Chemical, physical and nutritional changes in soybean meal as a result of toasting and extrusion cooking

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen
in het openbaar te verdedigen
op vrijdag 1 mei 1998
des namiddags te vier uur in de Aula

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The research described in this thesis was performed at:
- the laboratory of Food Chemistry and at the pilot plant of the Food and Bioprocess Engineering Group, both at the department of Food Science, Wageningen Agricultural University, the Netherlands,
- the department of Animal Nutrition, Wageningen Agricultural University, the Netherlands,
- Hendrix' voeders, Boxmeer, the Netherlands and
- Trouw Nutrition, Putten, the Netherlands.

The research described in this thesis was financed by Nutreco (Boxmeer, the Netherlands)

Cover photo: torpedo element with six rows of flights on the screw

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Marsman, Gerrit, J.P.

Chemical, physical and nutritional changes in soybean
meal as a result of toasting and extrusion cooking
Thesis, Wageningen Agricultural University. - With ref. -
With summary in Dutch
ISBN 90-5485-849-4
Subjects headings: soybean meal / extrusion cooking / toasting / protein /
chicken / enzymes

STELLINGEN

1. Het is onwaarschijnlijk dat trypsineremmers onder normale proces condities een belangrijke rol spelen in het verklaren van de verbeterde verteerbaarheid van geëxtrudeerd, getoast sojameel.

   Dit proefschrift, Hoofdstuk 2

2. Tijdens toasten worden hoofdzakelijk non covalente interacties verbroken. Hierdoor zal de eiwitdenaturatie waarschijnlijk niet volledig zijn en zouden de eiwitten na toasten hun originele structuur kunnen hervinden. Het verbreken van zowel non covalente interacties als disulfide bindingen zal eerder leiden tot irreversibele eiwit denaturatie.

   Dit proefschrift, Hoofdstuk 7

3. De urease activiteit en de eiwit dispergeerbaarheid mogen dan duidelijk het verschil tussen toasten en extruderen aantonen, voor de evaluatie van het extrusieproces hebben zij geen enkele waarde.

   Dit proefschrift, Hoofdstukken 2 en 3

4. Afschuifkrachten tijdens het extrusieproces hebben een additioneel effect op de denaturatie van glycinin en β-conglycinin, maar ook op de denaturatie van de trypsineremmers.

   Dit proefschrift

5. Hoewel rond de pH-STAT methode, bekend als zijnde een *in vitro* eiwit verteerbaarheids methode, vraagtekens geplaatst mogen worden, vertoont deze snelle methode op zich vaak een trend die overeenkomt met *in vivo* resultaten.

   Dit proefschrift

6. Met het verbieden van de jacht op schadelijk wild, zoals vastgelegd in de voorgestelde nieuwe flora en fauna wet, wordt het jachtbedrijf in Nederland een belangrijke doelstelling onmogelijk gemaakt, namelijk het in stand houden van een gezonde en gevarieerde wildstand.

7. De alsmaar toenemende lengte van het NOS of RTL weerpraatje lijkt vaak omgekeerd evenredig met de betrouwbaarheid daarvan.

8. De introductie van de Bega-claim op de Nederlandse Effectenbeurs zal voor veel mensen een bevestiging zijn van het idee dat Beursplein 5 één groot gokpaleis is.

9. Handelen in effecten is niets anders dan het vinden van een goede balans tussen angst en hebzucht.

10. De alsmaar strengere eisen rond de microbiologische kwaliteit van voedingsmiddelen, maakt van de mens uit de westerse landen een kasplantje.

11. De opmerking van oudere mensen dat de tijd sneller gaat naarmate men ouder wordt is eigenlijk niet juist, aangezien men relatief minder snel oud wordt naarmate de leeftijd toeneemt.

12. De negatieve opmerkingen aan het adres van gereformeerde mensen over het oprichten van eigen scholen en andere gereformeerde instellingen staat haaks op het algemeen aanvaard zijn van de oprichting van homo-bejaardentehuizen.

13. Doordat steeds meer politieke partijen zich naar het politieke midden begeven om op deze manier zoveel mogelijk kiezers te trekken, vervaagt hun kleur en helderheid en neemt de kans op een toekomstig 'grijs-1' sterk toe.

Stellingen behorende bij het proefschrift "Chemical, physical and nutritional changes in soybean meal as a result of toasting and extrusion cooking".

Gerard Marsman

Zutphen, april 1998.

VOORWOORD

Ondergetekende beseft terdege dat het tot stand komen van een proefschrift nooit het werk van één persoon geweest kan zijn. Ik wil daarom, zonder de intentie te hebben volledig te willen zijn, de volgende personen oprecht bedanken voor hun medewerking.

Fons Voragen, toen ik nog niet eens afgestudeerd was, wist jij mij enthousiast te maken voor dit project. Je was op zoek naar een kandidaat die theoretisch kon zijn, maar ook niet vies was van smerige handen. Honderden kilo's sojameel extruderen in de proceshal, microgrammen monsters injecteren op allerlei soorten kolommen op LMC, ratten voeren in het proefdierencentrum en kippen slachten op De Haar. Het hoorde er allemaal bij. Het maakte van ondergetekende niet de gemiddelde promovendus die je doorgaans op LMC ziet rondlopen. Ik ben je, desondanks, dankbaar dat je keuze op mij is gevallen. Het op deze manier mogen werken aan vele aspecten van de wetenschap is mij zeer goed bevallen.

Het werken in multidisciplinair verband komt ook tot uiting in het hebben van een tweede promotor, Martin Verstegen. Martin, jouw inbreng in het project was onmisbaar. Jij wist ons heel duidelijk te maken dat er een levensgroot verschil zit tussen de termen *in vitro* en *in vitro*. Jouw visie hierop was zeer verhelderend en werd niet alleen door mij maar ook door de overige participanten in het project zeer gewaardeerd.

Veel dank ben ik verschuldigd aan Harry Gruppen, mijn co-promotor. Weet je, het is precies zo gegaan zoals men mij toentertijd al vertelde. Het eerste jaar loop je mee aan het handje van de begeleiders, het tweede jaar loop je gelijk op en de laatste twee jaren neem je zelf het voortouw. Ik moet bekennen dat ik alle zeilen heb moeten bijzetten om bij jou ook dat laatste te bewerkstelligen. Desondanks was het onder jouw bezielende supervisie goed toeven. Het was geruststellend te weten dat er een collega aanwezig was die op alle fronten echt mee wou het diepe in. Ik wil je ook bedanken voor de manier waarop we tot publiceren zijn gekomen.

Een speciaal woord van dank gaat uit naar Aart Mul van Nutreco. Aart, jij was er van het begin af aan bij, eigenlijk al eerder toen het project alleen nog maar als idee in hoofden van enkele personen borrelde. Jij was ook degene die bij de overgang van BP Nutrition naar Nutreco je hard hebt gemaakt om het, toen nog maar kort lopende, project binnen het nieuwe Nutreco te behouden. De inbreng tijdens de vele vergaderingen heb ik zeer gewaardeerd. Ik hoop dat de inhoud van dit proefschrift voldoet aan je verwachtingen en dat het je ex-collega's nieuwe aanknopingspunten kan bieden voor de nimmer ophoudende drang om op alle relevante vragen een antwoord te vinden. Intussen zijn we collega's geworden, wie weet komen we elkaar in één of ander project wel weer tegen.

Naast het vele werk op de vakgroep van LMC was het op meerdere fronten verfrissend om eens een poos te gast te mogen zijn op de vakgroep Veevoeding. Het werken in het toen nog knusse gebouw aan de Haagsteeg was toch heel wat anders dan de twee strakke gangen op de vijfde verdieping van het Biotechnion. Diverse mensen van de Vakgroep Veevoeding ben ik dank verschuldigd. René Kwakkel, ik wil je bedanken voor het coördineren van de groeiproef met de kippen, de interpretatie van de resultaten en de hulp bij het op papier zetten van deze resultaten, Peter van der Togt voor het uitvoeren van de statistische analyses, Tamme Zandstra voor zijn hulp tijdens het verzamelen van chyme aan het eind van de tweede kippenproef en Thomas van der Poel voor het opstellen van de kippen dieten en zijn inbreng tijdens de reguliere vergaderingen.

Een woord van dank gaat ook uit naar de vakgroep Proceskunde. Zonder de adviezen van Dick van Zuilichem was hoofdstuk drie van dit proefschrift, en alles wat daaruit voortvloeide,

niet tot stand gekomen. De ontwikkeling van de speciale schroefconfiguraties kwam mede door jouw tot stand. Het was uiteindelijk de Technische Dienst die dit precisie werkje aandurfde. Ik wil hierbij ook Willem Stolp bedanken voor de uitleg die hij mij gegeven heeft tijdens het extruderen.

Jan Willem Resink en Alfred Bonte van Trouw Nutrition wil ik beide hartelijk bedanken voor hun inbreng tijdens de vele voortgangsvergaderingen die we gehad hebben. Daarnaast heeft Jan Willem mij de mogelijkheid gegeven ook in Putten bij Trouw Nutrition extrusieproeven uit te voeren en heeft hij de eerste kippen groeiproef, die uitgevoerd is in Boxmeer, gecoördineerd. Alfred, nog bedankt voor het uitvoeren van de viscositeitsmetingen na de tweede kippen groeiproef.

De bedrijfsleider van De Haar Pluimvee, Roel Terluin, en zijn collega's wil ik dank zeggen voor hun hulp tijdens de tweede groeiproef met kippen. Ik heb, helaas, maar een korte tijd van jullie kunnen leren.

Dhr. de Haas en zijn collega's van het proefdierencemtrum wil ik ook bedanken voor hun hulp en begeleiding tijdens de rattenproef. Het samenwerken met deze afdeling was een hele bijzondere ervaring.

Aan dit proefschrift hebben ook enkele doctoraalstudenten hun medewerking verleend. In chronologische volgorde waren dit: Corinne Closs (ETH Zürich), Peter Leij, Marjolein Bemelmans, Jolanda Laurijsen, Marjan Bredius en Ana Patricia Moreira (Instituto Superior de Agronomia, Lissbon). Jullie onderzoek heeft zeker bijgedragen aan het tot stand komen van dit proefschrift. Mijn dank daarvoor.

Toen bleek dat, na enig rondzwerven, kamer 526 mijn vaste stek zou worden werd ik de kamergenoot van Margien Mutter. Hoewel onze projecten weinig raakvlakken vertoonden konden we het goed met elkaar vinden, zeker als het ging om gesprekken over die andere dingen in het leven die ons zo bezig hielden. Henny en Edwin van 'next door', je kon geen morgen zo vroeg aanwezig zijn of de koffie was al gezet. Bedankt voor de gezelligheid! Jolan, jouw jarenlange ervaring met het werken met eiwitten is niet alleen mij goed van pas gekomen, ook vele anderen maken daar dankbaar gebruik van.

Berty, Margareth, Lex, Henk, Ben, Jan ..., of het nu wel of niet over het werk ging, jullie maakten deel uit van een tijd in mijn leven waar ik zeker goede herinneringen aan over zal houden.

Lieve Henca, zonder jouw steun was dit proefschrift niet tot stand gekomen. Jij gaf mij die mogelijkheid en je cijferde jezelf daarbij zes jaar lang weg. Het kostte jouw je baan in Assen, Ede en nu, gezien de laatste ontwikkelingen, waarschijnlijk ook in Zutphen. Daarnaast wist jij mij toch weer te stimuleren in de laatste fase van het project toen we beide heel slecht nieuws kregen te verwerken. Het enige wat ik je nu kan geven is mijn aanwezigheid, niet meer maar ook zeker niet minder. Samen weten wij dat er Eén is die ons beide, in goede en slechte tijden, steun heeft gegeven en steun zal blijven geven. Ik beloof je het de eerst komende tijd wat kalmer aan te gaan doen.

ABSTRACT

The effect of soybean meal extrusion and the development of shear forces during single-screw extrusion was compared with the toasting process of soybean meal. Attention was focused on chemical, physical and nutritional changes during these thermo-mechanical treatments.

Monitoring target parameters were tested for their usefulness during processing. It appeared that the nitrogen solubility index in potassium hydroxide, pH-STAT *in vitro* protein digestibility, trypsin inhibitor activity, differential scanning calorimetry and specific mechanical energy were parameters which can be used to evaluate the protein quality between the different extrusion conditions. On the other hand, the protein dispersibility index and the urease activity were of no use in monitoring the extrusion process.

Extrusion significantly increased the *in vitro* protein digestibility and the apparent ileal digestibility of crude protein in broiler chickens when compared with toasted soybean meal. Also, the feed conversion ratio was significantly improved after extrusion. Thermal studies revealed that toasted soybean meal was only partly denatured, while after extrusion a completely denaturation of the main storage proteins in soybean meal was achieved. This was explained by the fact that during toasting mainly non covalent interactions were involved in protein structure formation, while after low shear extrusion both non covalent and disulfide bonds were broken during extrusion.

The development of shear forces during extrusion resulted in a maximum *in vitro* protein digestibility as well as the apparent digestibility of crude protein ($P<0.1$). At high shear extrusion, the nutritional value of the soybean meal started to deteriorate again. This was seen *in vitro* as well as *in vivo*. Under these high energetic conditions, also covalent cross linking reactions may occur. Also, the amount of soluble non starch polysaccharides and the water holding capacity in the chyme was increased after high shear extrusion, resulting in a significant higher chyme viscosity. It is shown, theoretically and experimentally, that shear forces may have an additional effect on protein denaturation during extrusion cooking at different shear levels.

The use of hydrolytic enzyme preparations on toasted and extruded soybean meals resulted in a higher apparent ileal digestibility of crude protein and non starch polysaccharides. An *in vitro* study showed that after extrusion both β-conglycinin as well as glycinin were rapidly and completely degraded by proteases (Neutrase) when compared with toasted soybean meal. The hemicellulase preparation (Energex) was able to release up to two thirds of the water unextractable solids in extruded soybean meal. However, these results were not translated to a better growth performance in broiler chickens.

It was concluded that the trypsin inhibitor activity after extrusion of commercial available toasted soybean meal is only a minor factor in explaining the obtained increased nutritional value of this soybean meal. The development of shear forces may be responsible for an additional inactivation effect of trypsin inhibitors.

LIST OF ABBREVIATIONS

ADG	Average daily gain
ANF	Antinutritional factor
BBI	Bowman-Birk trypsin inhibitor
BE fraction	Buffer extractable, non dialyzable fraction
BR fraction	Buffer retentate fraction
Br	Brinkman number
BW	Body weight gain
CP	Crude protein
D fraction	DTT extractable fraction
DC_{CP}	Apparent ileal crude protein digestibility
DC_{NSP}	Apparent ileal non starch polysaccharides digestibility
DC_{Fat}	Apparent ileal fat digestibility
DC_{Starch}	Apparent ileal starch digestibility
DM	Dry matter
DSC	Differential scanning calorimetry
DU fraction	DTT and urea extractable fraction obtained after extraction of the residue obtained after extraction with DTT
DU_{res} fraction	The remaining residue after extraction with DTT and urea
ExTSBM	Extruded toasted soybean meal
ExUSBM	Extruded untoasted soybean meal
Ex-0, Ex-4, Ex-8	Untoasted soybean meal extruded with a torpedo element containing zero, four and eight rows of flights on the screw, respectively
FCR	Feed conversion ratio
FDNB	1-fluor-2,4-dinitrobenzene
FI	Feed intake
HPAEC	High performance anion-exchange chromatography
HPSEC	High performance size-exclusion chromatography
HTST	High temperature short time
H_v	Transition enthalpy
L/D	Length to diameter ratio of the screw
ME	Metabolizable energy
Mw	Molecular weight
NPN	Non protein nitrogen
NSI	Nitrogen solubility index
NSP	Non starch polysaccharides
PDI	Protein dispersibility index
PER	Protein efficiency ratio
PI	Protease inhibitor
P_n	Net motor power
RSM	Rapeseed meal
RTD	Residence time distribution
SBM	Soybean meal
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SME	Specific mechanical energy

STI	Kunitz trypsin inhibitor
T_d	Denaturation temperature
TI	Trypsin inhibitor
TIA	Trypsin inhibitor activity
TLSS	Twin lead slotted screw
TSBM	Toasted soybean meal
TRSM	Toasted rapeseed meal
UA	Urease activity
U fraction	Urea extractable fraction
UD fraction	Urea and DTT extractable fraction obtained after extraction of the residue obtained after extraction with urea
UD_{res} fraction	The remaining residue after extraction with urea and DTT
USBM	Untoasted soybean meal
WHC	Water holding capacity
WUS	Water unextractable solids

CONTENTS

chapter 1

General introduction

Origin and production

Soybean (*Glycine max*), which belongs to the family of the Leguosae, is of Eastern Asian origin. It moved from the countries of the Far East in ancient times to Europe and was introduced in the United States in the early 19th century[1]. There are three species of soybean; *Glycine ussuriensis* (wild), *Glycine max* (cultivated), and *Glycine gracilis* (intermediate). *Glycine max* is commonly grown throughout the world[2].

In terms of total production and international trade, soybean is the world's most important oilseed. Soybean is mainly cultivated for its seeds, used commercially as human food and livestock feed and for the extraction of oil. Table 1 shows the world oilseed production from 1980 to 1992. Soybeans are dominating the world oilseed production with a share of 52.3 and 51.5% in 1980 and 1992, respectively (Table 1). World production of soybeans has increased by 44% to 116.5 millions tons from 1982 to 1992. The forecast for 1996/1997 is almost 134 million metric tons of soybeans[3].

TABLE 1
World oilseed production in 1980 and 1992 in [million metric tons][4]

Crop	1980	1992	change (%)
Soybean	81.0	116.5	44
Cottonseed	24.8	31.6	27
Rapeseed	11.1	25.2	127
Sunflower seed	13.2	21.5	63
Peanut/groundnut	16.3	23.2	42
Copra	4.8	4.7	- 2
Palm kernel	1.5	4.0	167
Total	154.9	226.8	46

Most of the increases in soybean production has been achieved by Brazil and Argentina. Since the early 1980s their combined production jumped by 75% and presently accounts for more than 30% of the world production (Table 2). Also, China and India have become important producers of soybeans in the past decade[5]. A part of the world's oilseed production is exported in the form of seeds or processed products e.g. protein meal and vegetable oil. The major exporting regions are the United States, Brazil and Argentina, while the EU and Japan are the major import markets. In 1992 approximately 17% of the total oilseeds were traded, with trade of soybeans at 25% of the total soybean production, exceeding that of other oilseeds[4].

Global demand for protein meals has almost tripled since 1970. The major importing countries are highly industrialized countries with high livestock populations.

The EU accounts for half of the total world import demand for protein meals.

Soybean meal (SBM) dominates the export market for protein meals due to its high protein content (close to 50%) as well as its good availability. This makes SBM a very suitable component for feedstuffs. While about only 36% of SBM production is traded, it accounted for 66% of the total protein meal traded in 1992[4]. In the United States, SBM accounts for about 80% of all protein meals fed to livestock. SBM is especially important to poultry and hog feeding. Poultry (broilers and layers) and young pigs require the highest ration of high-protein feeds in their diets. Ruminants are able to derive proteins from other sources, such as microbial protein released in the rumen from roughage. Therefore, SBM is not as importance in ruminant nutrition as in monogastric nutrition.

TABLE 2
Top 1992 soybean producing nations [in millions metric tons][5]

Country	1992
United States	54.0
Brazil	18.6
Argentina	10.1
China	9.8
India	2.3
Paraguay	1.6
Canada	1.4
Italy	1.4
Indonesia	1.3

A small percentage of SBM (less than 1%) is used for human and industrial consumption. SBM for food use includes high protein derivatives of meal used in cake mixes, breads, snack food, and baby foods. Industrial uses of SBM are found in the dietetic health and cosmetics industry as well as in the production of antibiotics[4].

Soybean processing

The most commonly used process of soybean processing to SBM and soya oil is shown in Figure 1. Seed cracking is used to separate the hulls from the soybean cotyledons. After dehulling, the soybean is subjected to preconditioning at 65-70°C for 30 min in order to make the soybeans more pliable for flaking[6]. Flaking is necessary to make oil extraction of the soybean more rapid and more efficient. In extraction, the oil is washed with a solvent, mostly hexane, in order to separate the oil from the flakes. Batch extractors as well as continuous extractors are used[7].

Desolventization or toasting is normally performed at a temperature of 70-80°C for about 20 min[6]. This is the most critical stage in soybean processing. To retain high solubility of the protein, control of processing variables is essential with respect to temperature, pressure, presence of moisture and residence time[8]. In the last step, grinding and classification is necessary to meet special standards e.g. defatted SBM or soy grits.

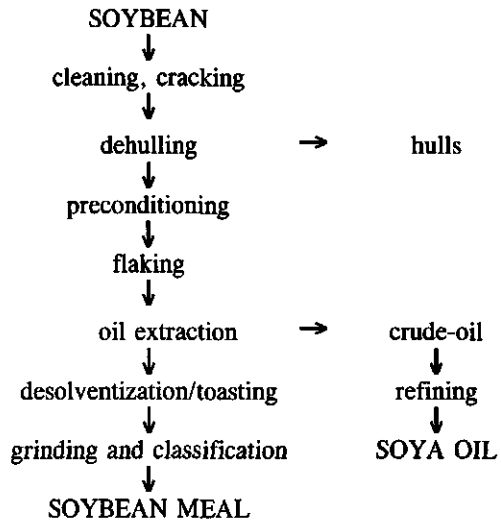

FIGURE 1
Soybean meal processing[2,6,7]

Chemical composition of soybean meal

A proximate composition of SBM is given in Table 3. These data indicate that the proximate composition of the meal is influenced by genetic as well as environmental factors.

TABLE 3
Average composition of soybean meal[2,6,10,12,13]

Constituent	% on dry weight basis
Carbohydrates	30 - 40
sucrose	6 - 9
raffinose	1 - 1.5
stachyose	5 - 8
starch	0 - 5
non starch polysaccharides	16- 22
Crude protein	50 - 58
Fat	< 1
Ash	6

Carbohydrates

Sucrose, stachyose and raffinose are the main oligomeric carbohydrates in SBM. Raffinose and stachyose are responsible for the flatulence and abdominal discomfort often experienced after ingestion of soybean and their products[2]. While it is said that soybean seeds lack starch[2], some others found a starch content of SBM of up to 5%[10]. In the fiber or non starch polysaccharides (NSP) fraction, galactose, glucose, arabinose and galacturonic acid are the prevailing sugar residues. The cell wall of soybeans is composed of cellulose, pectins, galactan, arabinogalactan, (highly) branched arabinan and galactomannan[10,11,12].

Protein

Seed proteins have been classified traditionally through the sequential extraction of proteins by a solvent series[14]. The fraction extracted by water is defined as albumins, the fraction by dilute salt as globulins, the fraction by ethanol as prolamines and the fraction by acid or alkali as glutenins.

Soybean proteins can be divided in albumins (10%) and globulins (90%)[15]. Soybean globulins are composed of 4 major components, 2S (15.0%), 7S (34.0%), 11S (41.9%) and 15S (9.1%), according to their sedimentation rates in a pH 7.6, 0.5 M ionic-strength buffer[16]. The 2S fraction consist of the Bowman-Birk- and Kunitz trypsin inhibitor, cytochrome C and α-conglycinin[17,18]. In the 7S fraction, β-conglycinin is the most important protein. Also small amounts of γ-conglycinin ($< 3\%$) can be found in this fraction. The proteins in the 11S fraction are called glycinins and the proteins in the 15S fraction are polymers of glycinin[18]. β-Conglycinin and glycinin are the two most important storage proteins in soybean. The native β-conglycinin and glycinin proteins differ significantly in many important physicochemical properties (Table 4).

