by the enzyme-extract but this incubation reaction went more slowly and less efficient. However, results from FLIA-experiments indicated 80% of the functional lectins to be eliminated after the incubation of bean flour with enzyme extract.
It is remarkable that lectins and phaseolin are cleaved simultaneously by the enzym-extract. Also during germination, the cleavage of both phaseolin and lectins starts on the third day of germination as was shown by results from earlier research (Chapter IV). The reason for this could be the fact that phaseolin and lectins are both glycoproteins and therefore cleaved by the same cystein proteases but because also other proteins, such as the Kunitz trypsin inhibitor in soya beans (Wilson et al, 1988), are cleaved by these enzymes, other reasons cannot be excluded.Boylan and Sussex (1987) already purified an enzyme, responsible for the cleavage of phaseolin during germination of beans (*Phaseolus vulgaris*, var. Taylor's Horticultural). They tested the enzyme only with purified lectins. But the enzyme-specificity

on phaseolin and lectins in bean flour was not tested by these and other researchers (Nielsen and Liener, 1984). Our results indicated that the purified enzyme was able to break down lectins. However when bean meal was used instead of purified lectins, the lectins in the beanflour were only partly broken down (Chapter IV). Probably inhibitors in the bean flour could have disturbed the enzyme substrate reaction.

Effects of bioprocessing of lectins and phaseolin

Germination can function as the ideal model for studying effects of proteases on phaseolin and lectins, but this seems not an optimal procedure to be used in practice, for instance in the feed industry. Therefore alternative bioprocesses were studied in order to compare results for lectin- and phaseolin cleavage. Various options were screened, including incubations of lectins, phaseolin or bean flour from raw beans with plant enzymes, animal enzymes, microbial enzymes and intact microbes. As best alternatives the plant enzymes papain from *Papaya latex* and the enzyme-extract from germinated kidney beans were found. Of the microbes *Bacillus subtilus*, mixed rumen bacteria and Rhine water micro-organisms were found to be active (Chapter V). This observation is not too surprising, because a relationship in action of papain and a microbial cystein protease has been observed by Ryan and Walker-Simmons (1981). They detected a similarity between the amino acid sequence of the peptide regions near the active site cysteines.

Changes of amino acids

Many of the storage proteins (e.g. globulins) in seeds are deposited in an water-unsoluble form. To make them fully susceptible to hydrolysis they must first be modified. One way in which this can be achieved is by limited endopeptidic attack on the stored protein to yield large, but more soluble, components (Bewley and Black, 1985). In faba, soya and kidney beans the different storage proteins were hydrolysed into smaller fragments during the first days of germination.

Mazelis (1980) reviewed the several types of reaction which are involved in the breakdown of amino acids. For many amino acids the process of catabolism involves removal of nitrogen from the carbon skeleton, which then undergoes breakdown or interconversion. The fate of the carbon skeleton may be to provide the basis for an alternative amino acid, a respiratory substrate, or other non-nitrogenous metabolic components such as keto acids or even sugars (Lea and Joy, 1985). Transamination and deamination are the two main reactions involved in removal of nitrogen from simple amino acids.

The bulk hydrolysis during which reserve proteins are hydrolysed into amino acids for the growing seed (Chapter I and VI) generally appears to occur in two stages, according to Bewley and Black (1985): modification of the large components (subunits) of the storage polypeptides, followed by their degradation into amino acids and small peptides. The decrease of proteonaceous nitrogen in the white kidney beans during germination is obviously caused by the further metabolization of amino acids into ammonia, which is used in the interconversion from one amino acid to another.

Proteolysis during germination is followed by further breakdown of the released amino acids (Chapter VI). Major forms in which amino groups from the storage organs into and throughout the growing seedlings are changed are amides, i.e., asparagine and glutamine. This means that amino acids liberated from storage proteins must be further metabolized, including the conversion of amino nitrogen to amido nitrogen.
There is an increase in the activity of enzymes involved in glutamate, glutamine and asparagine synthesis in cotyledons of various legumes seeds at a time when the major protein reserves are being hydrolyzed. Some of the amino acids released from the storage protein by hydrolysis, e.g., aspartate, glutamate, alanine, glycine and serine, can be converted to saccharides for transport and use in other regions.