β-Conglycinin

β-Conglycinin is a glycoprotein with a trimeric quaternary structure. It is composed of seven different combinations of three subunits, α'(M_w, 57,000-72,000), α (M_w, 57,000-68,000), and β (M_w, 42,000-52,000), resulting in a molecular weight of 141,000-204,000. The seven combinations, B_0-B_6, are, $\beta\beta\beta$, $\beta\beta\alpha'$, $\beta\beta\alpha$, $\beta\alpha\alpha'$, $\beta\alpha\alpha$, $\alpha\alpha\alpha'$ and $\alpha\alpha\alpha$, respectively[19]. The subunits are non covalently associated via hydrophobic and hydrogen bonding without any disulfide bonds[22]. β-Conglycinin has no -SH groups and two disulfide bonds per molecule[19]. The subunits contain about 5% carbohydrates (mannose and glucosamine) and are associated with each subunit attached to Asn[19,21].

Glycinin

Glycinin is made up of six subunits, each consisting of a basic polypeptide (B) and an acidic polypeptide (A) which are connected by a single disulfide bond forming the AB subunit. Glycinin consist of two hexagonal rings stacked on top of the other, giving two identical half-molecules of glycinin. The heterogeneity of the glycinin molecule is expressed in its molecular size which ranges from 320,000 to 375,000. At least six acidic (A_{1a}, A_{1b}, A_2, A_3, A_4 and A_5) and five basic (B_{1a}, B_{1a}, B_2, B_3 and B_4) polypeptides have been determined from glycinin[20]. Glycinin has 2 -SH groups and 18-20 S-S bonds per molecule[19].

TABLE 4

Physicochemical properties of glycinin and β-conglycinin[20-22]

Property	glycinin	β-conglycinin
Molecular weight	± 350,000	± 175,000
Subunits	12	3
M_w subunits	A ; 37,000-45,000	α'; 57,000-72,000
	B ; 22,500	α ; 57,000-68,000
		β ; 42,000-52,000
Secondary structure	6% α helix	6% α helix
	40% β structure	34% β structure
	55% random coil	60% random coil
Carbohydrate content	0	4,94
SH-groups	0-2	0
Disulfide bonds	18-20	2
Stokes radius	58.5 A°	59°
Isoelectric pH	4.64	4.9
Denaturation temperature	80°C	67°C

Antinutritional factors

The nutritional value of untreated SBM is negatively influenced by the presence of antinutritional factors (ANF). The most important ANF are protease inhibitors, lectin, phenolic compounds and phytate[2,23,24]. The presence of goitrogenes, antivitamins, alkaloids and saponins are reported but are less important in soybeans[23]. Whether or not classified as ANF, also oligosaccharides and allergenicity of storage proteins in soybeans should be considered as factors influencing the nutritional value of SBM.

Protease inhibitors (PI)

PIs in soybean include trypsin and chymotrypsin inhibitors. Two families are known; the Kunitz (STI) and Bowman-Birk (BBI) trypsin inhibitor family. The STI has a molecular weight of about 21 kDa with a sequence of approximately 180 amino acids including generally four cysteines forming two disulfide bonds[23,25]. It primarily inhibits trypsin, single headed with the reactive site being located at residues Arg 63 and Ile 64, and weakly inhibits chymotrypsin. The BBI family is characterized by molecular weights around 8-9 KDa (71 amino acids) and a high content of cysteines involved in seven disulfide bridges. The unique feature of the BBI family is that they have two independent binding sites: a trypsin reactive site (Lys 16 and Ser 17) and a chymotrypsin reactive site (Leu 43 and Ser 44)[23]. Both inhibitors cause an increase in synthesis and secretion of the pancreatic enzymes trypsin and chymotrypsin into the gastro intestinal tract (duodenum), which may cause hypertrophy of the pancreas in some species, resulting in retardation of growth by loss of endogenous protein[2]. The trypsin inhibitors are heat labile. The tight compact structure of the BBI as a result of the seven disulfide bridges may explain the fact that the BBI is less sensitive to heat compared to the STI[25]. However, there are conflicting reports about the stability of the BBI[24]. It is stated that PIs seemed to account

for only 40% of the growth inhibition produced by raw soy flour[23]. This means that also other antinutritional or non-antinutritional factors should be considered with respect to the nutritional value of SBM.

Lectins

Haemagglutinins (lectins) are mainly located in the seeds of plants. Their function is to protect the seed against fungi, bacteria and viruses[24]. Most lectins are in the form of glycoproteins and are characterized by their unique capability to bind carbohydrate containing molecules. Lectins in the diet survive gut passage and appear to influence many aspects of cellular metabolism[27]. For example, they are able to bind to surface receptors of the intestinal epithelium where they causes disruption of the nutrient absorption[23,27]. Lectins from *Phaseolus vulgaris* have been shown to be a strong growth inhibitor in diets for rats but especially for pigs[28]. However, lectins are also heat labile. Their inactivation by heat closely parallels the destruction of trypsin inhibitors[23].

Phenolic compounds

Within the group of phenolic compounds, tannins are the most important phenolic compounds. Tannins are heat stable water soluble substances having a molecular weight between 500 and 3000 Da[24]. It has been suggested that tannins play a major role in the plant's defence against fungi, insects and birds, because they have a bitter taste protecting them against glutton. The two major types of tannins, condensed tannins and hydrolyzable tannins, are chemically quite different[29]. Tannins complex strongly with proteins resulting in harmful nutritional effects. Digestive enzymes may also be inhibited by tannins. Tannins complex also with mucous membranes which result in increased endogenous losses of protein[29]. Soybeans have relatively low levels of tannins (45 mg/100g) compared with faba beans (2000 mg/100g)[23]. Therefore, the nutritional significance of tannins in soybeans is mostly ignored.

Phytate

Phytate is present in raw soybean at about of 1.5% of dry weight[2]. Almost 80% of the total phosphorus content is represented by phytate phosphorus. Phytic acid forms complexes with anions resulting in a reduced availability of Ca, P, Mg, Zn, Cu and Fe[24]. It is known that phytate strongly interacts with the basic residues of proteins also with those of digestive enzymes[22]. Therefore, its deleterious effect on growth and mineral status of monogastric farm animals has prompted efforts to reduce the phytate content of soybeans[23].

Allergenicity

Allergenicity of proteins in soybean is a well known problem. Especially the 2S fraction but also components of β-conglycinin and glycinin are suspected for the allergic response. In generally, it is believed that the immunochemical reactivity of most of the protein components is destroyed by heat treatment[23].

Flatulence producing factors

SBM contains substantial levels of sugars, particularly oligosaccharides containing α-galactosidic and β-fructosidic linkages[23]. The α-galactosides of sucrose (raffinose,

stachyose and verbascose) cannot be degraded in the small intestine of monogastric animals due to the absence of endogenous α-(1,6)-galactosidase. These sugars then pass into the large intestine where microbial fermentation converts them into CO_2 and H_2, the main components of flatus. Because these oligosaccharides are stable to heat, attempts have been made to eliminate them by enzymatic or chemical treatment. Removal of 90% of the α-galactosides of sucrose from SBM, using either ethanol extraction or exogenous α-galactosidase, did not result in a better nutritional value when fed to chickens[30].

Feed processing

Feed costs are the most important costs involved in the animal production system. Economic production is only possible, if the livestock is supplied with an adequate diet in terms of nutrient content and utilization allowing consumption without waste. Nowadays, feed processing methods are become more important in order to achieve feeds with a high nutritional value. The major thermo-mechanical processes used in the production chain of animal feed are listed in Table 5.

TABLE 5
Thermo-mechanical treatments in the animal feed industry[9,31]

Process	Temp. [°C]	Residence time	Temp./Time[3]	Moisture[4] content	Shear
Extrusion[1]	90-160	seconds	HTST	medium	+ +
Expansion[1]	80-140	seconds	HTST	medium	+
Toasting[1,2]	90-110	minutes	LTLT	low/medium	-
Steam flaking[1]	± 100	minutes	LTMT	medium	+
Steam explosion[1]	140-210	seconds	HTST	medium	+ +
Drying[1,2]	> 100	minutes	MTLT	low/medium	-
Preconditioning[2]	< 100	minutes	LTLT	medium	-
Pelleting[1]	60-100	seconds	LTST	low	+
Granulation[1]	50-95	minutes	LTLT	medium	-
Grinding[1,2]	± 20	seconds	LTST	low	+ +

[1] used as main process; [2] used as pretreatment; [3] H=high, M=medium, L=low; [4] low: ≤ 18%; medium: 18-30%

The ranges given in Table 5 are only indicative. For example, it is difficult to compare the shear forces developed during extrusion to the shear forces during grinding. The major process parameters are temperature, moisture content, residence time and shear forces. These conditions determine the reactivity of the various chemical entities contained in the materials, the type of reactions and the extent to which they will occur[2]. The two thermo-mechanical treatments used in our project, toasting and extrusion cooking, will be outlined in the next paragraphs.

Toasting of soybean meal

Toasting of SBM is mostly performed after oil extraction. This desolventization is the distillation process in which the extraction solvent is removed from the defatted flakes. Toasting is normally performed at a temperature of 70-80°C for about 20 min[6]. This is the most critical stage in soybean processing, in which damage to the protein can either be kept minimal, or otherwise make the protein unfit for further processing, e.g. for concentrates or isolates. To retain the high solubility of the protein, control of processing variables is essential with respect to temperature, pressure, presence of moisture and residence time[8]. Beside removal of the extraction solvent, toasting is also responsible for a certain increase in nutritional value of SBM due to inactivation of ANF[23]. In addition, also denaturation of the storage proteins should also be considered. Toasting is a non shear process, which mean that shear forces are absent during processing. This and the much longer residence time are the main differences between toasting and extrusion cooking.

Single-screw extrusion cooking of soybean meal

The extrusion process

Single-screw extrusion cooking is a high-temperature-short-time (HTST) process. In most cases, it is not a single-unit operation but it combines the operations of thermo-mechanical treatment, feed transport, mixing and forming. In general, extruders consist of a fixed metal barrel which contain one or two screws that conveys the raw material from the feed supply section to the die[31]. The extruder is viewed as a continuous chemical reactor processing biopolymers and feed mixes at high temperatures (90-160°C) for relatively short residence times (30-120 seconds) at high pressures and at relatively low moisture contents (below 30%)[32].

The unique process condition factor present during extrusion is the exposure of raw material to severe shear forces. The amount of shear forces developed during extrusion depends on numerous process conditions e.g. screw configuration, compression ratio, die hole, screw speed and raw material characteristics. Under these high energy conditions, a molten mass is formed in the metering section, which is the last section of the extruder near the die. The high pressure inside the extruder keeps the water in the liquid state. When the processed material exits the extruder through the die at the end of the barrel, the superheated entrapped liquid water vaporizes instantaneously, due to a sudden drop of pressure to normal atmospheric conditions. This water passes through the molten mass and produce a network of holes in the extrudates, resulting in a porous product[35].

While the effects of temperature, moisture content and residence time on the nutritional value of SBM and other raw materials are well documented[32-35], from the effects of shear forces on SBM components is much less known. Because protein denaturation is one of the most important reactions in SBM extrusion, it will be outlined in more detail later on in this chapter.

Systems analysis of extrusion cooking

The understanding and control of the extrusion process is very difficult. Meuser and Wiedman[36] have tried to examine extrusion cooking using a systems analysis approach. For this purpose, a model was developed that divides the extrusion process into three steps; process, system and target parameters (Table 6). The theoretical basis for the systems analysis is that the chemical and physical changes of the extrudates are dependent on the time-dependent mechanical and thermal energy inputs (Z=f(Y), in Table 6). This implies that the energy input must be functionally related to the process conditions and to the characteristics of the raw material (Y=f(X), in Table 6).

Process parameters such as screw speed, barrel temperature and mass flow can be adjusted and continuously measured. Also, the composition of the raw material is known, seasonal variations in the raw material composition not considered.

The system parameters, the mechanical and thermal energy inputs into the mass as well as the residence time in the extruder, can also be measured, but adjustment is only possible by changing one of more process conditions or by changing the raw material composition. In most cases, process parameters are heavily interrelated. For example, an increase in residence time can be achieved by increasing the screw speed or by increasing the mass flow. However, more energy will be necessary and the specific mechanical energy (SME) will increase, the pressure may rise and more friction will occur, resulting in an increase in temperature.

External and internal features of the extrudates, which are the result from the energy input, can generally only be characterized by physical, chemical and physiological analysis after extrusion. These are the target parameters, which will be explained in more detail in the next paragraph.

TABLE 6

Model used to describe the extrusion-cooking process[36]

Process parameters Y=f(X)	**System parameters** Z=f(Y)	**Target parameters**
↓	↓	↓
X	Y	Z
Process conditions	Specific mechanical energy	Protein dispersibility
- barrel temperature	Product temperature	Nitrogen solubility
- screw speed	Residence time	Antinutritional factors
- screw configuration	Pressure at the die	Urease activity
- compression ratio	Net Power	*In vitro* digestibility
- die hole		*In vivo* digestibility
- mass flow		Color
		Thermal behavior
Raw material composition		Reactive lysine
- moisture content		Hardness
- protein, NSP, fat		Maillard reaction
- pH		

Target parameters: Monitoring of the extrusion cooking process

Proper extrusion of raw materials includes precise control of temperature, residence time, moisture content and shear forces. Adequate screening methods are necessary to monitor the chemical, physical and physiological changes as a result of processing. Optimal processing means a destruction of ANFs and, if possible, complete denaturation of proteins. Under- and overprocessing can result in a product with a lower nutritional quality. Underprocessing results in a failure to inactivate heat labile ANFs, while overprocessing may result in a reduced availability of lysine.

In-vitro protein digestibility

The most important parameter in evaluating protein quality as a result of processing is the nutritional value. For that reason all kinds of *in vitro* methods have been developed. A rapid multi-enzyme technique for estimating the *in vitro* protein digestibility was suggested by Hsu et al.[37]. They measured the pH drop after adding proteolytic enzymes to samples containing the processed proteins. The results they obtained were closely related with the *in vivo* digestibility. Later, this method was modified by keeping the pH constant during enzyme incubation. The amount of sodium hydroxide titrated, in order to neutralize the production of hydrogen ions, was an indication of the *in vitro* protein digestibility[38]. Shear forces are suspected to play an important role in the final protein structure formation during extrusion. Increasing shear forces denatured proteins more easily and they are, therefore assumed to be, more accessible for enzyme attack[39]. Theoretical calculations and experimental results indicated that shear has an influence on the denaturation of the main storage proteins in SBM[40]. If energy is in excess, all kinds of cross-linking reactions of protein molecules can occur e.g. Maillard reaction or lysinalanin formation, resulting in a decreasing nutritional value of the meals[41].

The relation between *in vitro* and *in vivo* protein digestibility has always been questioned. However, determination of the *in vivo* digestibility is time consuming and mostly very expensive. The same, to a lesser extent, can also be concluded for the determination of the *in vitro* digestibility. In the past, some rapid screening methods were developed to monitor protein denaturation and, therefore, the nutritional value.

Urease activity

In the feed industry the urease activity, measured as a pH rise in an ammonia solution, has long been used to monitor the heat treatment on soy products. It is said that destruction of urease parallels that of the trypsin inhibitor inactivation[42]. It should be pointed out that this may not be valid throughout the whole inactivation process. While the urease activity is a satisfactory target parameter after extrusion of soy products up to the point of optimum heating, it is of no value in detecting possible overprocessing[43].

Protein Dispersibility Index (PDI)

As stated before, protein denaturation is accompanied by a decrease in protein solubility. By measuring the protein solubility, one can get a good impression to what extent proteins are denatured. The protein dispersibility index (PDI) is often used to characterize raw and processed materials. This method, also called the 'fast stir' method, measures the amount of proteins which are dispersible in water. Untreated raw materials have PDIs of about 80%, while thermo-mechanical treated materials have PDIs of 20-

40%. It appeared that after extrusion of untoasted SBM at 120°C and at low shear levels, the PDI dropped to levels below 10%[44]. In another study, the PDI also decreased after spray drying, toasting and extrusion of defatted soy products[45]. However, the use of the PDI seems to depend on the type of material. After extrusion of peas, the PDI was a much better indicator in evaluating protein quality[46] than after extrusion of SBM.

Nitrogen solubility index (NSI) in potassium hydroxide

In quality programs in the American poultry industry, an alternative method of evaluating the protein quality as a result of thermo-mechanical treatment has been used frequently[43,47]. In this method the proteins are extracted in alkaline solutions using 0.042 M potassium hydroxide. Several studies were performed to evaluate the usefulness of the nitrogen solubility index (NSI) in potassium hydroxide. After autoclaving, the solubility ranged from approximately 73 to 85% and appeared to be consistent with optimal SBM processing[43]. In another study SBM was also autoclaved. Related to a growth experiment with broiler chickens, it was found that protein solubility in potassium hydroxide in excess of 85% or less than 70% indicate under- or overprocessing of SBM[47]. However, after autoclaving full-fat Kunitz trypsin inhibitor-free soybeans, the NSI in potassium hydroxide seems a less sensitive target parameter in indicating underprocessing[48]. On the other hand, after autoclaving canola meal[49] and sunflower meal[50], the NSI showed to be useful in indicating overprocessing.

There is a lack of information about the use of the NSI in potassium hydroxide as an indicator for protein quality after extrusion cooking.

Antinutritional factors

In SBM processing, the trypsin inhibitor activity (TIA) and lectin content are the most important ANFs. Both are heat labile and can be effectively inactivated by thermo-mechanical treatments, but their destruction in soy products by extrusion cooking is a function of temperature, residence time, moisture content and particle size[23,40,44,51,52]. For the TIA, the method of Kakade[53] is still the most widely used assay in SBM processing. Monitoring of the extrusion process by determination of tannins and phytate is less important after heat treatment of SBM.

Theoretical calculations show that some influence of shear forces on the inactivation of trypsin inhibitors can not be excluded. However, after developing a trypsin inhibitor inactivation model, Van den Hout[40] concluded that there was no indication that shear forces are involved in the inactivation of trypsin inhibitors during extrusion cooking of SBM.

Thermal behavior

The denaturation temperatures (T_d) of most proteins in solutions are usually below 100°C. However, the moisture content during extrusion, which is in general lower than 30%, affects the T_d. Since differential scanning calorimetry (DSC) has proven to be a valuable tool in the analysis and finger-printing of a wide array of proteins and proteinaceous materials, it has been applied to the study of protein denaturation. With this method the heat needed for denaturation of proteins, which is an endothermic process, can be measured. The lower the moisture content the higher the T_d[54]. From a study of Kitabatake and Doi, the T_d of the main storage proteins in SBM, β-conglycinin (7S) and

glycinin (11S), appeared to be 76.5 and 93.3°C, respectively at a moisture content of 94%. If the moisture content decreased to 29% or less no T_d of glycinin could be found and the T_d of the β-conglycinin shifted to a temperature exceeding 180°C[55]. In another study, toasting of soy flour at 137°C only caused a partial (about 40%) denaturation[56]. Proteins become stable towards heat when the moisture content is lower than 30%. It is expected that additional shear forces during processing can lower the T_d compared with no shear processes.

Maillard reaction

The processing conditions used in extrusion cooking, high temperature and a relative low water content, are known to favor Maillard reactions. Repercussions are both nutritional, through losses of available lysine, and sensorial, as they influence the appearance of products through the formation of colored compounds[57]. The reaction starts with a nucleophilic addition of the amino group of an amino compound to the electrophilic carbonyl groups of a reducing sugar, forming a glycosylamine. This is still a reversible reaction, which is followed by a re-arrangement of the glycosylamine to so called Amadori compounds. These latter compounds are rather stable and once formed the reaction is irreversible. Amadori compounds are further degraded, via complex reactions, to furfurals, aldehydes and melanoidines[9].

There are several ways to estimate the extent in which Maillard reactions may occur during extrusion cooking. It is possible to measure the decrease in total sugar content[58], the decrease in reactive lysine[33], the amount of hydroxymethylfurfural[59], or by measuring the color of the heat treated raw material[60]. By measuring the color of the extrudates after extrusion, Sgaramella and Ames[61] found that decreasing the moisture content from 18 to 13% had a greater effect on color intensity than increasing the die temperature from 125° to 135°C.

New trends in monitoring extrusion cooking in more detail

Both NSI and *in vitro* protein digestibility are methods giving a different, but overall view of the effects of extrusion on protein behavior. However, these methods are inadequate to study the different types of interactions in proteins as a result of processing. In the feed industry there is a growing need to study the influence of thermo-mechanical treatments on protein and carbohydrates components in more detail. If it is known that a certain protein or carbohydrate fraction of the total SBM may stay behind in nutritional value after a thermo-mechanical treatment, attention can be focused on that particular fraction, by changing process conditions, in order to try to increase its nutritional value.

Solubilization measurements in monitoring protein interactions

One topic is the study of the different types of interactions between proteins and how they are affected as a result of processing. The interactions involved in the process of folding and formation of proteins are both non covalent as well as covalent. Non covalent interactions, which stabilize the native conformation of proteins and, therefore, influence their functional behavior are: hydrophobic interactions, electrostatic interactions (hydrogen bonds and dipole-dipole) and van der Waals interactions. The strength of these interactions are relatively small, especially the dipole-dipole- and the van der Waals interactions, but the amount of non covalent interactions are numerous and are, therefore,

playing an important role in the protein structure.

The most important covalent bond is the disulfide bridge between two cysteine amino acids. The number of these bonds in a molecule is relatively small, however, a considerable amount of energy is needed in order to cleave these types of linkages.

During extrusion cooking, proteins are unfolded and thereby covalent and non covalent interactions are broken. The process of denaturation can be followed by aggregation or association reactions. One approach for studying protein-protein interactions is based on protein solubilization assays. Reagents with a known mode of action were used. Addition of urea will cause gross solubilization of denatured molecules and small aggregates held together by hydrophobic and hydrogen bonds. Sodium sulfite or dithiothreitol (DTT) can be added to cleave disulfide bonds in the larger aggregates, which remained insoluble after urea extraction[35].

Hager[62] applied this method to characterize extruded soybean concentrate. This study provides evidence that intermolecular disulfide bonding is an important factor contributing to extrudate structure, at least for extrusion temperatures below 150°C. Around temperatures of 140°C, the activation energy for breaking of peptide bonds was not attained[62]. In other studies on protein interactions in soy processing, also using the same kind of solvents as Hager, it is claimed that disulfide bonds were of negligible importance, suggesting that new peptide bonds were formed under severe process conditions[41,63,64]. However, recent studies on soy extrusion, carried out at 140 to 180°, showed that disulfide bonds followed by non covalent interactions were the prevalent types of protein-protein interactions in the extrudates[35,65,66].

Protein denaturation

Denaturation process

Extrusion cooking is often used to increase the nutritional value of proteins by inactivation of ANFs and protein denaturation. Inactivation of ANF only is insufficient to explain the increase in protein digestibility obtained after thermo-mechanical treatments[2]. This suggests that other influences like protein denaturation could be an important process in improving the protein digestibility.