Bewley and Black (1985) reviewed the amino acid composition (mole %) of the cotelydons after 4-5 days from the start of imbibition of mung beans and the composition of the major storage glycoprotein, vicillin. From the results they concluded that the level of the aspartate/asparagin fraction in the cotyledons increased and the level of the glutamate/glutamin fraction decreased when compared to the amino acid composition in vicillin.

It has been suggested by Bewley and Black (1985) that modifications in soybean cotelydons might be brought about through the removal by deaminases of amido groups from storage proteins prior to their hydrolysis: this activity could provide an early source of ammonia to the developing seedling. Many storage proteins are glycosylated but removal of the sugar components only occurs after cleavage of the peptide links, and after that the glycosyl units are released as complete oligosaccharides. Thus deglycosylation is probably not an early modification of storage proteins.

Nutritional evaluation in nonruminants

A lot of research on the *in vitro* protein digestibility of germinated legumes has been executed during the last years (Sawathar et al., 1981; Mostafa et al., 1987; Rahma et al., 1988). Possibilities of using germinated legumes in the feed of nonruminants however have only been studied very limited. The effect of germination of legume seeds on the digestibility of their proteins in rats has been studied by Palmer et al (1973). Diets containing 5% germinated kidney beans (*Phaseolus vulgaris*) in the total protein fraction were fed to rats. The N-content of the germinated beans was found to increase from 3.9 to 5.0 per cent (w/w) at day 8 of germination. The overall amino acid composition changed very little during germination. Germination improved the nutritive value of the beans in the rats, probably through the elimination of some of the toxic constituents of the seed.

In the last years research has been done on the effect of bioprocessed beans on the protein digestibility in nonruminants. Castanon and Marquardt (1989) tested the effect of the addition of cellulases and proteases to diets containing raw field beans (*Vicia faba*) in Leghorn chicks. Weight gains were improved by 6-10% with addition of enzymes. Näsi (1988) showed that the addition of an enzyme premix (containing proteases, cellulases and ß-glucanases) to diets containing soybean meal for growing pigs, improved the organic matter and protein digestibility of soybean meal.

In Chapter III studies on the *in vitro* protein digestibility by pepsin and pancreatin of white

kidney beans germinated for seven days have been described. Germination resulted in a protein hydrolysis percentage of 81.1. In the raw beans *in vitro* protein digestibility was only 67.2 %. When white kidney beans, germinated for seven days were used in an *in vivo* experiment, results were remarkable. The apparent protein digestibility in pigs, fed with the control diet, containing 7.5% raw beans was only 65.4% but it increased to 84.5% when instead of raw beans, germinated beans were used (Chapter VII). This means that due to the decreased lectin content, -trypsin inhibitor activity and-phaseolin content, beans seem to become more digestible for the pigs. These results are in agreement with the results found by Van der Poel (1990) who used thermally processed white kidney beans (*Phaseolus vulgaris*, var. Processor) in diets for pigs. An increase of the apparant ileal digestibility of protein in the small intestine of pigs was thereby established. This increased digestibility was thought to be related to the thermally inactivated lectins and -trypsin inhibitors in the Processor beans.

The apparent ileal digestibility of amino acids of raw beans treated with pancreatin was much higher than when raw beans were used without this pretreatment (Chapter VII). The elimination of trypsin inhibitors with pancreatin did not positively affect the work of the lectins.

The decrease of the content of essential amino acids during germination was counterbalansed by a considerable inactivation of antinutritional factors and storage protein thereby leading to an increase of the apparent ileal digestibility in pigs (Chapter VII). From a nutritional point of view germination appears to result in both negative and positive effects. A negative effect is the decrease of the content of essential amino acids during germination. Positive effects are a considerable inactivation of antinutritional factors and storage protein resulting in an increase in ileal digestibility in pigs. Production trials would be needed to see if the partial effects countrabalansed the negative effects.

Conclusions

Purified lectins, -trypsin inhibitors and -phaseolin, from white kidney beans can be cleaved in vitro by enzymes from the beans itself, or some plants, animals and microbes. However when bean flour from raw beans is used in stead of the purified products cleavage is less compared to purified products.

During germination of white kidney beans not only antinutritional factors decrease but also amino acids such as glutamin, lysin and arginin (Chapter VI). In contrast to these losses of amino acids the apparent ileal digestibility of ingested protein increased when germinated kidney beans were used in the diet of pigs in stead of raw beans. Further animal experiments are needed to investigate if and how much improved digestibility in the beans can compensate for the losses of amino acids.
Although germination of beans seems to be an attractive alternative for heat processes, germination on a large scale for animal feeding seems to have large practical problems. Compared to using germination of beans on a large scale only microbial biotechniques may have a perspective for the future.