Protein denaturation can be defined as any change in the conformation of a protein that does not involve the breaking of peptide bonds[34]. Most proteins undergo structural unfolding followed by aggregation when subjected to moist heat or shear. Unfolding is usually a reversible process and if the extrusion process is stopped before aggregation begins, the protein can return to its native conformation[67]. Thermo-mechanical treatment may also result in the breaking of covalent bonds. During denaturation, hydrophobic groups are uncovered resulting in a decreased solubility of the proteins in aqueous solutions. The process of unfolding and aggregation is in the literature often combined to one parameter called protein denaturation.

If during extrusion cooking the specific mechanical energy (SME) is high enough, protein denaturation can be followed by association and dissociation reactions and also by breaking or formation of some covalent bonds e.g. hydrolysis of peptide bonds,

modification of amino side chains (lysinalanine or Maillard products) and the formation of new covalent isopeptide cross-links[41]. Upon cooling large protein complexes can be formed by inter- and intra molecular interactions.

Factors determining protein denaturation during extrusion cooking

From Table 5 it can be concluded that temperature, moisture content and shear forces are the main factors influencing the denaturation process. To a lesser extent the residence time, pH and the presence of other components can be of some importance. Parameters like moisture content, temperature and shear forces are highly interrelated[68].

Moisture content, temperature and shear forces

During extrusion, mechanical dissipation of the energy input, by friction of the particles in the extruder channel, will result in an increase in the product temperature. If the initial moisture content of the raw material is increased, the viscosity and, therefore, the viscous dissipation will decrease resulting in lower product temperatures. Proteinaceous rich materials under extrusion conditions behave like non-Newtonian fluids. Therefore, viscosity is observed to be highly dependent on moisture content, temperature and shear rate. In those processes it is almost impossible to change one of the parameters while keeping the other process conditions constant.

Residence time distribution

Residence time is a process parameter which can relatively easy be adjusted during extrusion cooking. For extrusion, but also expansion, steam explosion, pelleting and grinding, the residence time is in the magnitude of seconds, while treatments like toasting, drying, granulation and precondition are processes in which the residence is mostly expressed in minutes. More important in extrusion cooking is the residence time distribution (RTD). A high RTD means that parts of the proteinaceous materials are exposed for a longer time to heat and shear than others resulting in deviated chemical, physical and physiological behavior of the extrudates. The RTD can be measured, but it is a result of the process parameters used during extrusion, like, mass flow, screw configuration and screw speed[69].

pH

The pH has also an influence on protein denaturation. Greater thermostability was seen in the isoelectric region (pH 4-5) where the net charge of the proteins is low. As one moves away from the pH region ($pH < 4$ or $pH > 9$) the T_d decreases considerably. These extreme pH values are not favorable in the feed industry, because of unwanted side reactions like the Maillard reaction[41].

The influence of other components on protein denaturation

Protein denaturation may also be influenced by the presence of other components. The presence of lipids in the raw material, e.g. full fat soybean meal, will lead to a more greasy dough in the extruder channel, which will result in a lower development of shear forces during the process. This may result in the prevention of expansion of proteins, which is necessary for a good texturized product[32]. In addition, lipids may be responsible for negative effects such as autoxidation, thermal degradation and polymerization

exhibited during extrusion. The denaturation of proteins during extrusion is also inhibited by the presence of cell wall polysaccharides. It is proposed that, in some way, the embedded carbohydrate effectively stabilizes the hydrophobic interactions or gives rise to additional stabilizing forces[12]. In the presence of carbohydrates, extrudates were more resistant to stress and the protein matrix was less disrupted than without carbohydrates[34]. Reducing sugars can play an important role in the Maillard reaction. The process conditions used in extrusion cooking, such as a high temperature and a low moisture content, are known to favor the Maillard reaction. Clearly, the interaction with other food components during processing on the protein denaturation merits further research.

Aim and outline of this thesis

The effects of moisture content, screw speed and product temperature during single-screw extrusion on chemical, physical and nutritional parameters are well documented. However, there is a lack of knowledge about the influences of shear forces during extrusion cooking, especially shear forces developed by the use of different screw configurations. It is also not known which target parameters can be best used in monitoring the extrusion process.

The aim of this thesis is therefore 1) to study the effect of different shear forces during SBM extrusion, 2) to develop new monitoring target parameters in extrusion technology and elucidate their usefulness and 3) to compare the chemical, physical and nutritional changes in SBM as a result of single-screw extrusion with changes obtained after toasting of SBM.

In a preliminary experiment (**chapter 2**), temperature, moisture content and shear forces (by changing the screw speed) were varied during extrusion of toasted and untoasted soybean meal (TSBM and USBM, respectively). This experiment was performed in order to confirm the presumption that in the extrusion process shear forces could be responsible for an additional effect in improving the nutritional value of extruded SBM. Several target parameters were tested for there usefulness as an indicator for protein quality.

Because of the close relationship between screw speed and residence time, special screw tips were developed in order to study in more detail the variation in shear forces, without too much disturbance in residence time. The effects of toasting and extrusion (with none, one or two twin lead slotted screws or torpedo elements with different lengths) of SBM and rapeseed meal on the target parameters PDI, NSI in potassium hydroxide and pH-STAT protein digestibility are described in **chapter 3**.

In **chapters 4 and 5**, the physiological effects of SBM toasting and extrusion at different shear levels by using twin lead slotted screws and torpedo elements, respectively, were studied. A growth experiment with broiler chickens was used to study growth performance and several *in vivo* apparent ileal nutrient digestibilities. In addition, the effect of adding hydrolytic enzyme preparations was studied. Growth performance and several *in vivo* apparent ileal nutrient digestibilities in broiler chickens were studied by adding proteases, carbohydrases and combinations of both to diets containing toasted and

extruded SBM.

In vitro experiments were performed in order to study the effect of proteases and carbohydrases on toasted and extruded SBM samples. Several techniques were used in monitoring *in vitro* breakdown of proteins and carbohydrates as a result of enzymic activity. The results are presented in **chapter 6**.

Finally, a study was conducted to study the effect of toasting and extrusion on protein interactions during SBM extrusion. Results are compared with the results obtained after toasting of SBM. Shifts in yields of extractable protein from differently processed SBM were measured by using two sequential extraction procedures. The protein fractions obtained were also analyzed for their *in vitro* protein digestibility and trypsin inhibitor activity. The results are given in **chapter 7**.

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chapter 2

Effect of extrusion on the *in vitro* protein digestibility of toasted and untoasted soybean meal

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In: Recent advances of research in antinutritional factors in legume seeds. Wageningen Press: Wageningen, 1993, 461-465.

ABSTRACT

Soybean meal (SBM) was extruded with a single-screw extruder at different extrusion conditions (initial moisture content, screw speed and temperature of the product at the die) in order to study the effect on protein solubility, trypsin inhibitor content, *in vitro* protein digestibility and urease activity.

The results shows that, besides trypsin inhibitor inactivation, also non-ANFs effects are responsible for an increasing *in vitro* protein digestibility. Furthermore, it is concluded that the urease activity is not a suitable parameter for evaluation of the extruded products. Instead of urease activity, the nitrogen solubility index in potassium hydroxide is a more promising quality parameter, because this method provides more distinct differences between extruded products as a result of processing even under extreme extrusion conditions.

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chapter 2

INTRODUCTION

Extruders, which are described as "high temperature short time reactors", are commonly used in the food and feed industry. In these extruders a wide range of biopolymer reactions may occur, such as gelatinization and cross linking of starch, browning reactions and protein denaturation including enzyme inactivation[1]. The process time, together with factors such as moisture content, shear forces, feed rate, temperature and screw configuration are decisive variables to these biopolymer reactions[2].

In the animal feed industry raw feed materials such as soybean meal (SBM) or rapeseed meal are extruded in order to increase their nutritional value by inactivating antinutritional factors (ANFs) like trypsin inhibitors, lectins and tannins[3,4].

It is known that overprocessing may result in undesired effects like the occurrence of Maillard reactions. The processing conditions used in extrusion cooking, such as a high temperature and a low moisture content, are known to favor this reaction[5]. Repercussions are both nutritional, due to losses in the availability of lysine resulting in a decreased availability of amino acids, and sensorial, as they influence the appearance of products through the formation of colored compounds[6].

The urease activity test has been frequently employed to determine the quality of processed SBM. However, there are some doubts about the accuracy of this method in treatments where it is necessary to detect a possible overprocessing[7]. An alternative method to evaluate the quality of processed SBM is the protein solubility in potassium hydroxide[8]. The protein solubility in potassium hydroxide is virtually 100% in raw SBM.

The purpose of this work was to study the effect of different extrusion conditions on the *in vitro* protein digestibility, the trypsin inhibitor activity, the protein solubility, and the urease activity in untreated and extruded SBM samples.

MATERIALS AND METHODS

Toasted soybean meal (TSBM) and untoasted soybean meal (USBM) were extruded on a single-screw Almex Battenfield-extruder at screw speeds of 80 and 140 rpm, initial moisture contents of 26 and 35%, and temperatures, measured in the product at the die, of about 90° to 140°C. After extrusion all samples were dried for 48 h at 40°C and ground to pass a 0.2 mm sieve.

The protein solubility was determined by the nitrogen solubility index (NSI) in 0.042 M potassium hydroxide[8]. The *in vitro* protein digestibility was performed with the pH-STAT method according to the method described by Pederson and Eggum[9]. In these experiments sodium caseinate was used as standard. The trypsin inhibitor activity (TIA) was performed with a modified Kakade method according to Smith et al.[10] and the urease activity (UA) was measured by pH change.

RESULTS AND DISCUSSION

The influence of extrusion on the TIA is given in Figure 1 (A and B) and it is evident that besides extrusion also toasting is an effective process for inactivation of the trypsin inhibitors, because extrusion of TSBM has no further effect on the inactivation of TIA.

The results of the *in vitro* protein digestibility determined with the pH-STAT method are given in Figure 1 (C and D). The untreated USBM showed a poor *in vitro* protein digestibility (18%), extrusion resulted in a large increase in *in vitro* protein digestibility (Figure 1; C). In TSBM the *in vitro* protein digestibility of the unextruded sample was 59% and toasting increased the protein digestibility by more than 40%, probable due to trypsin inhibitor inactivation. Extrusion of TSBM gave a further increase in the protein digestibility at temperatures exceeding 110°C (Figure 1; D). This effect cannot be ascribed to trypsin inhibitor inactivation, because there is no effect of extrusion on these ANFs (Figure 1; B). This suggest the presence of non-ANF effects resulting in a higher *in vitro* protein digestibility. The temperature is probably one of the processing variables involved with these non-ANF effects. In Figure 1 (C and D) it is evident that extrusion at a high screw speed in combination with a low moisture content results in a high *in vitro* protein digestibility, indicating that shear forces could be responsible for the increasing nutritional value of SBM after extrusion.

The effect of extrusion on the UA is shown in Figure 2 (E and F). It is obvious that the UA is not a very accurate method in evaluating the quality of extruded soybean products. Comparing of the UA with the *in vitro* protein digestibilities shows that meals with zero urease activities do not necessarily have impaired nutritive value.

The NSI in potassium hydroxide of the extruded samples are given in Figure 2 (G and H). For USBM as well as TSBM samples, the NSI in potassium hydroxide is a much reliable parameter in evaluating the processed soybean samples, because there are clear differences in NSI values even at higher extrusion temperatures. The NSI in untreated USBM is almost 100% and decreased to 35% under extreme extrusion conditions. In TSBM the unextruded sample showed a NSI value of 72% as a result of toasting and decreased to 45% during extrusion at 140°C. A high moisture content has more impact on the NSI than a low moisture content, probably due to a better heat conduction in the high moisturized samples. There was also a tendency to lower NSI values if the was screw speed decreased due to longer residence times in the extruder.

ACKNOWLEDGEMENT

This research was financially supported by Nutreco Agribusiness R&D, Boxmeer, The Netherlands.

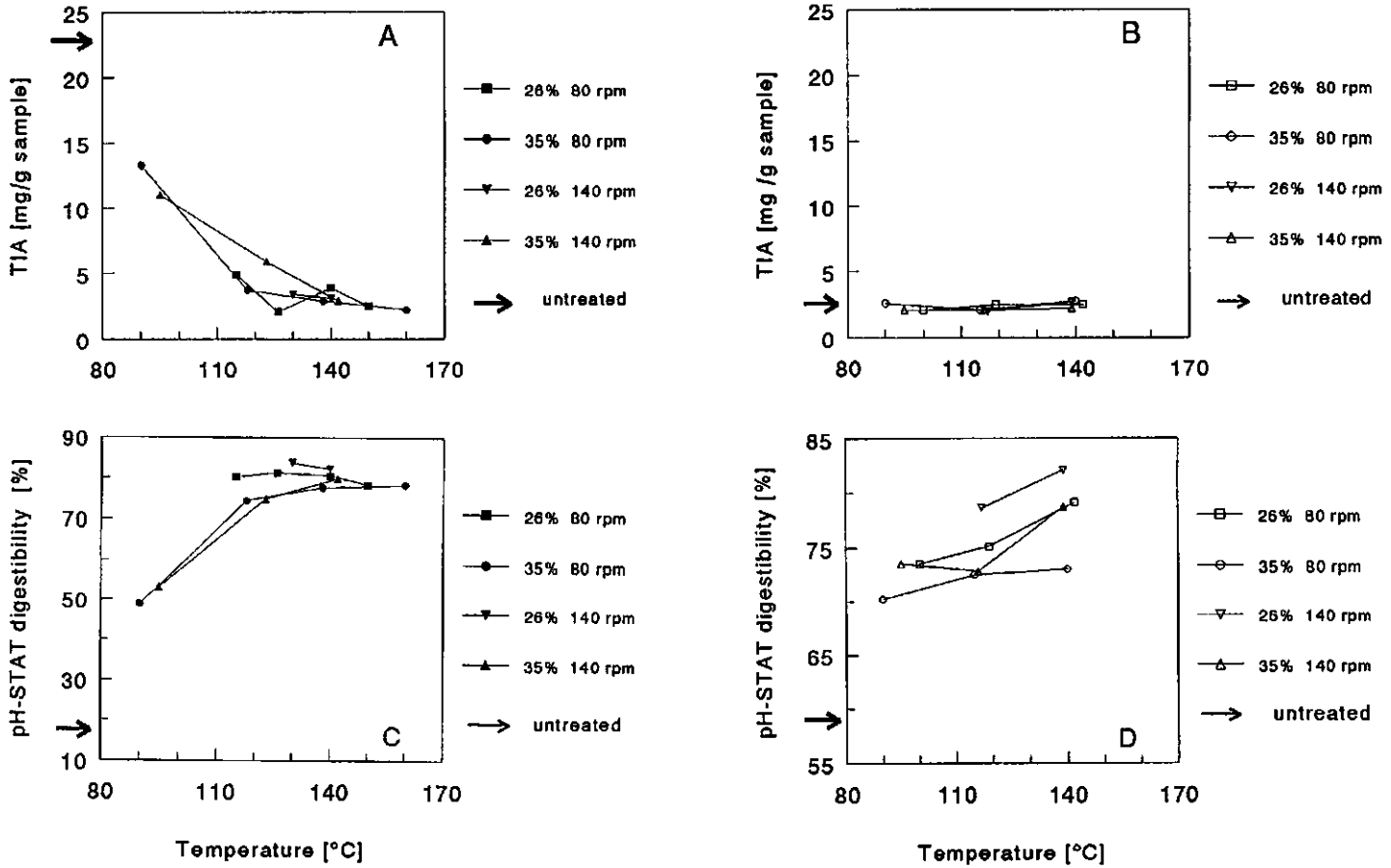

FIGURE 1

The influence of extrusion on the trypsin inhibitor activity (TIA) and pH-STAT digestibility (Digest.). A and C: USBM at different extrusion conditions, B and D: TSBM at different extrusion conditions

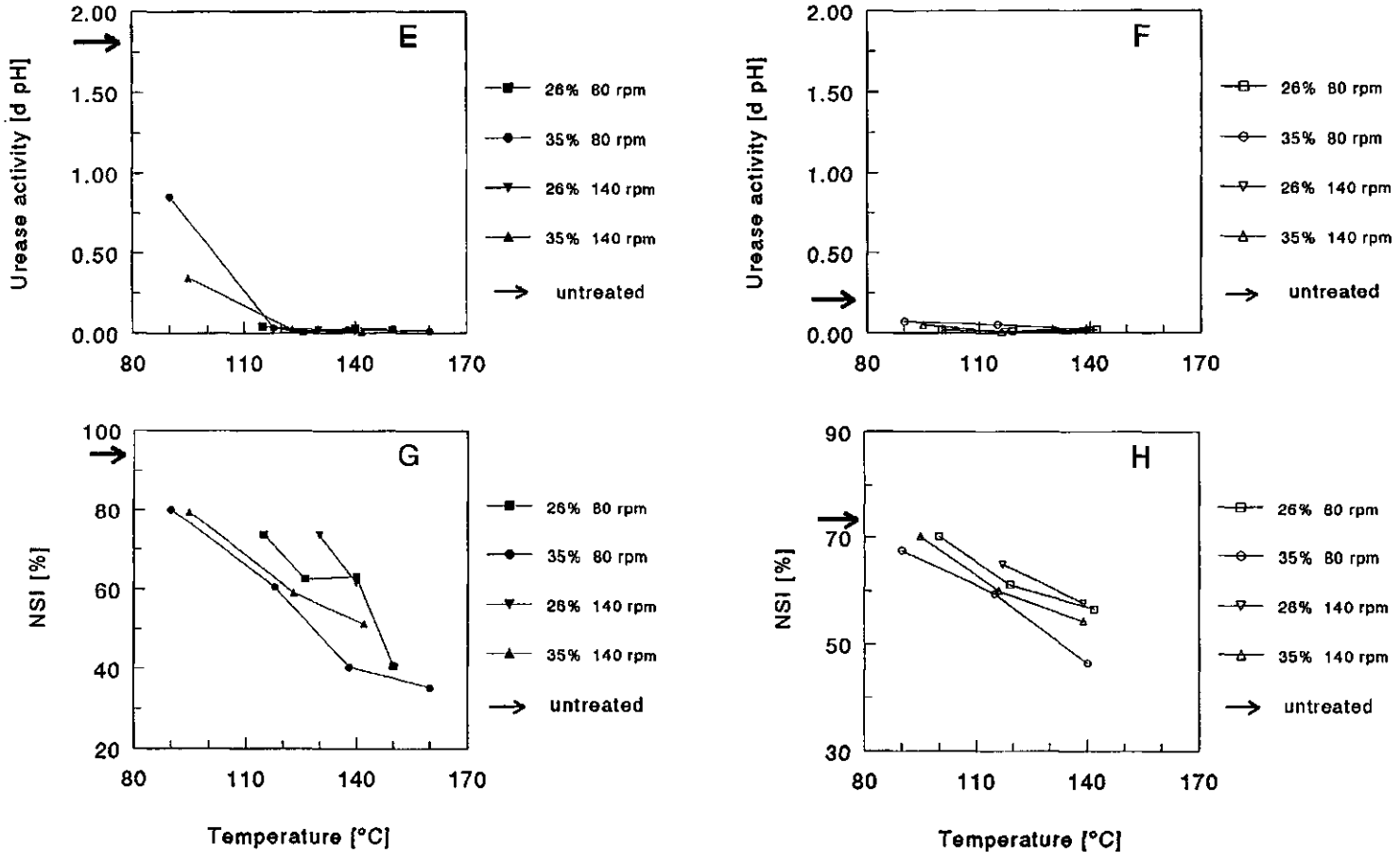

FIGURE 2

The influence of extrusion on the urease activity (d pH) and nitrogen solubility index in potassium hydroxide (NSI). E and G: USBM at different extrusion conditions, F and H: TSBM at different extrusion conditions

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chapter 3

The influence of screw configuration on the *in vitro* protein digestibility and protein solubility of soybean- and rapeseed meals

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Journal of Food Engineering, 26, (1995) 13-28

ABSTRACT

Toasted soybean meal (TSBM), untoasted soybean meal (USBM), and toasted rapeseed meal (TRSM) have been extruded in a single-screw extruder at various shear and mixing levels. Extrusion experiments were performed with different torpedo elements and twin lead slotted screws. Protein dispersibility index (PDI), nitrogen solubility index (NSI) in diluted potassium hydroxide, and *in vitro* protein digestibility, measured with the pH-STAT method, have been used as parameters to characterize the extrudates. It followed that PDI was not a suitable parameter to differentiate between extrusion conditions. The NSI was shown to be a better indicator to evaluate the effect of extrusion on protein solubility with various torpedo elements. Extrusion significantly increased the *in vitro* protein digestibility of all extruded samples. However, even higher values were obtained if TSBM and USBM were extruded with a torpedo mixing element provided with four rows of flights. Longer torpedo elements however, resulted in decreasing *in vitro* protein digestibilities. In practice twin lead slotted screws are commonly used. Extrusion of TSBM and TRSM with these screws resulted in an increasing *in vitro* protein digestibility. In these cases no optimum was noticed. NSI values remained unaffected as a result of extrusion with twin lead slotted screws.

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chapter 3

INTRODUCTION

Extrusion-cooking is a cooking process also used in the production of animal feed. The extruder can be considered as a continuous chemical reactor for processing feed mixes at high temperatures (up to 250°C) for relatively short residence times (usually 0.5-2 min). Other extrusion characteristics are high pressure (up to 25 mPa), development of shear forces, and also a wide range of moisture contents (below 30%).

Soybeans and rapeseed are two major oilseed crops. After oil extraction the meals can be heat treated by extrusion-cooking in order to increase their nutritional value by denaturation of proteins and/or inactivation of antinutritional factors. The effect of extrusion-cooking on soybean meal has been widely reviewed[1-3]. Less is known about extrusion-cooking of rapeseed meal. The amino acid content of the proteins in rapeseed meal compares well with that of soybeans. Both crops have a well-balanced amino acid composition and are especially rich in lysine and sulphur-containing amino acids[4]. However, a high level of phenolics, phytic acid, glucosinolates, and fibers prevents the full use of rapeseed proteins[4-7].

In order to obtain a good-quality product it is necessary to control the reactions by the extrusion conditions. Meuser and Wiedmann[8] classified the parameters in the extrusion-cooking as process, system, and target parameters.

Process parameters

These are variables of both extruder and raw material such as mass flow, moisture content, screw speed, barrel temperature, and screw configuration. Process parameters can be adjusted and continuously measured.