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SUMMARY

The main reason for setting up this research project was to find a reasonable alternative for technological elimination of antinutritional factors (ANF's) and storage proteins in beans in order to increase the protein digestibility of beans in pigs.

In our literature review it was concluded that proteinaceous ANF's such as lectins and trypsin inhibitors are degraded during germination by enzymes. From tannins, which are nonproteinaceous ANF's (polyphenols), no direct relationship between enzymatic activation and degradation was observed during germination. Effects of ANF-containing legumes such as faba beans (*Vicia faba*), soya beans (*Glycine max*) and white kidney beans (*Phaseolus vulgaris*), which are used in animal feed nutrition, were observed especially in the literature review (Chapter I).

High concentrations of ANF's such as tannins, trypsin inhibitors and lectins are present in specific varieties of faba- , soya- and white kidney beans respectively. These three legume varieties, containing high ANF-concentrations, were used in an *in vitro* experiment in which the effect of germination (0-7 days) of the protein digestibility of the beans was tested with a pepsin and pancreatin enzyme-complex. Germination resulted in a maximum increase in protein digestibility of soya and white kidney beans after already 12 hours of germination. The *in vitro* protein digestibility of faba beans achieved a maximum after five days of germination.

Protein pattern change was observed after two days of germination in soya and white kidney beans and 3 days in faba beans. However lectin and trypsin inhibitor content did not decreased during the first days of germination and tannin content remained even the same after 7 days of germination. An increased protein digestibility in the three legumes can therefore not be explained by the change in contents of ANF's or protein pattern changes but probably by a more susceptibility for hydrolysis by pepsin-pancreatin of the bean protein (Chapter II and III).

Trypsin inhibitor content decreased during germination of soya beans. Enzymes responsible for this effect have been studied thoroughly in the past. However up till now the degradation of lectins by enzymes in white kidney beans during germination has only been studied very limited. Therefore an experiment was started to study the effect of germination on proteolytic activities in white kidney beans. After three days of germination proteolytic activity increased in the beans. Also the cleavage of lectins and the storage protein phaseolin started at that time of germination. It was decided to isolate an enzyme-extract from beans germinated for four days. This enzyme-extract was able to degrade purified lectins and phaseolin. When complete raw beans were used as a substrate, lectins and phaseolin were also cleaved. Functional lectins, able to bind with brush border membranes from the pigs gut wall, were thereby degraded for more than 80% (Chapter IV).

Further enzymatic studies resulted in the conclusion that not only an enzyme-extract from white kidney beans but also proteases and glycosidases from other origins such as micro-organisms, other plants and animals were able to hydrolyse purified lectins and/or phaseolin as well as lectins and/or phaseolin in bean flour from raw beans (Chapter V).

In order for further use in *in vivo* experiments germinated (0-7 days) faba-, soya and white kidney beans were investigated for the effect of germination on their amino acid composition.
In animal nutrition the real value of a plant protein is namely determined by the ease with

which it can be digested and by its capacity to supply amino acids in a ratio close to what is needed for maintaining the animal or for the production of animal protein in meat, milk or eggs. The amino acids in a feed can thus be compared with the ratio in which they are ideally needed. In the concept of "ideal protein", the required amino acid composition is often expressed as the percentage in which an essential amino acid (Thr, Val, Meth+Cys, Ile, Leu, Phe+Tyr, Lys and Try) is needed in comparison with Lysine. The results showed that the amino acid composition of legune seeds before germination did deviate somewhat from the ideal composition, but not extremely. Germination did however considerably increased the deviation, particularly for soy protein and phaseolus protein. The results indicate a significant reduction in protein quality due to germination (Chapter VI).

The effect of feeding young growing pigs a semi-synthetic diet containing 7.5% beans, either germinated (diet GB), pancreatin treated (diet PTB), or untreated (raw beans; diet RB) on protein and amino acid digestibilities at the terminal ileum was studied. A ^{15}N-isotope dilution method was used to determinethe the amount of endogenous protein passing the terminal ileum and the true ileal protein digestibility. The apparent ileal crude protein of the RB, GB and PTB diets were 74, 87 and 75% and for the amino acid (AA) digestibilities 76, 89 and 78%, respectively. The true ileal protein digestibility was 88, 93 and 93% for the RB, GB and PTB diet respectively (Chapter VII).

It can be concluded that germination of the white kidney beans clearly improves the digestion of the protein by a) decrease in the content of bean antinutritional factors and b) increase in the true ileal bean protein digestibility. However it seems not advisable to germinate for longer than 2 or 3 days, without affecting the protein quality. This will not be long enough to eliminate ANF's so, other methods such as process technology and/or addition of enzymes are required additionally.

SAMENVATTING

De hoofdreden voor het opzetten van dit onderzoek was het vinden van een redelijk alternatief voor de technologische eliminatie van antinutritionele factoren (ANF's) en voor de ontsluiting van voorraadeiwitten ten einde de eiwitvertering na het voeren aan varkens te verhogen.

In de literatuurstudie werd geconcludeerd dat de hoeveelheid eiwit-ANF's zoals lectinen en trypsine-remmers afnemen tijdens kieming. Voor tanninen, die behoren tot de niet-eiwit ANF's (polyphenolen), werd geen direct verband tussen de enzymatische inactivatie en de afname geconstateerd. ANF-bevattende peulvruchten zoals faba bonen (*Vicia faba*), soja bonen (*Glycine max*) en voerbonen (*Phaseolus vulgaris*), in dierlijke voeding, werden speciaal onderzocht in het literatuuroverzicht (Hoofdstuk I).

Hoge concentraties van ANF's zoals tanninen, trypsine-remmers en lectinen zijn aanwezig in specifieke varieteiten van respectievelijk faba-, soja en voerbonen. Deze drie peulvruchtvarieteiten, die hoge ANF-concentraties bevatten, werden gebruikt in een *in vitro* experiment waarbij het effect van kieming (0-7 dagen) op de eiwitverteerbaarheid werd onderzocht met een pepsine en een pancreatine bevattend enzym-complex. Kieming resulteerde reeds na 12 uren in een maximale verhoging in eiwitverteerbaarheid van soja en witte kidney bonen. De *in vitro* eiwitverteerbaarheid van faba bonen bereikte haar maximum pas na vijf dagen van kieming.

De die electrophorese bepaalde verandering na electrophorese in eiwitpatroon bij soja- en voerbonen werd geconstateerd na twee dagen van kieming en na 3 dagen bij faba bonen. Echter de hoeveelheden lectinen en trypsine-remmers veranderden niet gedurende de eerste dagen van kieming en de hoeveelheid tanninen bleef constant zelfs na zeven dagen van kieming. Een verhoogde eiwitverteerbaarheid in de drie peulvruchtvarieteiten kan daarom niet verklaard worden door de verandering in ANF hoeveelheden of door een verandering in eiwitpatroon maar waarschijnlijk wel door een hogere gevoeligheid voor hydrolyse van het boneneiwit door pepsine-pancreatine (Hoofdstuk II en III).

De hoeveelheid trypsine remmer nam af gedurende de kieming van soja bonen. Enzymen die verantwoordelijk zijn voor dit effect zijn grondig bestudeerd in het verleden. Echter de afname van lectinen door enzymen in voerbonen gedurende de kieming is tot nu toe slechts zeer summier bestudeerd. Daarom werd een experiment uitgevoerd om het effect van kieming op de proteolytische activiteit in voerbonen te bestuderen. Na drie dagen van kieming nam de proteolytische activiteit in de bonen toe. Op dat moment van kieming werden ook de lectinen en het phaseoline gesplitst. Besloten werd om een enzym-extract te isoleren uit gedurende vier dagen gekiemde bonen. Dit enzym-extract kon gezuiverde lectinen en phaseoline afbreken. Wanneer volledig intacte rauwe bonen gebruikt werden als substraat bleken de lectinen en het phaseoline ook afgebroken te worden. Functionele lectinen, die in staat zijn te binden aan de borstelzoom membranen van de darmwand bij varkens, werden voor meer dan 80% afgebroken (Hoofdstuk IV).

Nader enzymatisch onderzoek resulteerde in de conclusie dat niet alleen een enzym-extract van gekiemde voerbonen maar ook proteasen en glycosidasen van andere oorsprong, zoals micro-organismen, andere planten en dieren in staat zijn om gezuiverde lectinen en of phaseoline alsook lectinen en/of phaseoline in bonenmeel van rauwe bonen te hydrolyseren (Hoofdstuk V).