System parameters

System parameters or technological parameters are e.g. the mechanical and thermal energy inputs into the mass as well as the residence time in the extruder. An extruder is a complex feed reactor. During extrusion two main energy flows have to be considered. The first one is the mechanical dissipation of the motor power by means of friction of the particles in the extruder channel. The second one is the flow of heat transferred through the barrel wall. For single-screw extruders, processing highly viscous materials with a high velocity, the second energy flow can be neglected[9]. In extruder technology the mechanical energy is an important system parameter. The amount of mechanical energy which works on 1 kg mass during extrusion is defined as the specific mechanical energy (SME). In formula form:

$$SME = \frac{P_n}{Q_m} = (C_p\Delta T + \epsilon\Delta H_w + H_v) \quad [J/kg] \qquad (1)$$

where:

P_n = net motor power [J/s]
Q_m = mass flow [kg/s]
C_p = specific heat of the material [J/°C]
ϵ = the fraction vaporized on the total mass [-]
ΔH_w = vaporizing enthalpy of water [J/kg]
H_v = phase transition enthalpy [J/kg]

Protein rich materials under extrusion conditions behave like non-Newtonian fluids. Therefore, viscosity (η) is observed to be highly dependent on shear rate ($\dot{\gamma}$), moisture content (M), and temperature (T). The relation between those parameters can be expressed as[10]:

$$\eta(\dot{\gamma},T,M) = m\dot{\gamma}^{n-1} \exp -\Delta E/RT \exp(KM) \quad [Pa.s] \quad (2)$$

where:

m = consistency factor [-]
n = flow behavior index [-]
ΔE = activation energy [J/mol]
R = universal gas constant [J/kg K]
K = constant [-]

An important element of extrusion cooking is that the material is brought under the influence of shear. A rough approximation of the total shear strain (γ) can be calculated as:

$$\gamma = \bar{t} * \dot{\gamma} = \bar{t} * \pi ND / H \quad [-] \quad (3)$$

where:

$\bar{t}$ = mean residence time [s]
N = screw speed in revolutions per second [s^{-1}]
D = barrel diameter [m]
H = channel depth [m]

Target parameters

The effect of extrusion-cooking on raw materials can generally be characterized by physical and/or chemical analysis. Those extrudate characteristics are the target parameters.

Solubility is an important target parameter. In the animal feed industry the protein dispersibility index (PDI) is often used to characterize the protein-quality of raw materials. With this method, which is also called the "fast stir" method, it is possible to measure the amount of proteins which are dispersible in water. During conventional toasting, in order to inactivate antinutritional factors, proteins are denatured and protein aggregates are formed. Raw materials which are untreated have PDIs of 70-90%, while heat treated materials have PDIs of 20-40%. An alternative method of evaluating the processing of the soybean meal is the protein solubility in a 0.042 M potassium hydroxide solution. This method has been used in quality control programs by several major feed companies in North America and is widely used by the Brazilian poultry industry[11]. Its usefulness as an indicator of protein-quality in rapeseed meal has not been thoroughly evaluated. Hafermann et al.[12] concluded that protein solubility in potassium hydroxide may be an useful indicator in establishing overprocessing of rapeseed meal and that protein solubility values of 35% and lower are suggestive of overprocessed rapeseed meal.

Nutritional value is another important target parameter in evaluating protein quality of extruded soybean- and rapeseed meal. Enzymatic hydrolysis of soybean proteins has been used as an *in vitro* measurement of digestibility. Hsu et al.[13] devised a

rapid multi-enzyme technique for estimating protein digestibility and they observed that these values correlated closely with *in vivo* studies. Rather than measuring the pH drop like Hsu et al.[13], other authors measured the production of hydrogen ions at a set pH using a pH-STAT method[14,15]. With this procedure the effects of changing pH on the proteolytic enzymes were minimized and the buffering capacity by newly released amino groups was eliminated[16].

In a previous study[17] it was observed that the effect of shear was important for both the solubility and the *in vitro* protein digestibility. The purpose of this work was to study the effect of different shear and mixing levels on the protein solubility and *in vitro* protein digestibility of soybean- and rapeseed meal. In addition, the effect of moisture content and product temperature has been studied.

MATERIALS AND METHODS

Materials

Commercial solvent-extracted and toasted soybean meal (TSBM) with a protein content (N x 6.25) of 58.5% on a moisture-free basis was supplied by Cargill, Amsterdam, The Netherlands. The toasted soybean meal had a PDI of 20%. From the same batch of solvent-extracted soybean meal a part was not toasted but air dried at room temperature. This untoasted soybean meal (USBM) had a PDI of 80%. Commercial double zero rapeseed meal (TRSM) was also supplied by Cargill. The protein content of the rapeseed meal was 44.0% (N x 6.25) on a moisture-free basis. The PDI of the TRSM was 10%. TRSM was ground before is was used for extrusion.

Extrusion-cooking

Extrusion trials with TSBM and TRSM were both performed on two different single-screw extruders, each at different shear and mixing levels. Extrusion of USBM was only performed with so called torpedo elements.

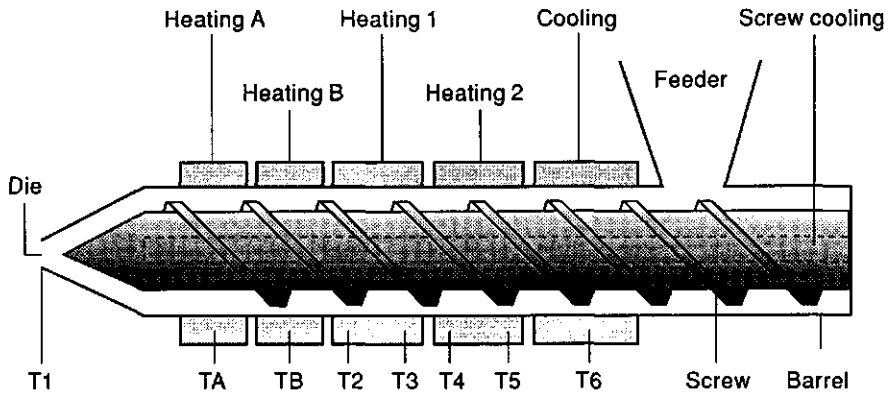

FIGURE 1
A simplified scheme of the Almex Battenfeld single extruder

Almex Battenfeld single-screw extruder

This extruder is shown in Figure 1. The length:diameter ratio (L/D) was 16. The compression ratio was 1.15. The trials were carried out with screws of a constant pitch of 32 mm and a diameter of 50 mm. The die diameter was 7 mm and the screw speed 100 rpm. Five especially designed torpedo elements (Figure 2) were used to create a difference in shear. These torpedo elements, which were assembled at the end of the screw, were equipped with: zero (0 L/D), two (½ L/D), four (1 L/D), six (1½ L/D), and eight (2 L/D) rows of flights. The row thickness was 6.5 mm. They are called torpedo screw 0, 2, 4, 6, and 8, respectively (Figure 2). Previous research showed negligible differences in residence time distributions (RTD) if a torpedo element was used compared with a smooth screw[18]. The temperatures in the different sections of the barrel were measured using eight thermocouples (Figure 1). With four heaters, which could be set individually, the temperature of the product at the die was adjusted to 120° or 140°C. Product temperature at the die was measured manually using a thermocouple, thus avoiding direct contact with the extruder wall or screws.

TSBM with a moisture content of 25%, 28%, 32%, and 35% was extruded with torpedo screws 0, 2, 4, 6, and 8 at product temperatures of 120° and 140°C. TRSM with moisture contents of 25% and 35%, and USBM with moisture contents of 25% and 30% were extruded

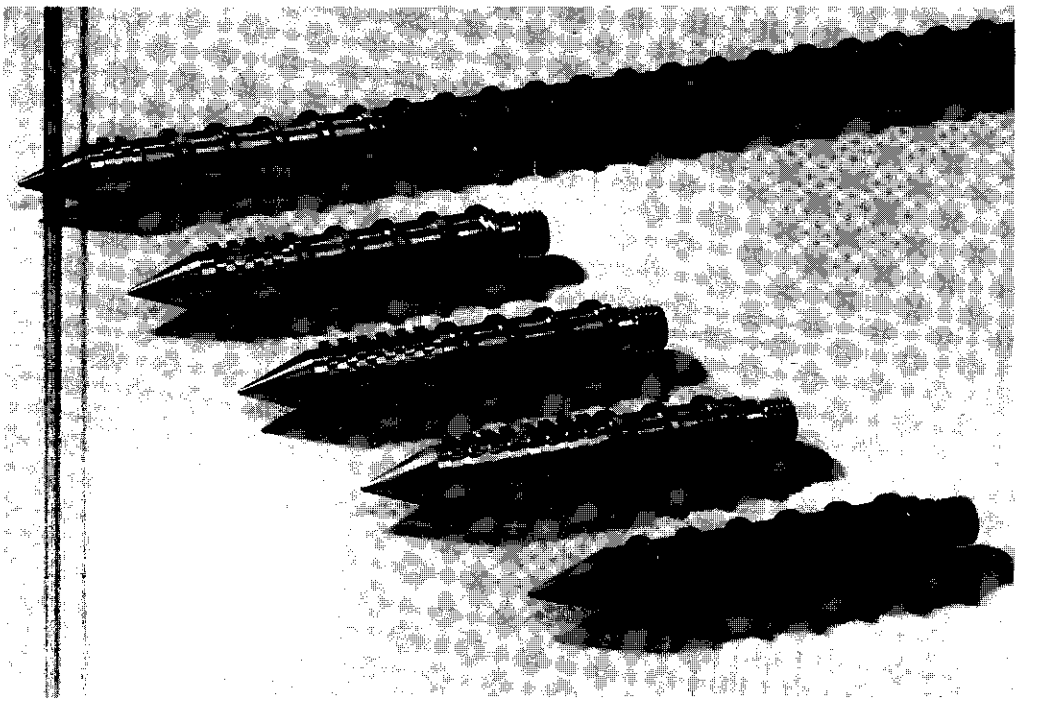

FIGURE 2

Torpedo elements. From top to bottom: torpedo screw number 2, 4, 6, 8, and 0 with, respectively, 2, 4, 6, 8, and 0 rows of flights on the screw

with the five different torpedo elements at 120°C. Moisturization was performed with a Sunther-Papenmeier mixer. It was impossible to extrude USBM with a moisture content of 35%, because a sticky dough was formed, resulting in extrusion inlet problems. The pre-moisturized materials were stored overnight at 4°C and brought to room temperature prior to extrusion cooking.

Wenger X-20 single-screw extruder

An eight-head Wenger X-20 single-screw extruder was also used. In trial 1 no twin lead slotted screws were used. In trial 2, the 8th head (element no.68321-1) at the end of the screw was replaced by a twin lead slotted screw (element no.68387-1) and in trial 3, besides the twin lead slotted screw on the 8th head, the 7th head (element no.68326-1) was replaced by a twin lead slotted screw (element no.68326-3). They are called twin lead slotted screw 0, 1, and 2, respectively. Those elements, shown in Figure 3, have the same constant pitch (13 mm) and channel depth (10 mm) as the normal elements on the screw with the exception that the twin lead slotted screws had a double row of flights interrupted by 3 axial slots on each row of flights on the same height. The other extrusion conditions were kept constant.

TSBM and TRSM were extruded with twin lead slotted screws 0, 1, and 2. The moisture content of both TSBM and TRSM was adjusted to 35% by adding water to the meals in the feeder just before extrusion. With eight steam jackets on the barrel, the temperature of the product at the die, manually measured using a thermocouple, was set at 120°C. The screw speed was 430 rpm and the die opening was 9.4 mm.

After the extrusion-cooking, the extrudates were dried at 45°C and ground to pass an 1-mm screen.

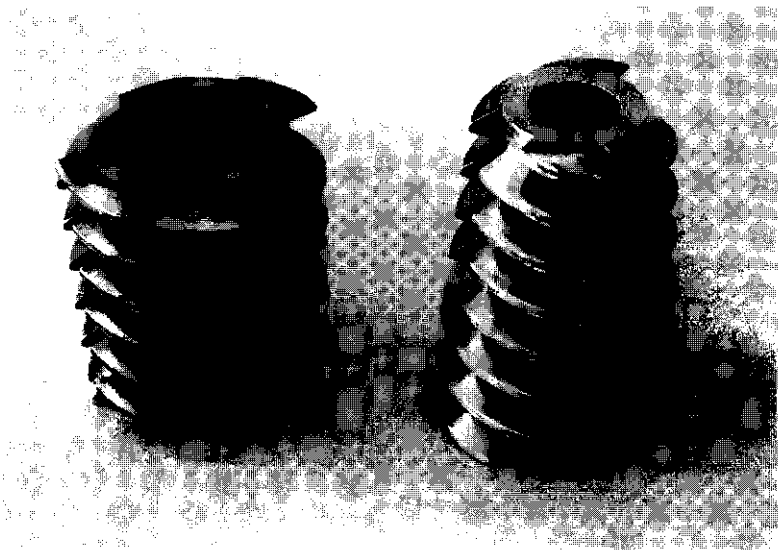

FIGURE 3

Twin lead slotted screws. Left: twin lead slotted screw number 68326-3. Right: twin lead slotted screw number 68387-1

Analytical methods

Moisture content

Moisture contents were determined according to the AACC method[19]. 5 g material was dried at 105°C for 3 h.

Protein solubility

The protein dispersibility index (PDI) was determined according to AOCS[20]. 20 g material was extracted for 10 min in 300 ml water at 8500 rpm using a Waring Blender. The temperature was maintained at 25°C using a blendercup with water cooling. The nitrogen solubility index (NSI) in 0.042 M potassium hydroxide was determined by the method described by Dale et al.[11] 500 mg material was extracted for 20 min in 25 ml 0.042 M potassium hydroxide at 500 rpm using a magnetic stirrer. The PDI and NSI were calculated as the ratio between the nitrogen content in the supernatant after extraction and the total nitrogen content of the material (%). The total nitrogen content and the nitrogen content in the supernatant after centrifugation for the NSI and PDI determinations were performed by a semi-automated micro-Kjeldahl method. Protein content was estimated by multiplying the N content by 6.25 for soybean- as well as for rapeseed meal.

In vitro protein digestibility

The *in vitro* protein digestibility was determined with the pH-STAT method with modifications[16]. Fifty ml of an aqueous suspension, containing 1 mg N/ml in distilled water, was allowed to stand at 4°C for at least one h, but no longer than 8 h. 10 ml of this suspension was adjusted to pH 8.0 using a Metrohm 614 Impulsomat and a Metrohm 665 Dosimat, containing 0.005 M NaOH under continuous stirring in a water bath at 37°C. The multi-enzyme solution, containing 1.6 mg/ml porcine pancreatic trypsin (Sigma, T0134), 3.96 mg/ml bovine pancreatic chymotrypsin (Sigma, C4129), and 1.04 mg/ml porcine intestinal peptidase (Sigma, P7500), was prepared and maintained in an ice bath and adjusted to pH 8.0 with 0.1 M NaOH until used. The titration was started, before adding the multi-enzyme to the sample solution, in order to assure the substrate was at pH 8.0 and to determine the stability of the base line. After 10 min 1.00 ml multi-enzyme solution was added and the NaOH consumption was recorded for exactly 10 min. To check the activity of the multi-enzyme solution, a control of sodium caseinate (DMV-Campina, Veghel, The Netherlands) was run for each new solution of enzymes. The amount of NaOH added during enzyme incubation has been corrected for the baseline uptake without the addition of the enzymes. The pH-STAT digestibility was calculated as the ratio between the NaOH consumption of the sample and the caseinate solution.

RESULTS AND DISCUSSION

System parameters

The relation between mechanical energy and heat transfer from the heaters to the product is given by the Brinkman number (Br):

$$Br = \frac{\mu V^2}{\lambda \Delta T} = \frac{\mu \pi^2 N^2 D^2}{\lambda \Delta T} \quad [-] \qquad (4)$$

where:

μ = dynamic viscosity [Pa.s]
V = screw velocity [m/s]
λ = heat conductivity [W/m°C]
ΔT = temperature difference between mass and barrel wall [°C]

If Br >> 1 then the heat added through the barrel wall can be neglected[9]. In order to check if the heat added through the barrel wall can be neglected when using the torpedo elements the following data: D = 0.048 m, N = 1.67 s^{-1}, μ = 2300 Pa.s for soybean meal with moisture content of 35%[21], λ = 0.43 and 0.48 W/m°C for soybean meal with a moisture content of 30% and 35%, respectively[22] were used in equation 4. From these calculations it could be concluded that if ΔT equals 30°C, Br would still be more than 10. Therefore, the electrical heat through the barrel wall can be neglected.

It is now possible to calculate the specific mechanical energy (SME). In equation 1, part of the SME is needed for heating the mass, expressed by $C_p\Delta T$. Wallapapan et al.[22] found C_p values of 2.90-3.40 kJ/°C kg for soybean meal with moisture contents of 25-35% measured at 130°C. The initial product temperature was 20°C and the temperature at the die was set on 120°C, which makes the expression $C_p\Delta T$ 290-340 kJ/kg for soybean meals with a moisture content of 25-35%. The same specific heat values have been used for TRSM. According to equation 1, another part of energy is used for vaporizing water into steam. Based on previous trials, it is assumed that maximal 4-6% of the mass is lost after extrusion at moisture contents of 25-35%. With a vaporizing enthalpy of 2300 kJ/kg for water, it can be calculated that 92-138 kJ/kg is needed for vaporizing the water from soybean- and rapeseed meal. The remaining energy can be used for other reactions, as expressed in equation 1 by H_v (phase transition enthalpy). An example of this is the denaturation of proteins which requires some energy.

The H_v values are given in Figure 4. It can be seen that extrusion with longer torpedo elements results in a higher SME surplus. The SME left for H_v in USBM is much higher than in TSBM and TRSM (Figure 4). If the moisture content increases less SME is left for H_v, which is the result of a decreasing viscosity (equation 2) of the doughs in the extruder. With increasing temperatures the viscosity will decrease, which means that less energy is necessary at temperatures of 140°C compared with 120°C (Figure 4). During extrusion with torpedo screw 0 and 2 not always a SME surplus was measured. Especially at high moisture contents the SME left for H_v appeared to be negative during extrusion of TSBM and TRSM.

TABLE 1

The influence of extrusion with torpedo elements at 120°C on the PDI [%] of TSBM and TRSM (moisture content: 35%) and USBM (moisture content: 30%)

Screw number	TSBM	USBM	TRSM
Untreated	21.2	90.8	9.6
0	7.0	8.0	5.6
2	6.8	7.1	5.2
4	7.2	7.6	5.2
6	7.5	7.2	5.2
8	6.9	6.6	5.1

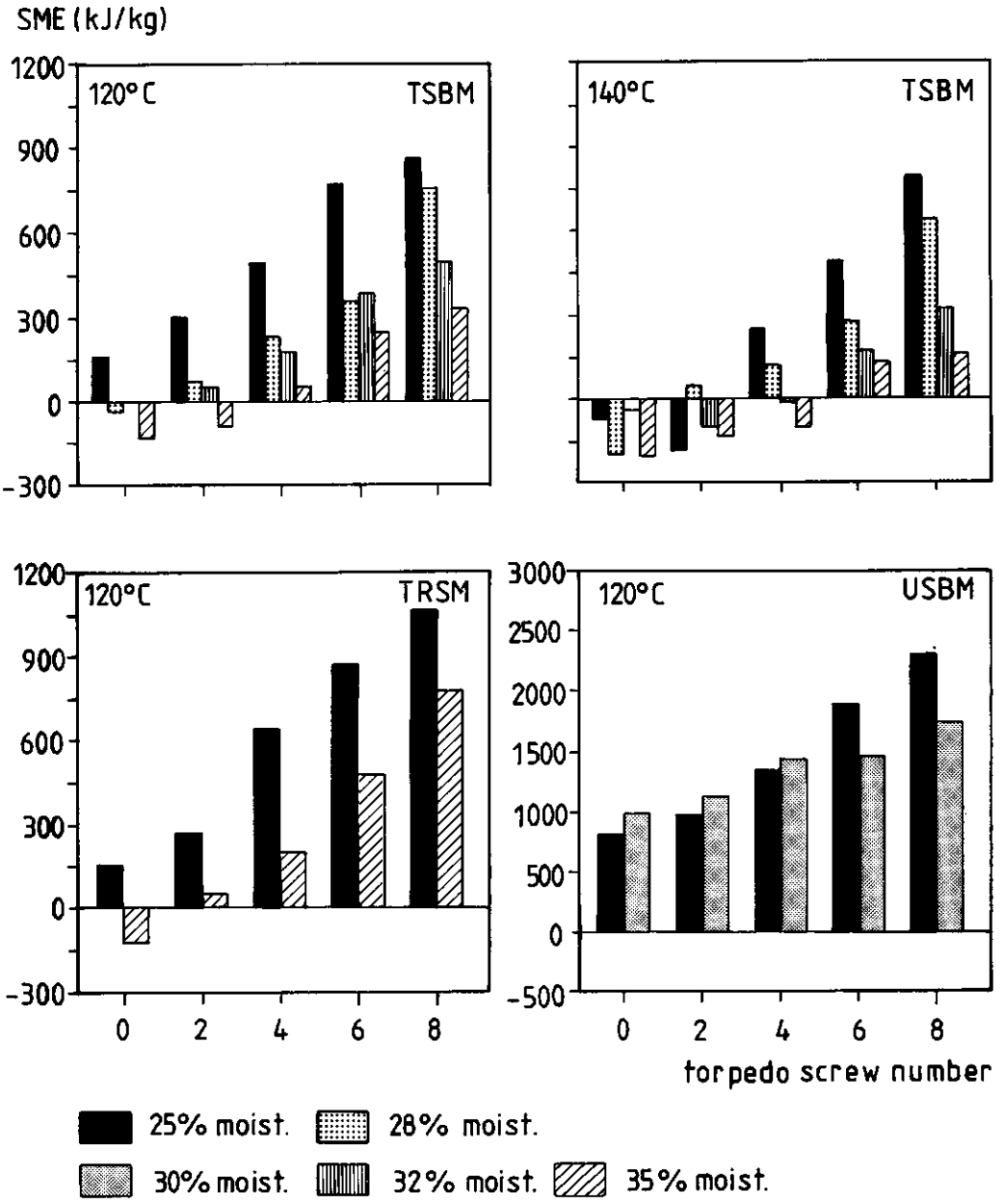

FIGURE 4

Specific mechanical energy (SME) [kJ/kg] left for transition enthalpy (H_v) after extrusion with torpedo elements at different moisture contents. TSBM at 120° and 140°C, USBM and TRSM at 120°C

TABLE 2

The influence of extrusion with twin lead slotted screws at 120°C and a moisture content of 35% on the PDI [%], NSI [%], and *in vitro* protein digestibility [%] of TSBM and TRSM

Screw number	TSBM			TRSM		
	PDI	NSI	pH-STAT	PDI	NSI	pH-STAT
Untreated	21.2	72.5	60.7	9.6	40.4	44.9
0	8.3	54.0	78.3	8.1	30.1	58.9
1	8.1	51.0	80.4	8.1	30.0	61.2
2	8.0	55.2	81.1	7.8	30.7	65.2

Protein solubility

The PDI of USBM, TSBM, and TRSM extruded with the torpedo elements and the twin lead slotted screws are given in Tables 1 and 2, respectively.

The PDI of untreated USBM was 90.8% but rapidly declines to 8.0% if it is extruded with torpedo screw 0. Extrusion with torpedo screws 2-8 did not change the PDI of USBM compared to torpedo screw 0 (Table 1). The PDI of unextruded TSBM was 21.2% and extrusion with torpedo screw 0 and twin lead slotted screw 0 was sufficient to decrease its value to 7.0% and 8.3%, respectively. Extrusion trials with TSBM with torpedo screws 2-8 (Table 1) and twin lead slotted screw 1 and 2 (Table 2) gave no further changes in PDI values. From Tables 1 and 2 the same conclusion can be drawn for extrusion of TRSM. Whereas Van der Poel et al.[23] used the PDI as a protein quality parameter during twin-screw extrusion-cooking of raw peas, in the present extrusion experiments the PDI was not a suitable parameter to differentiate between different shear and mixing levels. It is most likely that shear and heat during extrusion of soybean meal not only denatured proteins but also was responsible for the formation of large protein aggregates, which could not be made dispersible in the PDI determination. Therefore we also determined the NSI for the different extrudates. This parameter showed larger variations as a result of extrusion under different conditions (Figure 5). The NSI values of the unextruded USBM, TSBM, and TRSM were 91.1, 72.5, and 40.4% respectively, but NSI values dropped to 49.3, 36.7, and 26.3% after extrusion with torpedo screw 0 at 120°C (no further results shown). However, the use of different torpedo screws resulted also in clear differences in NSI values. In Figure 5 the NSI of TSBM extruded at 120°C and 140°C are given. It can be seen that there is an optimum in NSI values after extrusion with torpedo screw 4. Extrusion of TSBM at 120°C with moisture contents of 32 and 35% showed an NSI optimum after extrusion with torpedo screw 6 (Figure 5). At 120°C, the corresponding SME values left for H_v varied from 250 to 500 kJ/kg (Figure 4) and at 140°C from -50 to 250 kJ/kg (Figure 4). This means that a certain amount of transition enthalpy (H_v) is necessary to obtain a maximum in the NSI value, but that too much transition enthalpy will result in decreasing NSI values. Extrusion with increasing moisture contents at 120°C resulted in decreasing NSI values (Figure 5). At 140°C the obtained NSI values were, in general, lower than at 120°C, but the influence of moisture content at this temperature was not clear (Figure 5). However, with an increasing torpedo

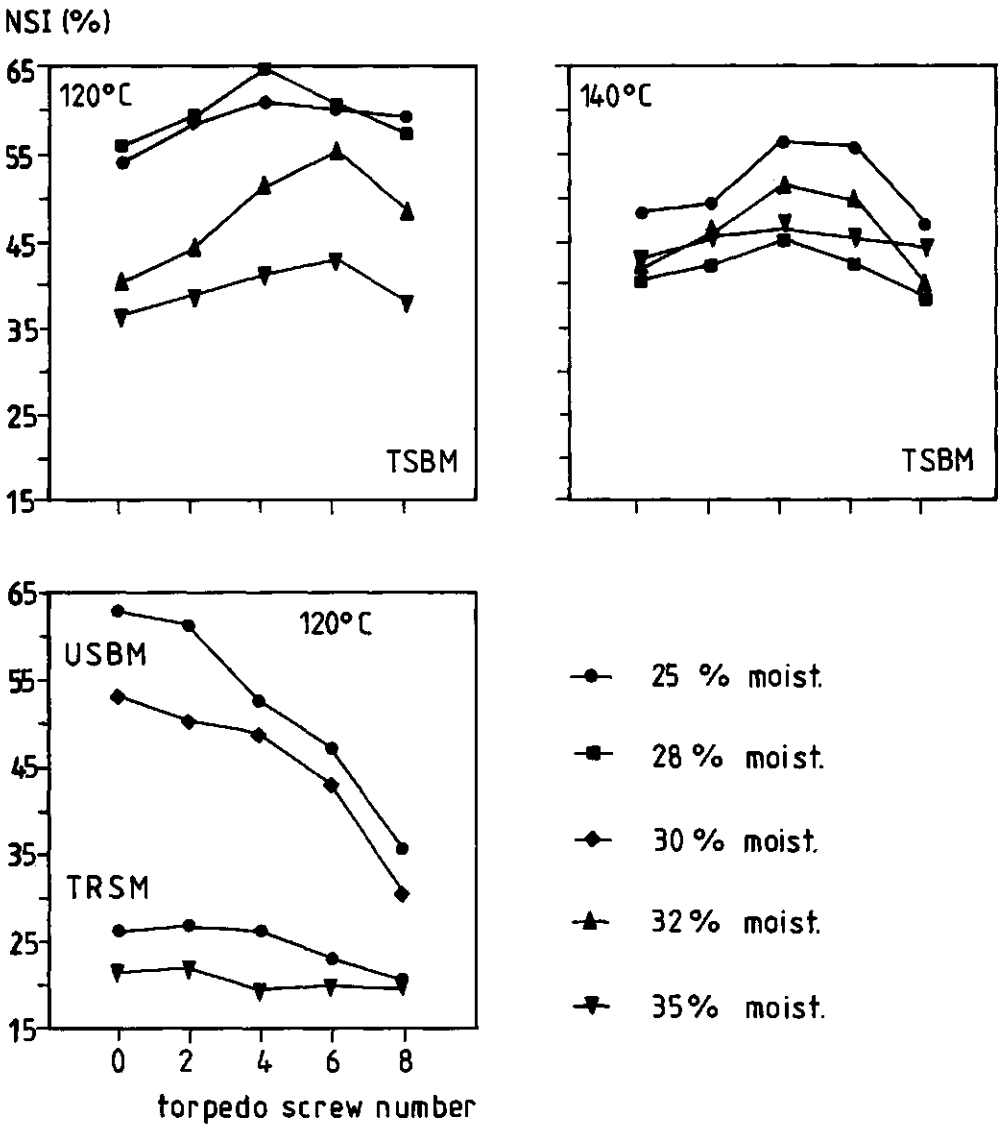

FIGURE 5

Nitrogen solubility index (NSI) [%] after extrusion with torpedo elements at different moisture contents. TSBM at 120° and 140°C, USBM and TRSM at 120°C

screw number the NSI of USBM showed a further decrease (Figure 5). This decrease in NSI values corresponded with sharply increasing H_v values (Figure 4). The surplus in SME during extrusion of USBM with torpedo screw 0 was already more than 800 kJ/kg, a value which is only reached during extrusion of TSBM and TRSM with torpedo screw 8 at low moisture contents (Figure 4). From Figure 5, it can also be seen that a high moisture content results in lower NSI values than extrusion at lower moisture contents. Those findings are in line with results from others[24]. In contrast to the effect of longer torpedo elements, there was no influence of more twin lead slotted screws on the NSI of TSBM (Table 2).

The effect of longer torpedo elements on the NSI of TRSM are also given in Figure 5. It can be seen that the NSI showed limited changes as a result of extrusion at different shear and mixing levels. The NSI values obtained after extrusion with twin lead slotted screws were lower than the NSI values obtained after extrusion with the torpedo elements, but also in these trials limited changes in NSI values were determined (Table 2).

It can be assumed that proteins in TSBM and TRSM were already denatured to a certain extent, due to exposure to heat during solvent removal. However, USBM contains proteins which are not damaged and are highly soluble. Therefore, it is very likely that USBM reacts, during extrusion, differently from TSBM and TRSM. During extrusion cooking a wide range of interaction energy is available for protein cross-linking with itself or other macromolecules[3]. Denaturation of proteins during extrusion cooking can be followed by association and dissociation reactions. Also, the formation of covalent bonds at high temperatures and the formation of non covalent interactions and disulfide bonds upon cooling are possible[3, 25-27].

The NSI values of TRSM were significant lower than the NSI values obtained with the soybean meal samples (Figure 5). In rapeseed meal also tannins may form soluble but also insoluble complexes with proteins resulting in a lower NSI value[28]. Extrusion at 130°C showed no significant effect on reducing tannin content[6], but autoclaving (1.5 h at 121°C) reduced tannin content by 40%[29]. However, different protein characteristics may also explain the differences obtained in NSI values between rapeseed- and soybean meal.

A large part of the specific mechanical energy input is converted into heat by viscous dissipation. This has an effect on the temperature profile along the barrel and, therefore, also an effect on the NSI values. In Figure 6 the temperatures of the product, measured with the thermocouples along the barrel of the Almex Battenfeld extruder (Figure 1), are given for USBM, TSBM, and TRSM extruded with torpedo screw 0 and 8. Extrusion trials with torpedo screw 0 needed higher temperatures in the first part of the extruder than the trials with torpedo screw 8, in order to reach the desired product temperature of 120°C at the die. This would mean that the meals were longer exposed to heat than extrusion with longer torpedo screws. This can also explain the lower NSI values with torpedo screw 0.

***In vitro* protein digestibility**

In vitro protein digestibility as measured by the pH-STAT is given in Figure 7 for TSBM extruded with the torpedo elements. The *in vitro* protein digestibility of unextruded TSBM was 61%. It can be seen that extrusion with torpedo screw 0 at different moisture contents increased the digestibility value to 75%. However, when TSBM is extruded with

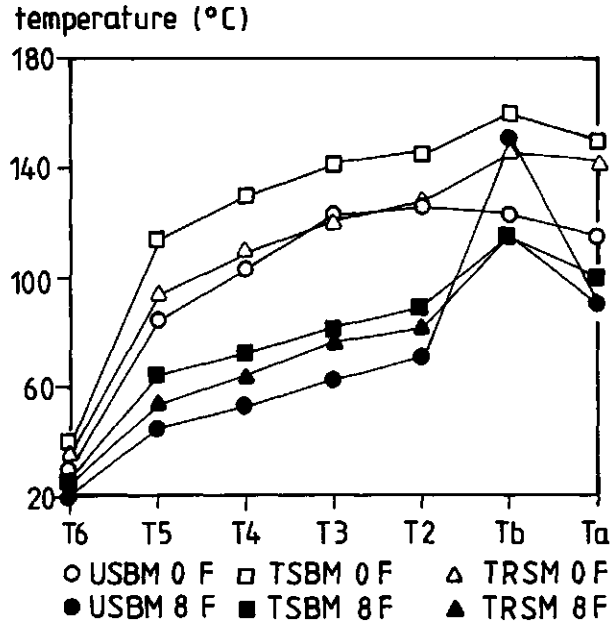

FIGURE 6

Temperature profiles along the barrel after extrusion of USBM, TSBM, and TRSM with torpedo elements with 0 rows of flights (0F) and 8 rows of flights (8F)

torpedo screws 2-8 the *in vitro* protein digestibility increased to 80% after extrusion with torpedo screw 4 and decreased after extrusion with longer torpedo elements. An exception was TSBM extruded at a moisture content of 25%, where the maximum protein digestibility value was obtained after extrusion with torpedo screw 6 (Figure 7). Most striking however, is the fact that the NSI values of TSBM (Figure 5) showed similar pattern as the *in vitro* protein digestibilities (Figure 7). Therefore, it can be concluded that after extrusion of TSBM with torpedo elements, the NSI would be a good parameter in screening the nutritional values of the extrudates. However, this relation between NSI and pH-STAT digestibility seems to be dependent on moisture content and product temperature. Such a conclusion cannot be taken for TSBM extruded with twin lead slotted screws. A slight increase in protein digestibility from 78.1 till 81.3% was obtained, whereas the NSI values showed no differences (Table 2). The protein digestibilities of TSBM, obtained after extrusion with twin lead slotted screws, were comparable with the digestibility values obtained after extrusion with the torpedo elements. According to Dale et al.[11], who determined the NSI of heat treated soybean meal in relation to the *in vivo* digestibility of broiler chickens, a NSI value of 67% or less appeared to impair growth and feed conversion of the chickens and is therefore considered to be overprocessed. On the other hand, soybean meal with a NSI value exceeding 85% was considered to be underprocessed. Feeding trials with rats are in progress to determine the *in vivo* digestibility

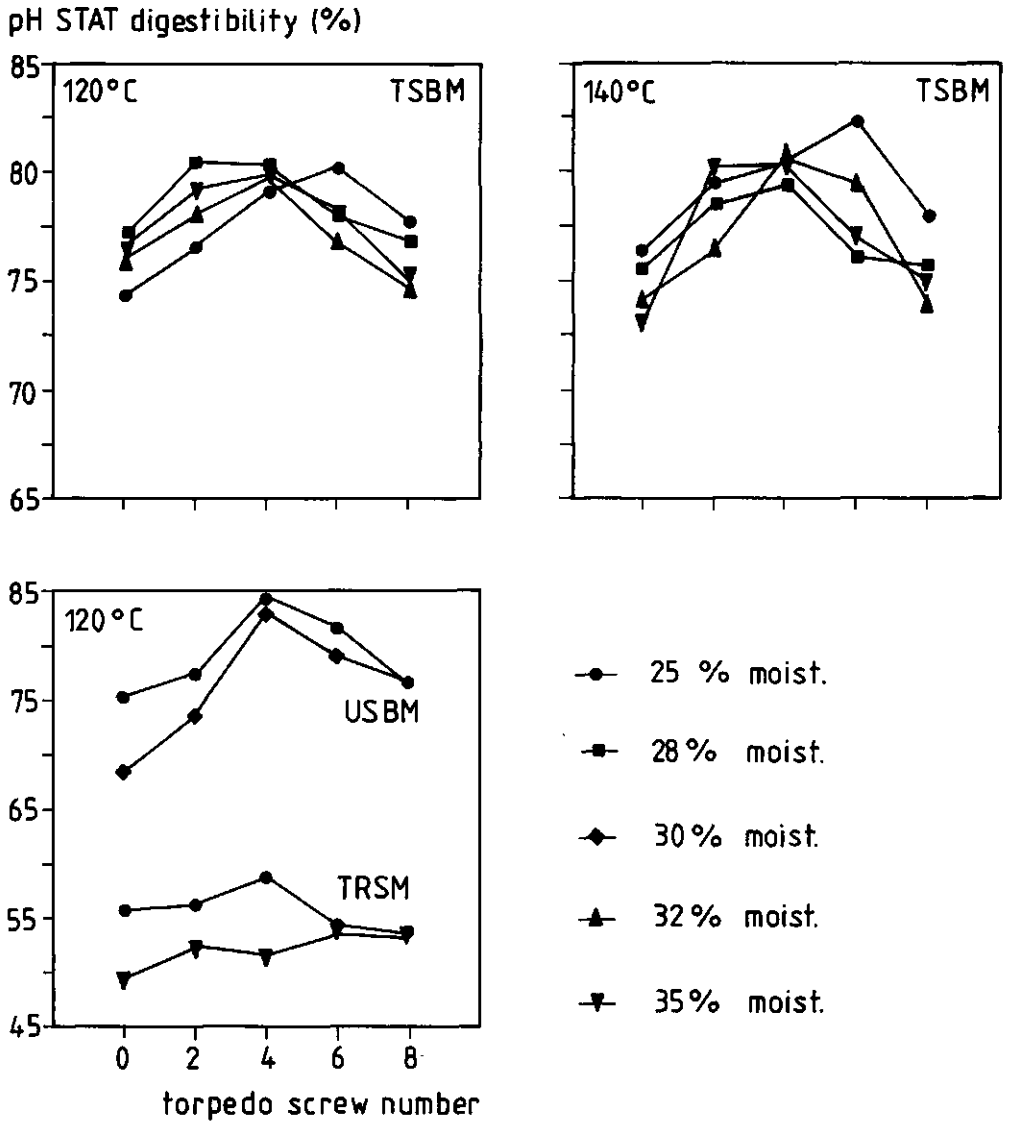

FIGURE 7
pH-STAT digestibility [%] after extrusion with torpedo elements at different moisture contents. TSBM at 120° and 140°C, USBM and TRSM at 120°

of soybean meal extruded at different shear and mixing levels. The *in vitro* protein digestibility of unextruded USBM was 19.8% (no further results shown). Extrusion with torpedo screw 0 sharply increased the protein digestibility till values of 68.4 and 75.7% at moisture contents of 30 and 25%, respectively (Figure 7). However, it can be seen that after extrusion with torpedo screw 4 the protein digestibility increases further to 83.6 and 84.8% at moisture contents of 30 and 25%, respectively (Figure 7). Extrusion with torpedo screws 6 and 8 decreased the *in vitro* protein digestibility significantly. While the increase in protein digestibility in USBM extruded with torpedo elements is the result of trypsin inhibitor inactivation, the increase in protein digestibility values obtained after extrusion of TSBM with torpedo screws 2 and 4 can most likely not be ascribed to trypsin inhibitor inactivation[17]. It is possible that during extrusion of TSBM with torpedo screw 0 and 2, the amount of energy input was insufficient to alter proteins in such a way that they are maximum accessible for enzyme attack. It was already concluded that SME values of TSBM and TRSM were sometimes negative (Figure 4). Bhattacharya and Hanna[30] found that an increase in shear forces in the extruder denatured the proteins more easily thereby facilitating enzymic hydrolysis. But, if energy is in excess all kinds of transformation of protein molecules can occur. The processing conditions used in extrusion cooking, such as a high temperature and a low moisture content, are also known to favor the Maillard reaction[31].

The *in vitro* protein digestibility of unextruded TRSM was 44.9%. Extrusion with torpedo screw 0 at 25 and 35% moisture content increased the protein digestibility to 55.2 and 49.4%, respectively (Figure 7). With longer torpedo elements, it seems that there is an optimum in protein digestibility after extrusion of TRSM with a moisture content of 25% with torpedo screw 4 (58.6%), but after extrusion with TRSM at a moisture content of 35% the results are not clear (Figure 7). The *in vitro* protein digestibilities of extruded TRSM with the twin lead slotted screws were higher (58.9 to 65.2%; Table 2) than the values obtained after extrusion with the torpedo elements (Figure 7). If protein digestibility values of TRSM are compared with NSI values, it appeared that there is no relation between those parameters (Table 2). Anderson-Hafermann et al.[12], showed that in rapeseed a NSI value of 35% or less stands for overprocessed rapeseed meal. This would mean that not only extruded TRSM samples in this study were overprocessed, but also the unextruded TRSM. The *in vitro* protein digestibility of TRSM is much lower than found in the soybean meals. This may be due to the high content of tannins and phytic acids, which can form complexes with the rapeseed proteins[28,32].

CONCLUSIONS

Single-screw extrusion cooking of proteinaceous material can be considerable influenced by the design of the shear heads on the screw. *In vitro* protein digestibility, measured with the pH-STAT method, showed that maximum digestibilities were obtained in USBM and TSBM after extrusion with torpedo screw 4. After extrusion of TSBM, the NSI values showed the same pattern as the corresponding protein digestibility values. Therefore, it is concluded that the NSI in potassium hydroxide can be used as an indicator for the nutritional value of the soybean meals after extrusion cooking. The PDI was not a

suitable parameter to differentiate between different shear and mixing levels during extrusion. *In vitro* protein digestibilities and NSI values of TRSM were significantly lower than values obtained with the soybean meal samples and they showed no or limited changes as a result of extrusion with longer torpedo screws. Extrusion with screws provided with twin lead slotted screws slightly increased the *in vitro* protein digestibility of both TSBM and TRSM, but the influence on the NSI was not clear. Much more specific mechanical energy (SME) is left for all kinds of reactions during extrusion of USBM compared with TSBM and TRSM.

ACKNOWLEDGEMENT

The authors wish to thank Mrs C.B. Closs from the ETH in Zurich and Mr P. Leij from the Wageningen Agricultural University for technical assistance during the extruder experiments and for the analysis of the NSI and *in vitro* protein digestibility as well as the Technical Service Department of the Wageningen Agricultural University for developing the torpedo elements. This research was financially supported by Nutreco Agribusiness R&D, Boxmeer, The Netherlands.

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chapter 4

The effect of shear forces and addition of a mixture of a protease and a hemicellulase on chemical, physical and physiological parameters during extrusion of soybean meal

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Animal Feed Science and Technology, 56, (1995) 21-35

ABSTRACT

The influence of shear forces during extrusion of toasted soybean meal (TSBM) and the addition of enzymes before and after extrusion on some chemical, physical and physiological parameters were studied. Shear forces were introduced by the use of 0, 1 and 2 twin lead slotted screws (TLSSs) during extrusion of TSBM with a single-screw extruder. The experiment included 6 treatments: 1. unextruded TSBM, 2-4. TSBM extruded with 0, 1, and 2 TLSSs, respectively, 5. addition of a mixture of a protease and a hemicellulase before extrusion of TSBM with 1 TLSS and 6. the same amount of enzymes added after extrusion of TSBM with 1 TLSS.

Chemical analysis showed a decrease of 25-41% in trypsin inhibitor activity (TIA) and a decrease in lectin content below the detectable level as a result of extrusion. The *in vitro* protein digestibility increased from 60.7% in TSBM to 81.1% after extrusion with 2 TLSS and seemed negatively correlated with TIA. Different shear levels during extrusion gave no correlation between growth performance in broiler chickens and TIA. The protein dispersibility index (PDI) and nitrogen solubility index in potassium hydroxide (NSI) decreased as a result of extrusion but both failed to differentiate between the different

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shear levels. Using NSI as a quality parameter after soybean meal extrusion, it was concluded that NSI levels of 50-55% improved *in vitro* protein digestibility and feed conversion of broiler chickens. The content of lysine which reacted with 1-fluor-2,4-dinitrobenzene at 435 nm (FDNB-reactive lysine) did not change as a result of extrusion at different shear levels.

A growth experiment with 578 female and 528 male one day-old broiler chickens showed that extrusion significantly improved feed conversion ($P < 0.001$). Also the addition of enzymes before and after extrusion improved feed conversion compared with the unextruded TSBM ($P < 0.001$). Enzyme addition after extrusion also increased weight gain of mainly the male chickens ($P < 0.0256$). No significant differences in animal performance between the different shear levels nor between the addition of enzymes before and after extrusion were obtained. A digestibility experiment with 30 rats fed with diets containing TSBM and TSBM extruded with 0, 1, and 2 TLSSs gave no significant differences in total tract digestibility of nitrogen as a result of extrusion at different shear levels.

INTRODUCTION

Soybean meal (SBM) is a potentially valuable protein source in animal diets, because of its high protein content and relatively well balanced amino-acid pattern. Extrusion cooking is a widely used technology for heat treatment of soybeans. It is well known that trypsin inhibitors and other antinutritional factors (ANFs) are inactivated by extrusion cooking[1-3]. The reported effects of extrusion of soybeans or SBM on animal performance are not uniform. Pigs fed moist extruded soy flakes and toasted SBM had an improved average daily gain (ADG) and gain:feed ratios compared with pigs fed non-extruded soy flakes and toasted SBM[4,5]. However, extrusion of SBM did not affect the ileal and faecal digestibilities of lysine in barrows, but the ileal and faecal digestibilities of nitrogen and energy showed a significant improvement[6]. No correlation was observed between protein nutritive value as protein efficiency ratio (PER) and net protein ratio (NPR) and residual ANFs in rats of extruded mixtures of maize and rice with dehulled soybeans[7]. Extrusion of untoasted soybeans at 138 and 154°C yielded weight gains and gain:feed ratios of broiler chickens similar to that of toasted unextruded SBM[8]. Data involving the effect of different screw configurations during extrusion on growth performance of animals are scarce.

During extrusion cooking shear forces are suspected to play an important role in changing the nutritional value of proteineous materials[9,10]. Normally, shear forces during extrusion are varied by changing the screw speed. However, by changing the screw speed also the residence time is largely affected[11]. Little attention has been given to the development of shear by various screw configurations. Yam et al.[12] found an improved conversion of starch in maize meal when more shear was introduced by one or two reverse screw elements. At a given screw speed, 30 forwarding paddles generate the highest rate of shear, followed by feed screws and single lead screws during twin screw extrusion[13].

In addition, supplementation of animal diets with hydrolytic enzymes is another possibility to remove ANFs from the diet and to improve the digestion and absorption of

nutrients. The inclusion of phytase, for example, resulted in an improvement in growth rate and feed conversion efficiency in both chickens and pigs[14]. Hemicellulolytic enzymes showed an improvement in weight gain and feed:gain ratio in broiler chickens fed with wheat and barley diets[15]. The improvement in chick performance by supplementation of non starch polysaccharides (NSP) degrading enzymes, like pentosanases and glucanases, to wheat and rye diets is generally ascribed to a decrease in viscosity in the gastro intestinal tract of the chicken[16]. So far, multi-enzyme preparations designed to act on SBM NSP substrate failed to induce an improvement in the growth of broilers fed SBM diets[17].