Voor de verdere toepassing in *in vivo* experimenten werden (0-7 dagen) gekiemde faba-,

soja- en voerbonen bestudeerd met betrekking tot het effect van kieming op de aminozuur samenstelling. In de diervoeding wordt de werkelijke waarde van het planteneiwit namelijk bepaald door de snelheid waarmee dit kan worden verteerd en door haar mogelijkheid om te voorzien in aminozuren en met name of de verhouding dicht in de buurt zit van wat nodig is om het dier in stand te houden of in de productie van dierlijk eiwit in vlees, melk of eieren te voorzien. De aminozuren in het voedsel kunnen zo vergeleken worden met de verhouding in welke ze in het ideale geval nodig zijn. In het ideale eiwitconcept wordt de verantwoordelijke aminozuursamenstelling vaak uitgedrukt als percentage in welke een essentieel aminozuur (Thr, Val, Meth+Cys, Ile, Leu, Phe+Tyr, Lys en Tyr) nodig is in verhouding tot Lysine. Uit de resultaten blijkt dat de aminozuursamenstelling van peulvruchten voor de kieming in bepaalde mate, maar niet buitengewoon, afwijkt van de ideale samenstelling. Kieming echter deed de afwijking toenemen, speciaal voor soja-eiwit en voerboneneiwit. De resultaten impliceren een significante reductie in eiwitkwaliteit als gevolg van kieming (Hoofdstuk VI).

Het effect van het voeren aan jonge biggen van een semi-synthetisch dieet, dat 7.5% bonen bevatte die gekiemd (dieet GB), of pancreatine behandeld (dieet PTB), of onbehandeld (rauw; dieet RB) waren, op de eiwit- en aminozuur-verteerbaarheden op het einde van het ileum werd bestudeerd. Een ^{15}N-isotoop verdunningsmethode werd gebruikt om de hoeveelheid endogeen eiwit die langs het einde van het ileum passeert en de echte ileale eiwit verteerbaarheid te meten. De schijnbare ileale ruw eiwitverteerbaarheid van het RB, GB en PTB dieet was respectievelijk 74, 87 en 75% en voor de aminozuur (AA) verteerbaarheden 76, 89 en 78 %. De ware ileale eiwit verteerbaarheid was respectievelijk 88, 93 en 93% voor het RB, GB en PTB dieet (Hoofdstuk VII).

Er kan geconcludeerd worden dat de kieming van witte voerbonen duidelijk de verteerbaarheid van het eiwit verbetert door a) een afname in de hoeveelheid antinutritionele factoren in de boon en b) een toename in de ware ileale booneiwit-verteerbaarheid. Echter het lijkt niet raadzaam om langer dan 2 of 3 dagen te kiemen, zonder de eiwitkwaliteit te beinvloeden. Dit zal niet lang genoeg zijn om ANF's voldoende te elimineren dus andere methoden zoals procestechnologie en/of enzymtoevoegingen zijn hiervoor nodig.

Curriculum vitae

Frans Savelkoul werd op 6 juli 1962 geboren te Grevenbicht. Zijn middelbare schooltijd begon in 1974 op de Mavo St. Jacobus te Born en eindigde op het Bisschoppelijk College St. Jozef te Sittard alwaar hij in 1981 zijn Atheneum-B diploma behaalde. In datzelfde jaar werd begonnen met de studie Biologie aan de K.U. te Nijmegen. In 1984 vervolgde hij deze studie op de L.U. te Wageningen waar hij in 1988 afstudeerde in de Celbiologie met als hoofdvak Immunologie en als bijvak Biochemie. Vanaf 1 januari 1989 was hij aangesteld als assistent in opleiding (AIO) bij de vakgroep Veevoeding van de L.U. waar het onderzoek beschreven in dit proefschrift is verricht. In 1993 begon hij met een studie 1e graads Biologie aan het UNILO-instituut van de K.U. te Nijmegen en in 1994 met een studie 1e graads Scheikunde aan de Katholieke Leergangen te Tilburg. In augustus 1994 werd hij benoemd tot docent Biologie aan de scholengemeenschap St. Ursula te Horn en tot docent Scheikunde aan de scholengemeenschap Sophianum te Gulpen.