In this study toasted soybean meal (TSBM) was extruded on a single-screw extruder. Shear forces were introduced by the use of 0, 1, and 2 twin lead slotted screws (TLSSs). Diets containing the extruded meals were fed to broiler chickens and rats in order to evaluate the effects of shear forces during extrusion on animal performance. In addition, a combination of a protease and a hemicellulase was added to SBM before and after extrusion cooking with a screw supplied with 1 TLSS.

MATERIALS AND METHODS

Materials

Commercial solvent extracted and toasted soybean meal (TSBM) was supplied by Cargill, Amsterdam. The protein content (Nx6.25) was 58.5% on a moisture-free basis.

Extrusion cooking

A eight-head Wenger X-20 single-screw extruder was used. Treatment 1 was the unextruded TSBM. In treatment 2-4 TSBM was extruded without twin lead slotted screws (TLSSs), with 1 TLSS placed on the 8th head, and with 2 TLSSs placed on the 7th and 8th head, respectively[18]. In treatment 5, a batch of TSBM containing a mixture of a liquid protease and a hemicellulase, added during moisturization just before extrusion, was extruded. The other extrusion conditions were kept constant. In all treatments the moisture content of TSBM was adjusted to 35% by adding water to the meal in the feeder just before extrusion. With eight steam jackets on the barrel, the temperature along the barrel was gradually increased from 20°C till a final product temperature at the die, measured manually six times per treatment using a thermocouple, of 120 $\pm$ 3°C. The mean residence time of the sample in the extruder was 30-35 s. The screw speed was 430 rpm and one die hole of ϕ 9.5 mm was used. The throughput of each treatment was 100 $\pm$ 5 kg/h. After extrusion cooking the extrudates were dried on a belt drier for 3 . The temperature of the extrudates did not exceed 70°C. The extrudates were ground to pass an one mm sieve. After grinding a part of the TSBM extruded with one TLSS was treated with an amount of enzymes equal to the addition of enzymes before extrusion, giving treatment 6.

Growth experiment with broiler chickens

A total of 576 female and 528 male one day-old broiler chickens (Hydro-G) obtained from a commercial hatchery were randomly distributed into 96 groups (cages). In the cages, wood-shavings were used as litter. The experimental design included 6 treatments as given in the previous paragraph with 16 replicates per treatment, 8 cages with 12 female and 8 cages with 11 male birds. Ambient temperature was gradually decreased from 32 to 22°C at day 42 and continuous light regime was required to enable a steady feed intake. After two weeks the light intensity was slightly deceased. The broilers were fed a starter diet (day 1-14), a grower diet (day

15-35), and a finisher diet (day 36-42). The compositions of the diets are shown in Table 1. The starter diet was fed in mash form and the grower and finisher diet in pellets. Feed and water were supplied *ad libitum*. Feed intake and the weight of the birds were recorded on day 14, 35, and 42.

TABLE 1

Compositions (g/kg) of the starter, grower, and finisher diets used in the growth experiment with broiler chickens

Ingredients	Diets		
	Starter	Grower	Finisher
TSBM[1-6]	281	267	235
Wheat	434	446	588
Tapioca	168	150	49
Soybean oil	54	48	33
Lard	20	49	63
Monocalcium phosphate	15.2	12.2	8.1
Limestone	11.6	9.3	7.3
Salt	2.1	2.0	2.1
Sodium bicarbonate	1.0	1.0	1.0
DL-Methionine	2.4	2.4	1.8
L-Lysine	2.7	3.0	2.8
Choline-chloride	4.0	4.0	4.0
premix[a]	4.0	5.0	5.0
Calculated concentrations:			
CP	195	192	190
Lys	12.5	12.4	11.7
Met	5.2	5.1	4.6
Cys	3.3	3.2	3.3
Ca	8.9	7.0	5.3
AVP	4.6	3.9	3.0
MSL (kcal kg^{-1})	2946	3057	3070

[1-6] Treatments: 1. Unextruded toasted soybean meal (TSBM). 2-4. TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs), respectively. 4. Enzyme addition (a mixture of a protease and a hemicellulase) before extrusion and 6. enzyme addition after extrusion with one TLSS. [a] premix contains vitamins and minerals and on feed basis in the starter diet: 125 ppm nicarbazin, 15 ppm avoparcin, in the grower diet: 70 ppm salinomycin-sodium, 20 ppm virginamycin, and in the finisher diet: 20 ppm virginamycin.

Balance experiment on rats

30 male weanling Wistar rats, 6 weeks of age and with an average weight of 170 g, were supplied by Harlan CPB. The rats were randomly divided in five groups of six rats. One group was fed a standard diet, which did not contain SBM. Four groups were fed diets containing a mixture of the rat standard diet and the rat experimental diet (70/30 w/w). Diet 1 contained the unextruded TSBM and diet 2-4 contained TSBM extruded with 0, 1 and 2 TLSSs, respectively. The compositions of the diets are given in Table 2. This procedure permits the calculation of the digestibility coefficients of the soybean extrudates under investigation from the digestibility coefficients of the diet by difference[19]. The rats were housed individually in metabolic cages

allowing separate collection of both faeces and urine. Room temperature was maintained at 21°C. Feed was provided at a level of 2.0 times the energy requirement for maintenance. The rats were adapted to the feed during a period of 7 days before the experiment was started. Faeces and urine were collected during the following 4 days, weighed, stored (-20°C) and freeze-dried prior to analysis. All rat diets were administered in meal form. Water was given *ad libitum*.

TABLE 2

Compositions (g/kg) of the standard and experimental diets used in the balance experiment on rats

Ingredients	Diets	
	standard	experimental
TSBM[1-4]	-	300
Gelatinized starch	609.4	426.58
Casein	151	105.7
Glucose	100	70
Cellulose	30	21
Corn oil	25	17.5
Coco oil	25	17.5
Calcium carbonate	12.4	8.68
Sodium dihydrogenphosphate	15.1	10.57
Potassium bicarbonate	7.7	5.39
Magnesium carbonate	1.4	0.98
Potassium chloride	1.0	0.7
Mineral premix[a]	10	7.0
Vitamin premix[b]	12	8.4

[1-4] Treatments: 1. Unextruded toasted soybean meal (TSBM). 2-4. TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs), respectively. [a]174 mg $FeSO_4$, 79 mg MnO_2, 33 mg $ZnSO_4$, 13 mg $NiSO_4$, 2 mg NaF, 0.2 mg KI, 15.7 mg $CuSO_4$, 0.3 mg Na_2SeO_3, 1.5 mg $CrCl_3$, 1.9 mg $SnCl_2$, 0.2 mg NH_4CO_3 per kg feed and corn starch (carrier). [b]vitamins: 4 mg B_1, 3 mg B_2, 6 mg B_6, 50 mg B_{12}, 0.05 mg K, 60 mg E, 8 mg A, 2 mg D, 20 mg niacin amide, 17.8 mg calcium pantothenate, 2000 mg choline chloride, 1 mg folic acid, 2 mg biotin per kg feed and corn starch (carrier).

Analytical methods

Moisture content was determined by drying at a temperature of 105°C[20]. The protein disperibility and solubility was measured by the protein dispersibility index (PDI)[21] and the nitrogen solubility index (NSI) in potassium hydroxide[22], respectively. The total nitrogen content in the diets, freeze dried faeces, and urine and the nitrogen content in the supernatant after centrifugation for the NSI and PDI determinations were performed by a semi-automated micro Kjeldahl method[23].

The *in vitro* protein digestibility was determined with the pH-STAT method principally according to Pederson and Eggum[24] with some slight modifications[18].

The trypsin inhibitor activity (TIA) was performed according to Smith et al.[25]. Lectins were analyzed by a lectin immunoassay as described by Vretblad[26].

Available lysine was determined according to the method of Carpenter[27] as modified by Booth[28]. The amount of free ϵ-amino groups of lysine, obtained after hydrolysis in 8.1 M HCL, which reacted with 1-fluor-2,4-dinitrobenzene (FDNB) and was measured at 435 nm was called FDNB-reactive lysine. All analysis were performed in duplicate.

Statistical analysis

The results from the growth experiment with broiler chickens obtained after each period and for the whole period and the results of the balance experiment with the rats were statistical analyzed using the GLM procedure with a SAS program[29]. In the case of the rats the student Newman-Keuls test was used.

RESULTS AND DISCUSSION

Chemical analysis

In Table 3 the PDI, NSI, *in vitro* protein digestibility, trypsin inhibitor activity, lectin, and reactive lysine content are given for TSBM and TSBM extruded with 0, 1 and 2 TLSSs.

It can be seen that extrusion without TLSS decreased the TIA by 25% to 0.85 mg/g. A further decrease (up to 41%) was obtained after extrusion with 2 TLSSs. Lectin content was lowered below the detection level as a result of extrusion. It should be noted that the unextruded TSBM used in this experiment had already a very low TIA and lectin content compared with the corresponding untoasted SBM which had a TIA of 22.1 mg/g and a lectin content of 3.3 mg/g (no further results shown).

TABLE 3

Effects of extrusion on the nitrogen solubility index (NSI), protein dispersibility index (PDI), *in-vitro* protein digestibility (pH-STAT), trypsin inhibitor activity (TIA), lectin content and FDNB-reactive lysine (FDNB-lys) content of toasted soybean meal (TSBM) and TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs)

	NSI [%]	PDI [%]	pH-STAT [%]	TIA [mg g^{-1}]	Lectin [mg g^{-1}]	FDNB-lys [mg g^{-1}]
TSBM	72.5	21.2	60.7	1.13	0.3	22.5
0 TLSS	54.1	8.3	78.3	0.85	< 0.1	20.9
1 TLSS	51.0	8.1	80.1	0.72	< 0.1	22.9
2 TLSS	55.2	8.0	81.1	0.67	< 0.1	24.3

The *in vitro* protein digestibility measured with the pH-STAT method showed the opposite effect. Extrusion without TLSSs sharply increased the *in vitro* protein digestibility by 30% from 60.7 to 78.3% and increased it further to 81.1% after extrusion with 2 TLSSs (Table 3). The results suggest that TIA and lectin content in this experiment were negatively correlated with the *in vitro* protein digestibility.

The FDNB-reactive lysine in the TSBM was 22.5 mg/kg. Extrusion with or without TLSSs gave no clear losses in FDNB-reactive lysine (Table 3). These findings were in line with Hendriks et al.[30], who did also some experiments with SBM on a single-screw extruder. In the latter study extrusion of SBM at moderate temperatures (140°C) and high moisture levels (27-40%) resulted in a reduction in FDNB-reactive lysine ranging from 10 to 14%. However, at an initial moisture content of 30% and a

temperature of 114°C the decrease in FDNB-reactive lysine was only 4%. In general, the Maillard reaction is favored by a low water content and a high temperature[2]. The temperature used in our experiments (120 ± 3°C) was not high enough to have an impact on the FDNB-reactive lysine content.

The protein dispersibility index (PDI), often used as a parameter to measure the protein quality of SBM, was 21.2% in the unextruded TSBM and decreased to 8.0% as a result of extrusion with 0 TLSSs and remained constant with the use of 1 or 2 TLSSs (Table 3). From these results it can be concluded that differences in protein quality of extruded SBM can not be identified by the PDI. A decrease from 20.2 to 8% as a result of extrusion of SBM was also found by Hendriks et al.[30]

The nitrogen solubility index (NSI) in potassium hydroxide decreased from 72.5 to 54.1% as a result of extrusion. It decreased further to 51.1% after extrusion with 1 TLSS, but then increased to 55.2% after extrusion with 2 TLSSs, respectively (Table 3). The NSI in potassium hydroxide is introduced as an alternative of the urease activity test. The latter was not able to evaluate SBM for overprocessing[31]. Several studies have been performed to test its usefulness. A NSI of 67-70% or less, achieved after autoclaving SBM at 121°C at different times, appeared to impair chick performance and is, therefore, considered to be overprocessed. On the other hand, SBM with a NSI value exceeding 85% was considered to be underprocessed[22,31]. In our study this would mean that TSBM extruded with 0, 1 and 2 TLSS were all overprocessed. However, results from the *in vitro* protein digestibility method suggested that NSI values of 50-55% did not negatively effect these values (Table 3). Therefore, it is likely that different heat treatments such as extrusion, with a high shear input or toasting and autoclaving, where shear forces do not play an important role, do all have a different impact on protein solubility. More research is necessary to evaluate the usefulness of the NSI in potassium hydroxide as an indicator for nutritional value in SBM processing.

Growth experiment with broiler chickens

Results of the growth experiment with the broiler chickens are summarized in Tables 4 and 5. In Table 4 weight gain, feed intake and feed conversion efficiency of male (M), female (F) and mixed flock (T) broiler chickens over the whole period of 42 days are presented. The performance of the chickens (male and female) for each period, starter, grower and finisher period are given in Table 5.

From Table 4 it can be seen that diets containing extruded TSBM significantly improved feed conversion efficiency of both male and female chickens compared with the diet containing unextruded TSBM. This improvement is achieved in both the starter and grower period but disappeared in the finisher period (Table 5). No improvement in weight gain for either male or female chickens was found as a result of extrusion at different shear levels (0, 1 and 2 TLSSs) compared with the diet containing the unextruded TSBM (Table 4). Feed intake of male and female chickens fed with TSBM extruded with 2 TLSSs, however, was significantly lower compared with the unextruded TSBM. This decrease in feed intake was mainly obtained in the starter and finisher period, but not in the grower period (Table 5).

Where over the whole period only limited effects in chick performance were observed as a result of extrusion of TSBM at different shear levels (0, 1, and 2 TLSSs), some significant differences were obtained considering the different feeding periods. From

TABLE 4

Growth performance (Mean ± SD) of male, female and mixed sexes broiler chickens over the total period (42 days), fed diets with different processed soybean meals

Treatment[1]	Weight gain (g)			Feed intake (g)			Feed conversion (g g^{-1})		
	male	female	mixed sexes	male	female	mixed sexes	male	female	total
1	$1956^{a} \pm 98$	1663 ± 50	$1809^{a} \pm 169$	$3330^{a} \pm 137$	$2876^{a} \pm 97$	$3103^{a} \pm 261$	$1.70^{a} \pm 0.03$	$1.73^{a} \pm 0.04$	$1.71^{a} \pm 0.04$
2	$2009^{ab} \pm 52$	1699 ± 58	$1854^{ab} \pm 169$	$3275^{ab} \pm 64$	$2847^{ab} \pm 128$	$3061^{ab} \pm 242$	$1.63^{b} \pm 0.02$	$1.67^{b} \pm 0.02$	$1.65^{b} \pm 0.03$
3	$1998^{ab} \pm 64$	1696 ± 49	$1847^{ab} \pm 165$	$3264^{ab} \pm 92$	$2830^{ab} \pm 85$	$3047^{ab} \pm 240$	$1.63^{b} \pm 0.02$	$1.67^{b} \pm 0.04$	$1.65^{b} \pm 0.03$
4	$1957^{ab} \pm 101$	1653 ± 78	$1805^{ab} \pm 180$	$3211^{b} \pm 15$	$2761^{b} \pm 114$	$2986^{b} \pm 266$	$1.64^{b} \pm 0.03$	$1.67^{b} \pm 0.02$	$1.65^{b} \pm 0.03$
5	$2012^{ab} \pm 77$	1678 ± 58	$1845^{ab} \pm 185$	$3294^{ab} \pm 78$	$2802^{ab} \pm 133$	$3048^{ab} \pm 274$	$1.64^{b} \pm 0.04$	$1.67^{b} \pm 0.04$	$1.65^{b} \pm 0.04$
6	$2035^{b} \pm 77$	1697 ± 59	$1866^{b} \pm 187$	$3298^{ab} \pm 135$	$2823^{ab} \pm 107$	$3061^{ab} \pm 271$	$1.62^{b} \pm 0.04$	$1.66^{b} \pm 0.04$	$1.64^{b} \pm 0.04$

[1]Treatments: 1. Unextruded toasted soybean meal (TSBM), 2-4. TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs), respectively. 5. Enzyme addition (a mixture of a protease and a hemicellulase) before extrusion and 6. enzyme addition after extrusion with 1 TLSS.

[abc] Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 5 it can be concluded that although extrusion at different shear levels induced some differences in the performance of chickens in the starter and grower period, they did not induce significant differences in chick performance over the whole period (Table 4). This may be explained by the fact that the diets were fed *ad libitum* so that every nutrient was abundantly available. There was also no decrease in FDNB-reactive lysine (Table 3).

Data in the literature suggest that extrusion of raw soy materials can improve animal performance and protein digestibility compared with the unextruded raw soy materials[1,5,7]. Whereas toasting of SBM had no effect on protein digestibility of pigs, extrusion significantly improved the protein digestibility[4]. Not only heat but also shear forces are considered to play an important role during extrusion. Proteins were more easily denatured when shear forces were increased during extrusion, thereby facilitating more enzymic hydrolysis[9].

It was concluded that TIA and lectin content in this experiment were negatively correlated with the *in vitro* protein digestibility (Table 3). However, results obtained in the *in vivo* experiment with the broiler chickens showed only a better feed conversion efficiency as a result of extrusion. The use of 0, 1 and 2 TLSSs did not have any relation with TIA and lectin content. No correlation between nutritive value and residual ANFs in soybeans extruded at different temperatures and fed to rats was also observed by Molina and co-workers[7].

In vitro protein digestibility and animal performance were partly correlated. Both parameters showed clearly the positive effect of extrusion, but *in vivo* data could not differentiate between the different shear levels (Table 4), while the *in vitro* method showed an improving protein digestibility if the TSBM was exposed to higher shear levels during extrusion. Also other factors than TIA and lectin contents may be responsible for differences in *in vitro* and *in vivo* digestibilities, especially shear forces which are quite unique for the extrusion process[10,32].

Enzymes added after extrusion of TSBM with 1 TLSS gave a significant higher weight gain and feed conversion efficiency over the whole period compared with the diet containing unextruded TSBM (Table 4). Most striking however, is that only the male chickens were responsible for the increase in weight gain. From Table 5 it can be seen that this effect by the male chickens is achieved in the starter and grower period. A better feed conversion was also observed with enzyme addition before extrusion compared with the diet containing the unextruded TSBM. Considering the whole period no significant differences in chick performance between enzymes added before or after extrusion were found.

Comparison of the performance of chickens fed with diets where enzymes were added before or after extrusion with the extrusion trials where different shear levels were used (0, 1, and 2 TLSSs), showed no that significant differences in weight gain, feed intake and feed conversion efficiency were obtained if the whole period is considered (Table 4). However, some significant effects were observed in the different periods, especially in the starter and grower period but, in general, those differences disappeared in the finishing period (see Table 5).

Several reports suggested that addition of enzymes to diets can increase animal performance. Enzymes which had high xylanase and cellulase activities considerably improved weight gains of young chicks when added to diets containing barley and rye, but not when added to a maize diet[33]. If broiler chickens were fed diets containing SBM as the sole dietary protein concentrate the birds showed poor growth in a number of feeding trials[34].

TABLE 5

Growth performance (Mean ± SD) of broiler chickens (male and female) over the starter (day 1-14), grower (day 15-35) and finisher (day 36-42) period

Treatment[1]	Weight gain (g)			Feed intake (g)			Feed conversion (g g^{-1})		
	starter	grower	finisher	starter	grower	finisher	starter	grower	finisher
1	$339^{a}\pm17$	$980^{a}\pm122$	$491^{a}\pm65$	$380^{a}\pm16$	1799 ± 172	$925^{a}\pm97$	$1.12^{a}\pm0.03$	$1.84^{a}\pm0.08$	1.89 ± 0.13
2	$330^{b}\pm14$	$1066^{b}\pm123$	$458^{b}\pm51$	$365^{b}\pm14$	1808 ± 168	$888^{b}\pm74$	$1.10^{ac}\pm0.03$	$1.70^{b}\pm0.05$	1.95 ± 0.10
3	$350^{c}\pm17$	$1027^{ab}\pm121$	$470^{a}\pm54$	$378^{a}\pm19$	1780 ± 168	$888^{b}\pm74$	$1.08^{b}\pm0.02$	$1.74^{c}\pm0.05$	1.90 ± 0.11
4	$330^{b}\pm16$	$1018^{a}\pm113$	$457^{b}\pm68$	$369^{b}\pm17$	1748 ± 169	$869^{b}\pm90$	$1.12^{ac}\pm0.03$	$1.72^{bc}\pm0.04$	1.91 ± 0.12
5	$334^{b}\pm17$	$1045^{b}\pm130$	$466^{a}\pm51$	$369^{b}\pm19$	1788 ± 182	$891^{b}\pm82$	$1.10^{c}\pm0.03$	$1.72^{bc}\pm0.05$	1.91 ± 0.10
6	$342^{b}\pm16$	$1047^{b}\pm137$	$478^{a}\pm62$	$378^{a}\pm14$	1795 ± 189	$888^{b}\pm79$	$1.11^{ac}\pm0.03$	$1.72^{bc}\pm0.07$	1.87 ± 0.13

[1]Treatments: 1. Unextruded toasted soybean meal (TSBM), 2-4. TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs), respectively. 5. Enzyme addition (a mixture of a protease and a hemicellulase) before extrusion and 6. enzyme addition after extrusion with 1 TLSS.

[abc] Values with different superscripts in the same column are significantly different ($P < 0.05$).

The same authors also tried to improve the nutritional value of SBM by adding multi-enzyme preparations to the diets but no improvement in growth of broiler chickens could be found[17]. However, addition of enzymes, containing cellulase, protease and β-glucanase activities, to SBM significantly increased protein and dry matter digestibility when fed to pigs[4]. In all experiments the effects of the hemicellulytic enzymes are suspected to act in the gastro intestinal tract. High-molecular weight forms of β-glucans and arabinoxylans yield viscous solutions upon solubilization. An increased viscosity at the sites of nutrient absorption could physically hinder the efficient uptake of nutrients. This is mainly the case with raw materials which contain a relatively high content of β-glucans and arabinoxylans such as wheat, rye and barley[35]. The diets used in this experiment also contained considerable amounts of wheat (Table 1). To avoid the problem that our enzyme mixture acts on the wheat and, therefore, overshadows the effect of enzyme activity on extruded SBM, Bio-Feed Plus CT was added to all the diets to break down the arabinoxylans which will leak out from the wheat in the gastro intestinal tract. Viscosity problems due to SBM are not considered to play an important role. Only small amounts of arabinoxylans are present in soybean NSP. The increasing chick performance could be the result of the protease and/or hemicellulase activity in the enzyme preparation.

Data concerning the use of enzymes in combination with heat treatment, especially extrusion cooking are scarce. If raw lupins were autoclaved for 20 min and fed to broiler chickens, weight gain and feed conversion improved compared with the raw lupins. The same was concluded when a combination of Energex, Bio-Feed Pro and Novozyme was added to diets containing raw lupins. However, birds fed with autoclaved lupin diets in which the same enzymes were added failed to improve the performance of broiler chickens[36]. Czarnecki et al.[37] incubated pinto beans with different concentrations of Cellulase 4000 and papain prior to single-screw extrusion. Improvement in *in vitro* protein digestibility was noted for 2% papain and 2% cellulase 4000 treatments. It is clear that in our experiment enzymes added after extrusion can act in the intestinal tract of the bird, while enzymes which are added just before extrusion can only be active during extrusion. The suggestion that proteins in the extruder, unfolded due to heat in combination with shear forces, are more easily hydrolysed by the enzymes which are present at that moment and may, therefore, result in a better performance of the chickens could not be noticed. It must be realized that the extrusion conditions (35% moisture contents, high temperatures, and short residence times) are not favorable for enzyme activity.

Balance experiment on rats

The results of the balance experiment with the rats are shown in Table 6. The total tract digestibility of nitrogen (TTD-N), nitrogen retention (NR), total feed intake, total water intake, urine and faeces production, and nitrogen excretion in both urine and faeces gave no significant differences between unextruded and extruded TSBM nor between the different shear levels. Positive effects on rat performance as a result of extrusion were reported in literature[1,7]. Where some authors found an increasing rat performance with a decreasing TIA level[38], in this experiment it was not able to compare the TIA results obtained after chemical analysis with rat performance, because of the high standard deviations in the balance experiment on the rats. In general, rats are less sensitive to the negative effects of ANFs than chickens or pigs[19].

TABLE 6

Total tract digestibility of nitrogen (TTD-N), nitrogen retention (NR), feed- and water intake, urine- and faeces production and nitrogen excretion in the urine and faeces over the total period of 4 days (Mean ± SD) in rats fed with differently processed soybean meals.

Treatment[1]	TTD-N [%]	NR [%]	Total intake [g]		Total excretion [g]		N-excretion [mg]	
			feed	water	urine	faeces	urine	faeces
1	93.8±1.3	64.1±6.2	67.0±4.2	86.1±19.2	33.3±13.2	10.4±2.3	1047±112	175±22
2	92.4±1.1	65.2±4.0	68.4±2.8	74.7±7.6	27.9±5.0	10.8±0.8	992±83	198±24
3	92.6±1.0	65.8±6.2	66.1±4.7	78.0±21.8	31.3±12.5	10.4±2.6	966±109	190±23
4	93.9±1.1	59.8±4.5	67.8±3.5	87.4±11.1	38.7±7.8	9.9±1.0	1113±113	173±17

[1]Treatments: 1. Unextruded toasted soybean meal (TSBM). 2-4. TSBM extruded with 0, 1, and 2 twin lead slotted screws (TLSSs), respectively.
Results were statistical analyzed but no significantly differences ($P < 0.05$) were found.

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chapter 5

The effect of thermal processing and enzyme treatments of soybean meal on growth performance, ileal nutrient digestibilities and chyme characteristics in broiler chicks

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Poultry Science, 76, (1997) 864-872

ABSTRACT

Effects of thermal processing (toasting or extrusion) of untoasted soybean meal (USBM) on growth performance, apparent ileal nutrient digestibilities, and chyme characteristics were studied in broiler chicks fed diets with soybean meal (SBM) as the main protein source. Effects of increasing shear forces during extrusion as well as enzyme treatments (protease and carbohydrase) were also studied. When compared with toasting, extrusion significantly improved feed conversion ratio (1.56 vs 1.62) and apparent ileal digestibilities of CP and non starch polysaccharides (87.5 vs 82.2% and 26.7 vs 11.4%, respectively). Enzyme treatment improved apparent ileal digestibility of CP and non starch polysaccharide compared with no enzyme treatment (85.2 vs 83.7% and 20.6 vs 14.5%, respectively). However, enzyme treatments did not result in a better growth performance of the chicks. Among the enzyme treatments, no differences were found in growth performance and apparent ileal CP digestibility, whereas the carbohydrase significantly improved apparent ileal non starch polysaccharide digestibility compared with the other enzyme treatments. Extrusion of SBM at the highest shear level caused a significant increase in the water-holding capacity, chyme viscosity, and concentration of soluble non starch polysaccharides in the chyme compared with extrusion of SBM at lower shear levels. The increase in chyme viscosity did not affect growth performance nor did it influence apparent ileal nutrient digestibilities.

(*Key words*: soybean meal, toasting, extrusion, shear forces, enzymes)

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INTRODUCTION

The high content of proteins and a well balanced amino-acid pattern makes soybean meal (SBM) a valuable protein source in diets for livestock. However, the nutritional value of SBM is decreased by the presence of antinutritional factors (ANF). Thermal processing e.g., toasting and extrusion cooking, is frequently used to increase the nutritional value of SBM[1]. In general, process conditions like temperature, moisture content, screw speed, shear forces, and duration of heating will determine the effectiveness of inactivation of the heat-labile ANF and the degree of denaturation of the storage proteins in SBM[2,3]. The extent to which trypsin inhibitors are responsible for the variation observed within the *in vivo* and *in vitro* protein digestibility in heat treated SBM is uncertain. Other studies have shown that trypsin inhibitors are not the only factors determining the nutritional value of SBM[4-6]. At low trypsin inhibitor levels, the adjustment of process conditions like temperature, moisture content and shear forces, are more important than a further decrease in trypsin inhibitor activity (TIA) in order to achieve a SBM with an optimal nutritional value[7,8]. Structural characteristics of the main storage proteins in SBM may explain the differences in nutritional value in SBM after toasting vs extrusion cooking[9,10]. During toasting and, in particular, extrusion, the nutritional value of the compact folded proteins can be increased if both non covalent interactions and disulfide bonds are broken, resulting in irreversible protein denaturation. This process increases the accessibility of proteins to enzymatic breakdown[11,12]. On the other hand, overprocessing may decrease the nutritional value of SBM due to e.g., Maillard reactions[8,13].

In order to increase the nutritional value of SBM, several attempts were made by adding proteases and carbohydrases either before or after processing[14,15]. Enzymatic breakdown of arabinoxylans and β-glucans are known to decrease the viscosity in the gastro intestinal tract of broiler chicks fed rye-, wheat-, or barley-based diets[15]. Multi-enzyme preparations designed to act on soybean non starch polysaccharides failed to induce any improvement in the growth performance of broiler chicks fed diets containing SBM as the main protein source[16]. In a previous study, broiler chicks fed diets containing extruded SBM showed no differences in BW gain, feed intake (FI) and feed conversion ratio (FCR) as a result of hydrolytic enzyme treatment. However, all birds had better FCR when compared with toasted SBM[17].

The objective of this research was to study the effect of toasting and extrusion cooking of untoasted SBM (USBM) on growth performance, apparent ileal nutrient digestibilities and chyme characteristics in broiler chicks. Secondly, chick performance was studied using different shear levels during extrusion cooking. A final objective was to determine whether extrusion cooking of USBM alters the *in vivo* susceptibility towards proteolytic and cell wall degrading enzymatic activity compared with toasted SBM.

MATERIALS AND METHODS

Birds and management

Each of 520 1-d-old female Ross broiler chicks was randomly allocated to one of 10 dietary treatments. Each treatment consisted of four cages with 13 birds per cage. A cage was

considered as a replicate experimental unit. Feed and water were consumed *ad libitum*. Ambient temperature gradually decreased from 32°C at day 0 to 22°C at day 25. A light regimen of 23 h light and 1 h dark was set to enable a continuous FI. All diets were fed in pelleted form.

Processing of the experimental diets

Commercial solvent-extracted, toasted (85°C, 20 min) soybean meal (TSBM) with a CP content of 51% (N x 6.25, as is) was supplied by Cargill (Cargill B.V., Amsterdam, The Netherlands). Part of the solvent-extracted meal was not toasted, but air-dried resulting in the untoasted soybean meal (USBM). Protein dispersibility indices of TSBM and USBM, which are often used as a parameter to measure the protein quality of SBM, were 20 and 80%, respectively. The protease preparation (Neutrase) and the cell wall degrading enzyme preparation (Energex) were obtained from Novo-Nordisk (NOVO Enzyme Process Division, Novo-Nordisk a/s, Bagsvaerd, Denmark).

Extrusion of USBM (ExUSBM) was performed on laboratory-scale using an Almex Battenfeld single-screw extruder. The length to diameter ratio was 16 and the compression ratio 1.15. The trials were carried out with screws of a constant pitch of 32 mm and a diameter of 50 mm. The die diameter was 7 mm and the screw speed 100 rpm. USBM with an initial moisture content of 25% was extruded with a torpedo screw containing zero (Ex-0), four (Ex-4), or eight (Ex-8) rows of flights on the screw, enabling an increase in shear forces[8]. Moisturization of USBM was performed with a Sunther-Papenmeier mixer. Temperatures in the different sections of the extruder were measured using eight thermocouples. With heaters on the barrel wall, the temperature of the product at the die was adjusted to 120°C. The throughput was about 12 to 14 kg/h. After extrusion the samples were dried for 2 d days at 40°C.

Experimental design

The experiment comprised 10 treatments with four replicates per treatment. Treatments 1 to 4: TSBM (Treatment 1) and TSBM treated with Neutrase (Treatment 2), Energex (Treatment 3) or a combination of both enzymes (Treatment 4), respectively. Treatments 5 to 7: ExUSBM at different shear levels; Ex-0 (Treatment 5), Ex-4 (Treatment 6), or Ex-8 (Treatment 7), respectively. Treatments 8 to 10: ExUSBM at shear level Ex-4 supplied with; Neutrase (Treatment 8), Energex (Treatment 9) or a combination of both enzymes (Treatment 10), respectively. The liquid enzyme preparations were sprinkled on the diets after pelleting. From day 1 to 7, all broilers were fed the control diet with casein as the main protein source. At day 7 chicks were weighed and the number of chicks per cage was decreased to 12. From day 7 to 25 the chicks were fed the experimental diets. The composition of these diets is shown in Table 1.

Data recording and ileal chyme measurements

Body weight and FI of the birds were recorded at 7, 14, 21 and 25 d of age. From these data FCR were calculated (grams of FI per grams of BW gain). At d 25 all chicks were killed by an intravenous injection of T61, an aqueous solution containing 200 mg embutramide, 50 mg mebezoniumiodide, and 5 mg tetracainehydrochloride (Hoechst Holland NV, Amsterdam, The Netherlands). Chyme was collected for viscosity determinations from the anterior segment of the ileum, 15 cm starting from the junction jejunum. The posterior segment of the ileum, 15 cm backwards from the ileocecal junction, was used for the collection of chyme for chemical analysis. Chyme samples for chemical analysis were pooled per cage, freeze dried and grounded to pass through a 0.2 mm screen.

Viscosity was measured on two pooled chyme samples of three chicks per cage. Both observations were averaged for statistical analysis. The method of Bedford and Classen[18] was used. Approximately 1.5 g of homogenized chyme was immediately placed in a microcentrifuge

tube and centrifuged (Microcen 13, Herolab, GmbH, Laborgeräte, D-6908 Wiesloch, Germany) at 12000g for 1 min. The supernatant was directly used for the viscosity measurement using a Brookfield viscosimeter (Brookfield Engineering Laboratories Inc., Stoughton, MA 02172), Model RVDV-II$^+$/CP with a CP40 spindle at 100 rpm. Viscosity was measured at 40°C and expressed in centipoise (cP).

TABLE 1

Composition (g/kg) of the experimental diets

Ingredients and analysis	Diets[1]
SBM	382.5
Cornstarch	435.2
Sugars (meritose, dextrose)	100.0
Soya oil	50.0
Sodium chloride	3.8
Calcium carbonate	11.0
Monocalcium phosphate	14.5
DL-Methionine	3.0
premix[2]	10.0
Chromic oxide	0.4
Calculated analysis	
CP (N x 6.25)	200
ME (kcal/kg)	3046
Total Lys	10.9
Total Met+Cys	9.0
Calcium	7.8
Available phosphorus	4.0

1. Treatments: 1-4. Toasted soybean meal (TSBM) and TSBM treated with Neutrase, Energex and a combination of both enzymes, respectively. 5-7. Untoasted soybean meal (USBM) extruded at different shear levels; 0, 4, and 8 rows of flights on the screw, respectively. 8-10. Extruded USBM, 4 rows of flights, treated with Neutrase, Energex and a combination of both enzymes, respectively.

2. Premix contained per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 2,000 IU; vitamin E, 20 mg E; riboflavin, 4 mg; niacin amide, 40 mg; pantothenic acid, 12 mg; choline chloride, 500 mg; vitamin B_{12}, 15 μg; vitamin K; 5 mg; folic acid, 0.75 mg; Biotin, 0.1 mg. minerals: $CoSO_4.7H_2O$, 1 mg; $Na_2SeO_3.5H_2O$, 0.15 mg; KI, 5 mg; $FeSO_4.7H_2O$, 300 mg; $CuSO_4.5H_2O$, 100 mg; MnO_2, 100 mg; $ZnSO_4.H_2O$, 150 mg and Ethoxyquin 100 mg using cornstarch as carrier.

Nitrogen of the collected chyme and diets were analyzed with the semi-automated micro Kjeldahl method using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Crude protein content was calculated by 6.25 x N. Trypsin inhibitor activity (TIA) of USBM, TSBM, and ExUSBM extruded at different shear levels was performed with a modified Kakade method according to Smith et al.[19]. Benzoyl-DL-arginine-p-nitroanilide hydrochloride (DL-BAPA) was used as a substrate for trypsin. The TIA is expressed in mg inhibited trypsin per gram of SBM. Starch in chyme and diets were determined enzymatically using a starch test kit (Boehringer Mannheim GmbH, Tutzing, Germany).

Non starch polysaccharides (NSP) were determined as alditol acetates of their corresponding monosaccharides constituents using inositol as internal standard. In order to avoid the disturbing effects of high amounts of mono-, di- and small oligosaccharides, chyme was

extracted with 80% ethanol or with water. After 2 h extraction at room temperature the chyme was centrifuged for 10 min at 4000g. The residue was washed three times with either 80% ethanol or water and air dried (40°C) and used for the NSP determination, including pretreatment with 12 M H_2SO_4[12]. Water-soluble NSP content was calculated by the difference between the results obtained after ethanol or water extraction. Although the starch content in the chyme samples was low and could be neglected, in the diets the starch was removed prior the NSP determination by dissolving the diets in dimethyl sulfoxide and incubation with α-amylase and pullulanase for 16 h at 40°C[20]. To determine the amount of pectins, the uronic acid content of the chyme and diets after extraction with ethanol and water were determined as anhydro-uronic acid by the meta-hydroxydiphenyl assay[21] using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Sodium tetraborate (0.0125 M) was added to the 96% (wt/wt) H_2SO_4 in order to quantify glucuronic acid as well as galacturonic acid residues. Dry matter was determined by drying at a temperature of 105°C.

The dried samples were used for chromium analysis using atomic absorption spectrophotometry. Fatty acid content analysis was performed with a gas chromatography method as described by Anness[22] with margaric acid as internal standard. Hydrolysis was performed in 6 M HCl at 60°C for 1 h. Samples were analyzed on a gas chromatograph equipped with a CPsil 88 column (50m x 0.25mm). Standard fatty acid methyl esters were used for recognition of the different peaks.

The water-holding capacity (WHC) of the water-insoluble solids of the chyme samples was determined according to Robertson and Eastwood[23]. In a weighed tube (A), approximately 500 mg of sample was suspended in water (1:20) and stirred for 2 h. After centrifugation, excess water was decanted and the wet sample was weighed (B). After freeze drying the sample was weighed again (C). The WHC was calculated as (B-A)/(C-A) and expressed in grams of water per grams of sample.

The molecular weight distribution of the soluble NSP fraction was determined using high performance size-exclusion chromatography (HPSEC). Chyme samples were extracted with 80% ethanol and washed two times with 80% ethanol to remove mono- and small oligosaccharides. After adding TCA (to a final concentration of 12%) in order to precipitate the proteins and centrifugation (10 min at 4000g) the supernatant was subjected to HPSEC analysis as described elsewhere[12]. Dextran standards with molecular weights (Mw) of 10 to 500 kDa were used to estimate apparent molecule masses.

For determination of the apparent ileal nutrient digestibility, chromic oxide was added (0.04%) to each diet as a marker. Apparent ileal nutrient digestibilities (percentage) of protein (DC_{CP}), NSP (DC_{NSP}), fatty acid (DC_{Fat}) and starch (DC_{Starch}) were calculated with the following formula:

$$DC_X = 100 - \left(\frac{\%\ Cr\ in\ diet}{\%\ Cr\ in\ chyme} * \frac{\%\ 'X'\ in\ chyme}{\%\ 'X'\ in\ diet} * 100\%\right)$$

Statistical analysis

In a 2x2 factorial arrangement, the effects of heat treatment (toasting vs extrusion cooking) and enzyme addition (no addition vs addition *per sé*) were studied. Analyses of variance of data were performed using the contrast statements of the General Linear Models procedure of SAS®[24], with cage mean as experimental unit. Moreover, effects of different shear forces as well as the use of different enzyme preparations were studied by using Tukey's multiple comparison test[24].

chapter 5

RESULTS

The first objective was to study differences between toasting (TSBM) and single-screw ExUSBM on growth performance, apparent ileal nutrient digestibilities and chyme characteristics in broiler chicks (Treatments 1 to 4 vs Treatments 6, 8 to 10). In addition, a possible enzyme effect was also studied in this experimental design (Treatments 1 and 6 vs Treatments 2 to 4 and 8 to 10). The results are presented in Table 2 including possible interaction terms between thermal processing and enzyme treatment. Despite the fact that there were no significant differences in BW gain and FI between chicks fed TSBM or ExUSBM, FCR on the extruded diets were significantly improved ($P < 0.05$). Enzyme treatment did not affect BW gain, FI, and FCR. No interaction between thermal processing and enzyme treatment was found for these trials.

Apparent ileal digestibility of CP, starch and NSP were significantly improved in the ExUSBM compared with the TSBM ($P < 0.05$). Also, enzyme treatment significantly increased apparent ileal CP and NSP digestibility compared with no enzyme treatment ($P < 0.05$). Apparent NSP digestibility also showed an interaction between thermal processing and enzyme treatment (Table 2). The WHC of the water-insoluble solids in the chyme of ExUSBM birds was significantly increased compared with the chyme of birds fed TSBM ($P < 0.05$). The chyme viscosity and the concentrations of soluble NSP in the chyme did not change as a result of thermal processing or enzyme treatment. However, there was an interaction in chyme viscosity between thermal processing and enzyme treatment (Table 2).

Differences between the enzymes in animal characteristics are summarized in Table 3. No significant effects were found in growth performance. Apparent ileal digestibility of CP showed no differences between the enzyme additions. Treatment with the protease preparation gave higher apparent ileal digestibilities of starch and fatty acid compared with the addition of both the protease preparation and carbohydrase preparation, although no differences where found compared with the carbohydrase preparation treatment alone. The apparent ileal digestibility of NSP was significantly increased after the separate treatment with the carbohydrase preparation compared with combined treatment with the protease preparation or the protease preparation alone ($P < 0.05$). If the carbohydrase preparation was supplied to the diets (alone or combined with the protease), the concentration of soluble NSP in the chyme was significant lower than that of the separate protease preparation treatment ($P < 0.05$). The WHC of the water insoluble solids in the chyme was the lowest after treatment with both the protease preparation and carbohydrase preparation and differed significantly with the WHC obtained after treatment with the carbohydrase preparation alone (Table 3). Chyme viscosity was not altered by the addition of the different enzymes.

The results of shear forces during single-screw extrusion of USBM on growth performance, apparent ileal nutrient digestibilities and chyme characteristics in broiler chicks are shown in Table 4. Different shear forces had no significant effect on growth performance ($P < 0.05$). However, FCR seemed to be negatively affected in birds fed a diet containing Ex-8 ($P < 0.10$). Apparent ileal nutrient digestibilities showed no significant differences between shear levels. It should be mentioned that in chickens fed the diet containing Ex-4, the apparent ileal digestibility of CP had the tendency to give a

TABLE 2

Growth performance, apparent ileal nutrient digestibility and chyme characteristics (± SD) in broiler chicks fed diets with thermal processed SBM and diets treated with a protease, carbohydrase or a combination of both

Item	Thermal processing		Enzyme treatment		Probabilities		
	toasting	extrusion	No enzyme	Enzyme	Thermal processing(T)	Enzyme	T x E Treatment(E)
Chick performance 7-25 d of age							
BW gain, g	1319 ±46	1344 ±48	1321 ±53	1335 ±47	NS	NS	NS
FI, g	2141 ±92	2098 ±68	2104 ±64	2125 ±88	NS	NS	NS
FCR, g:g	1.62 ±0.03	1.56 ±0.03	1.59 ±0.05	1.59 ±0.04	0.001	NS	NS
Apparent ileal nutrient digestibility							
DC_{CP}, %	82.2 ±1.4	87.5 ±2.0	83.7 ±3.3	85.2 ±3.1	0.001	0.024	NS
DC_{Starch}, %	99.22±0.07	99.33±0.08	99.29±0.08	99.28±0.09	0.002	NS	0.013
DC_{Fat}, %	77.0 ±5.4	76.6 ±8.0	75.0 ±5.5	77.4 ±7.1	NS	NS	NS
DC_{NSP}, %	11.4 ±5.5	26.7 ±2.8	14.5 ±10.8	20.6 ±7.8	0.001	0.001	0.034
Chyme characteristics							
Soluble NSP, %	9.7 ±3.9	10.6 ±4.9	9.1 ±2.5	10.5 ±4.9	NS	NS	NS
WHC, g:g	5.14±0.13	6.24±0.28	5.61±0.58	5.72±0.61	0.001	NS	NS
Chyme viscosity, cP	3.52±0.48	3.41±0.41	3.53±0.42	3.44±0.46	NS	NS	0.016

maximum protein digestibility ($P < 0.10$). Extrusion at the highest shear level (Ex-8) significantly increased ($P < 0.05$) chyme viscosity, WHC and concentration of soluble NSP compared with extrusion at lower shear levels (Table 4).

TABLE 3

Growth performance, apparent ileal nutrient digestibility and chyme characteristics (±SD) in broiler chicks fed with diets treated with a protease, carbohydrase or a combination of both enzymes

parameter	Neutrase		Energex		Neutrase and Energex	
Weight gain, g	1316	±55	1327	±38	1362	±37
FI, g	2106	±76	2125	±85	2142	±109
FCR, g:g	1.60	±0.02	1.60	±0.03	1.57	±0.06
DC_{CP}, %	85.6	±3.7	85.7	±2.8	84.5	±3.0
DC_{Starch}, %	99.32[a]	±0.10	99.27[ab]	±0.08	99.24[b]	±0.10
DC_{Fat}, %	79.8[a]	±6.6	77.8[ab]	±6.6	74.7[b]	±7.9
DC_{NSP}, %	18.3[a]	±9.9	23.6[b]	±6.1	19.8[a]	±7.0
Soluble NSP, %	12.6[a]	±4.2	9.3[b]	±5.1	9.6[b]	±5.1
WHC, g;g	5.71[ab]	±0.71	5.86[a]	±0.64	5.61[b]	±0.47
Chyme viscosity, cP	3.35	±0.22	3.58	±0.38	3.41	±0.68

[a,b] Values in a row with no common superscript differ significantly ($P < 0.05$).

In order to study the molecular weight distribution of soluble NSP in the chyme, high performance size-exclusion chromatography (HPSEC) analysis was performed. Figure 1a shows the dextran standard. In Figure 1b are the results given for chyme of chicks fed diets containing TSBM and ExUSBM extruded at different shear levels (Ex-0, Ex-4 or Ex-8, respectively). With increasing intensity of the process (toasting, extrusion with zero, four and eight flights, respectively), the concentration of high molecular NSP (apparent Mw > 500 kDa) sharply increased. Also, NSP with an apparent Mw of 10 kDa increased with the intensity of the process. Carbohydrates with an apparent Mw of 1000, which are oligosaccharides, had the tendency to decrease after extrusion compared with toasting. If the protease, carbohydrase and a combination of both enzymes were supplied to diets containing TSBM (Figure 1c) or ExUSBM; Ex-4 (Figure 1d), the concentration of high molecular NSP (apparent Mw > 500 kDa) decreased. This result appeared especially after enzyme treatment of the ExUSBM diets, indicating that enzymatic breakdown of cell wall material had occurred. Mortality of the birds averaged 6.1%.

DISCUSSION

Growth performance

Extrusion of SBM improved FCR of broiler chicks compared with toasting (1.56 vs 1.62). In a previous study[17], it was shown that the FCR of chicks was also improved if

TABLE 4

Growth performance, apparent ileal nutrient digestibility and chyme characteristics (± SD) in broiler chicks fed with diets supplied with SBM extruded at different shear levels (Ex-0, Ex-4 and Ex-8)

parameter	Ex-0		Ex-4		Ex-8	
Weight gain, g	1384	±59	1357	±49	1330	±51
FI, g	2167	±37	2111	±79	2138	±95
FCR, g:g	1.57	±0.04	1.56	±0.03	1.61	±0.01
DC_{CP}, %	84.9	±1.4	86.6	±1.0	85.6	±0.6
DC_{Starch}, %	99.30	±0.06	99.28	±0.13	99.30	±0.05
DC_{Fat}, %	74.0	±6.1	71.9	±4.5	76.4	±5.8
DC_{NSP}, %	24.2	±3.6	24.4	±1.5	21.3	±2.6
Soluble NSP, %	4.3[a]	±3.5	7.7[a]	±1.9	13.4[b]	±1.8
WHC, g:g	5.65[a]	±0.20	6.12[ab]	±0.29	6.45[b]	±0.40
Chyme viscosity, cP	3.20[a]	±0.29	3.80[a]	±0.35	5.16[b]	±0.44

a,b Values in a row with no common superscript differ significantly ($P < 0.05$).

TSBM was extruded (1.65 vs 1.71) using a Wenger X-20 single-screw extruder. Both experiments clearly show the positive effect of SBM extrusion on growth performance. Conflicting results are reported in literature concerning the effect of extrusion on the nutritional value of soybeans. Meyer and Froseth[25] found an improved BW gain and FCR in broiler chicks fed a diet containing extruded vs toasted soybeans. However, FCR of broiler chicks fed extruded soybeans (138 or 154°C) were identical compared with those of birds fed a solvent-extracted SBM, but were significantly weaker after extrusion at 104°C[26]. Both studies do not report trypsin inhibitor contents. In the present study the TIA were 23.9, 2.9, 3.5, 1.7 and 1.1 mg/g SBM for USBM, TSBM, ExUSBM (Ex-0, Ex-4 and Ex-8), respectively (not tabulated). All thermal treatments were sufficient to decimate initial TIA levels. Therefore, the differences in growth performance between chicks fed TSBM vs ExUSBM diets can not fully be explained by residual trypsin inhibitor levels.

In general, the enzyme treatments did not improve growth performance of broiler chicks. Moreover, among the enzyme treatments no differences in growth performance were noticed. However, treatment with both the protease and carbohydrase showed a possible synergistic effect ($P > 0.05$) on BW gain and FCR as compared with the separate enzyme treatments. At a relatively stable FI, BW gain increased and FCR improved, indicating that by proteolytic and cell wall degrading activity some nutrients are better absorbed in the gastro intestinal tract of the chicken. Irish and co-workers[16] found no improvement in growth performance of 1 to 21 d old broiler chicks fed SBM diets, treated with a mixture of different carbohydrases. Although no differences in growth performance were found, *in vitro* experiments showed that several enzyme preparations were active on SBM. Treatment with Viscozyme™ and Xylanase X-250 preparations, which are both carbohydrases, increased nitrogen solubility in SBM caused

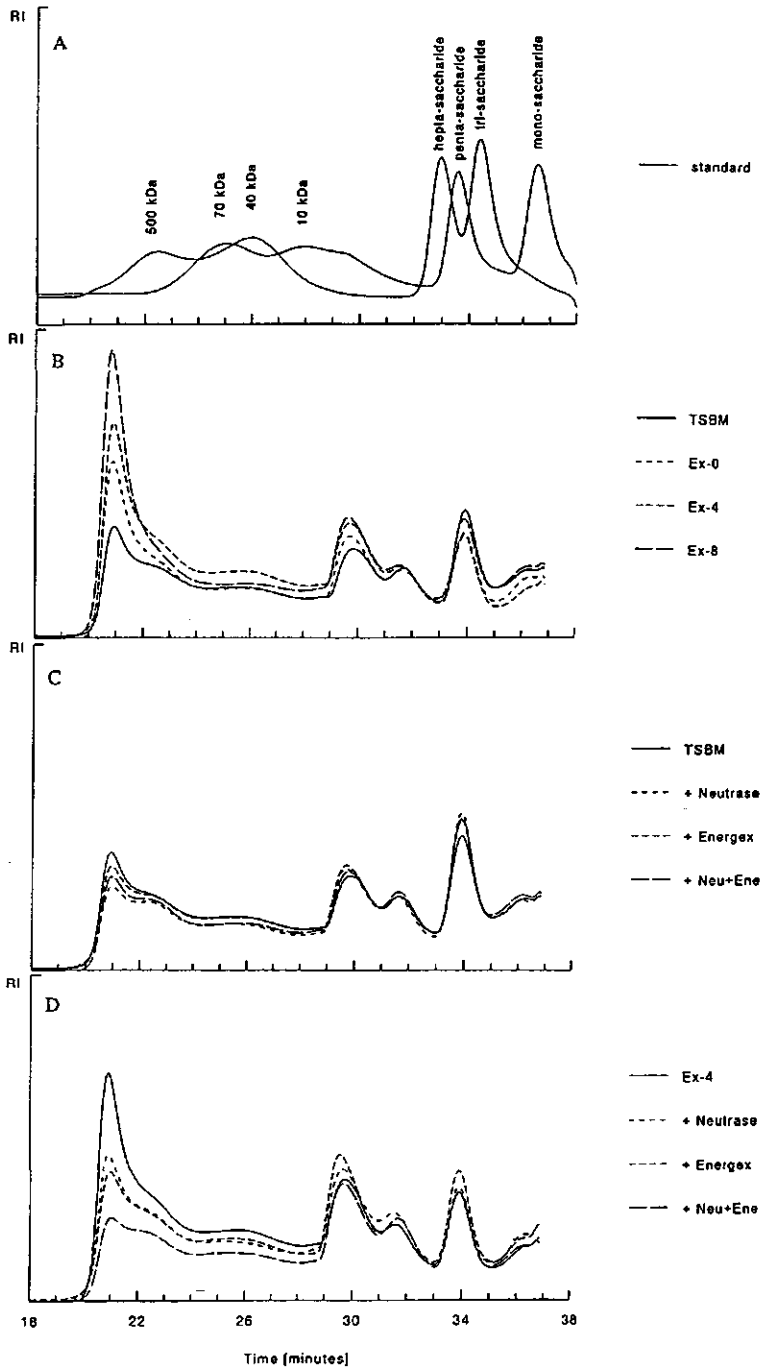

FIGURE 1

A: Elution pattern (HPSEC) of the standard. Elution pattern (HPSEC) of the soluble NSP fractions of chyme collected after feeding broiler chicks a diet containing **B**: TSBM and USBM extruded at different shear levels (Ex-0, Ex-4 and Ex-8, respectively), **C**: TSBM and TSBM treated with a protease preparation and carbohydrase preparation and a combination of both and **D**: extruded SBM (Ex-4) and Ex-4 treated with a protease preparation, carbohydrase preparation and a combination of both enzyme preparations

by release of proteins by cell wall degrading activities but also by some proteolytic activity in the crude enzyme preparations[27]. When the protease and carbohydrase preparations used in this research were studied in *in vitro* experiments with USBM, TSBM, or ExUSBM (Ex-4), it appeared that the protease preparation was able to solubilize higher amounts of proteins in the extruded samples compared with TSBM. On the other hand the carbohydrase preparation was able to solubilize considerable amounts of neutral sugars in both TSBM and ExUSBM (Ex-4)[12]. The protease preparation was also able to solubilize considerable amounts of neutral sugars, whereas the carbohydrase preparation could release proteins. This result may explain why it is difficult to find differences in growth performance between both enzymes. Nevertheless, the previously observed *in vitro* enzyme activities do not fully match the *in vivo* growth performance in broiler chicks in this experiment. This result must be described to other factors in the gastro intestinal tract of the chicken, such as pH, viscosity or the influence of other components, which may play an important role in the effectiveness of enzyme activity on the animal.

Extrusion at different shear levels did not alter growth performance of the chicks. Data on the effect of different screw-configurations on animal performance are scarce in the literature. Shear forces induced by specially designed screw elements such as the torpedo elements in this study or the so-called twin lead slotted screws in a previous study[17] were not powerful enough to result in significant differences in growth performance in broiler chicks. Exposure to higher shear forces with the single-screw extruder, is from a technical point of view, not feasible and economically less attractive.

Apparent ileal nutrient digestibility

In ExUSBM, a significantly higher apparent ileal CP digestibility was noticed compared with TSBM (87.5 vs 82.2%). Several studies showed that extrusion under mild conditions may improve *in vivo* CP digestibility. Extruded soybeans had superior CP digestibility in pigs compared with other thermal processes e.g., jet sploding and roasting[28]. With the presence of shear forces and a high energy input during extrusion, proteins are better unfolded and denatured compared with toasting[12]. The susceptible bonds for enzyme hydrolysis, which are buried in the interior of the proteins, are more easily accessible for enzyme attack after extrusion[11].

The amount of starch in the chyme was very low for all the treatments (12 to 22 g/kg DM, results not shown), whereas in the experimental diets the starch content was at least 435 g/kg. The apparent ileal starch digestibility appeared to be very high ($> 99\%$) for all thermal treatments, but evenso a significant increase ($P < 0.05$) was noticed after extrusion compared with toasting.

The apparent ileal digestibility of NSP increased as a result of extrusion compared with toasting (26.7 vs 11.4%). As monogastric animals lack the proper enzymes to breakdown cell wall materials, the higher NSP digestibility after extrusion may be the result of improved fermentation of cell wall components by bacteria in the gut. The effect has to be tremendous due to the fact that the major site for fermentation in birds is the ceca, which is 'post ileal'. However, chyme refluxing may provide bacterial populations in the used part of the ileum. In experiments with wheat based diets fed to rats, it is known that after extrusion the ratio of insoluble to soluble dietary fiber is lowered, resulting in a decrease in faecal recovery of arabinose, xylose, and glucose compared

with raw wheat flour[29]. The higher solubility is probably responsible for this increased fermentability. Lintas et al.[30] reported that soluble fiber content of extruded legumes increased compared with the raw materials, but the extent of solubilization depended upon the type of legume. The HPSEC analysis of the carbohydrate fractions in the chyme also showed that more soluble NSP components were present after extrusion compared with toasting. Therefore, disruption and homogenization of fiber by intense mechanical treatment during extrusion cooking could render dietary fiber more available to fermentation.

Enzyme treatment, which did not affect growth performance of the broiler chicks, significantly increased apparent ileal CP digestibility compared with no enzyme treatment (85.2 vs 83.7%). Among the enzymes no differences occurred in CP digestibility, which means that all enzymes, to some extent, increased *in vivo* CP digestibility. As mentioned before, in a previous study it was shown that the carbohydrase preparation was able to solubilize considerable amounts of protein, because it also exhibited proteolytic activity[12]. This finding may explain the overall increase in apparent ileal CP digestibility and also why no differences were found among the enzyme treatments. It could also mean that proteins were abundantly available in all diets. Therefore, the increased CP digestibility did not lead to a better chick performance.

Although enzyme treatment, in general, improved apparent ileal NSP digestibility compared with no enzyme treatment (20.6 vs 14.5%), it can be seen that, in particular, the treatment with the carbohydrase preparation increased the NSP digestibility (23.6%). From *in vitro* studies it is known that this enzyme preparation is very effective in solubilizing high amounts of cell wall material[12]. One might expect that a higher degree of solubilization, thus a higher fermentation rate in the gut, may contribute to a higher amount of ME in the diet which result in an improved growth performance[31]. The present study did not show this improvement in growth.

With respect to the apparent ileal NSP digestibility, there was an interaction between thermal processing and enzyme treatment. It appeared that addition of enzymes to TSBM increased apparent ileal NSP digestibility relatively better (13.6 vs 4.6%) than enzyme addition to ExUSBM (27.5 vs 24.4%). However, HPSEC analysis of chyme showed that enzyme treatment had more impact on the soluble NSP fractions in ExUSBM than TSBM. These results should be interpreted with care, because two factors are largely influencing the results. First, extrusion solubilized more cell wall material than toasting which resulted in a higher apparent ileal NSP digestibility. A certain amount of NSP was solubilized but could not be fermented by the bacteria, resulting in an accumulation of a high molecular undigestible NSP fractions (apparent MW > 500 kDa and 10 kDa, respectively). Secondly, enzyme activity was very effective in hydrolyzing those undigestible cell wall components into oligo- and monosaccharides. The HPSEC analysis shows the resultant of both phenomena, but it is clear that enzymatic breakdown of both soluble and insoluble cell wall components may contribute to improved NSP digestibility.

Chyme characteristics

The concentration of soluble NSP in the chyme of chicks fed TSBM or ExUSBM did not differ significantly (9.7 vs 10.6%). However, HPSEC analysis showed that after extrusion more high molecular weight NSP fractions and less oligosaccharides were obtained compared with toasting. It was expected that high molecular weight NSP

fractions may contribute more to the viscosity in the ileum than smaller ones[15]. However, no significant differences in ileal viscosities were obtained between toasting and extrusion (3.52 vs 3.41 cP). Therefore, it is difficult to conclude whether the lower growth performance and apparent ileal CP digestibility after toasting may be related to an increase in chyme viscosity, resulting in a decrease in nutrient absorption in the small intestine. A positive relationship existed between the concentration of soluble NSP and chyme viscosity in the chyme after feeding the chicks SBM extruded at different shear levels. Extrusion at the highest shear level significantly increased the concentration of soluble NSP (13.4 vs 4.3 and 7.7%) and chyme viscosity (5.16 vs 3.20 and 3.80 cP) compared with extrusion at lower shear levels (Ex-0 and Ex-4, respectively). The HPSEC analysis also showed that increasing shear forces resulted in an increasing concentration of high molecular NSP, which also explained the increase in chyme viscosity. The increase in chyme viscosity is also accompanied by an increase in WHC at the highest shear level compared with the lowest shear level. Nevertheless, this increase in viscosity did not significantly depress growth performance nor apparent ileal nutrient digestibilities. It should be mentioned that BW gain had the tendency to decrease with increasing chyme viscosity. Also, FCR and apparent ileal CP digestibility tended to worsen ($P < 0.10$) after extrusion with eight flights. This result may also be explained by the increase in ileal viscosity. Several studies are reporting an increase in viscosity in the small intestine due to high soluble molecular weight forms of e.g., β-glucans and arabinoxylans, which resulted in decreased diffusional rates of the nutrients[14,15]. Polysaccharides of legumes are more complex than their counterparts in cereals[32]. This fact makes it difficult to target these cell wall components for enzyme supplementation.

In conclusion, extrusion significantly improved FCR compared with toasting, which can be explained by higher apparent ileal digestibilities of CP, starch, and NSP. Enzyme treatment did not improve growth performance but, apparent ileal CP and NSP digestibility were significantly increased compared with no enzyme treatment. Among the enzymes no differences were found in growth performance, despite the increasing apparent ileal NSP digestibility after treatment with the carbohydrase preparation. The use of different torpedo elements in order to increase the amount of shear forces during extrusion were not powerful enough to induce significant differences in growth performance and apparent ileal nutrient digestibilities among the different shear levels. The increasing chyme viscosity caused by a higher concentration of NSP did not affect growth performance or apparent ileal nutrient digestibilities.

ACKNOWLEDGEMENTS

The authors wish to thank A. Bonte, Trouw International, Putten, The Netherlands for determining chyme viscosity, T. Zandstra, Department of Animal Nutrition, Wageningen Agricultural University, R.W. Terluin and his staff, Zodiac, Wageningen Agricultural University, and S. van Voorst, ID-DLO for their help during the experimental period and dissection, and J.W. Resink, Trouw International, Putten, The Netherlands for his advises. This project was financially supported by Nutreco Agribusiness R&D, Boxmeer, The Netherlands.

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chapter 6

In vitro accessibility of untreated, toasted and extruded soybean meal for proteases and carbohydrases

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Journal of Agricultural and Food Chemistry, 45, (1997) 4088-4095

ABSTRACT

The *in vitro* accessibility of the water unextractable solids (WUS) from untreated, toasted and extruded soybean meals (SBM) towards different enzyme activities was studied. WUS was incubated with seven commercial enzyme preparations. Two enzymes preparations were selected for further research. Upon addition of Neutrase, the extruded SBM yielded considerably more solubilized protein compared with the toasted and untreated SBM. Energex solubilized high amounts of neutral sugars after heat treatments compared with the untreated meals. Cell wall polysaccharides solubilized by the enzymes were released as small oligosaccharides and monosaccharides.

SDS-PAGE analysis showed that after enzyme addition to the extruded SBM, proteins were more rapidly and completely degraded compared with enzyme addition to the toasted and untreated SBM. Neutrase degraded both β-conglycinin and glycinin. Energex could only, partly, degrade β-conglycinin. The basic polypeptide from glycinin showed the highest resistance against proteolytic activity.

(*Key words*: soybean meal, toasting, extrusion, enzymes, soy protein, cell wall components)

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INTRODUCTION

Due to a good nutritional value and abundant availability as byproduct of the oilseed industry, soybean meal (SBM) has received considerable attention for the replacement of other crops in feed. However, the nutritional value of SBM is limited by the presence of antinutritional factors (ANFs), e.g. trypsin inhibitors and lectins, which negatively affects protein digestion[1]. Also, the compact structure of the most important proteins of SBM, β-conglycinin and glycinin, may hinder hydrolytic enzymes in the intestinal tract[2]. The presence of a high content of cell wall components may also limit the nutritional value of legumes[3,4].

To improve the nutritional value of SBM, heat treatments such as toasting and extrusion are frequently used. Heat-labile ANFs, e.g. trypsin inhibitors, are effectively inactivated[5], and proteins will denature to a certain extent, which makes them more susceptible to enzymic degradation. During extrusion, shear forces may play an important role in enhancing the *in vitro* protein digestibility[6]. However, too much heat[7] and/or shear force[8] may result in a decreased nutritional value of SBM.

Another approach for a further valorization of raw and processed materials is the application of feed enzymes. The main objectives are supplementation of the endogenous enzymes, removal of ANFs and to render certain nutrients more readily available for absorption[9]. Non starch polysaccharides (NSP) are known to exhibit an antinutritional effect, partly because monogastric animals lack the appropriate enzymes to hydrolyse them[10]. Also, the metabolic utilization of the sugars from NSP is limited in monogastric animals. Legumes contain numerous NSPs and they are more complex in structure than NSP from cereals. This makes it difficult to target the legume polysaccharides for feed enzyme supplementation[11]. The complexity of the polysaccharides and proteins in SBM makes it necessary to use multi-enzyme preparations for solubilization rather than addition of a single enzyme[12]. In most studies, the main attention has been focused on *in vivo* effects as a result enzyme addition[9,13].

In this research, untreated, toasted and extruded SBM were studied for their *in vitro* accessibility towards different enzyme activities. First, seven commercial enzyme preparations (proteases and carbohydrases) were studied for their ability to solubilize proteins and cell wall polysaccharides from untreated and toasted SBM. The differences between toasting and extrusion with respect to the *in vitro* accessibility towards a protease and a carbohydrase were studied in more detail.

MATERIALS AND METHODS

Materials

Commercial solvent-extracted, toasted (85°C, 20 min) soybean meal (TSBM) was used. From the same batch of SBM, after oil extraction, a part was not toasted but air-dried at room temperature, yielding untoasted soybean meal (USBM). The protein content of both meals was 51% (N x 6.25), and they were supplied by Cargill (Amsterdam, The Netherlands). The nitrogen solubility index (NSI) in potassium hydroxide was 98 and 73% for USBM and TSBM, respectively. Esperase, Neutrase, Bio-Feed Pro, Bio-Feed Plus, SP-249 and Energex were obtained from Novo Nordisk (Bagsvaerd, Denmark) and Driselase from Sigma (St. Louis, MO).

Extrusion experiments

Extrusion trials were performed with a Battenfeld single-screw extruder. USBM with an initial moisture content of 25% was extruded using a screw of a constant pitch, a compression ratio of 1.15, and a die diameter of 7 mm. At the end of the screw a torpedo element with four rows of flights was assembled. Temperature control was performed with one cooler, four heaters, and eight thermocouples, which were connected in the different sections of the barrel. The final product temperature, manually measured at the die using a thermocouple, was kept at 120°C. The screw speed was set at 100 rpm. Moisturization was performed with a Sunther-Papenmeier mixer a day before extrusion. The premoisturized meal was stored overnight at 4°C and brought to room temperature prior to extrusion. The extruded soybean meal (ExUSBM) was dried at 45°C and ground to pass a 0.2 mm screen.

Preparation of water unextractable solids (WUS)

To avoid the disturbance by mono- and oligosaccharides, e.g. sucrose, raffinose, and stachyose, which are present in SBM during sugar analysis, USBM, TSBM and ExUSBM were extracted with water to yield the WUS. Ten grams of SBM was extracted with 200 ml of water at room temperature for 2 h under continuous stirring. After centrifugation (20 min, 10000g), a residue and a supernatant were obtained. The residue was resuspended in 100 ml of water and centrifuged again. This procedure was repeated three times. The residues were resuspended in a small amount of water, dialyzed, and freeze-dried.

Enzyme incubations

In a first experiment, several commercial enzyme preparations were screened for their ability to solubilize proteins and to release fragments of neutral and acidic cell wall polysaccharides from USBM and TSBM. Esperase, Neutrase and Bio-Feed Pro (proteolytic enzymes) and Bio-Feed Plus, SP-249, Energex and Driselase (cell wall degrading enzymes) were added to 10% SBM WUS suspensions in a 0.05 M acetate buffer (pH 5.0). With Esperase incubations were also performed in a carbonate buffer (pH 9.0), the optimum pH of this enzyme preparation. The enzyme/substrate ratios were 0.025 and 0.25% (protein/protein basis) for all the enzymes. Incubations were performed under continuous stirring at 37°C for 0 min (blanks) and 24 h. After incubation and centrifugation (10 min, 3000g), the supernatant was directly used for determination of the amount of soluble proteins and neutral and acidic cell wall polysaccharides fragments analyzed as neutral sugars and uronic acids, respectively.

Second, Neutrase and Energex were used in to study the accessibility of USBM, TSBM, and ExUSBM toward these enzymes in more detail. Also, combinations of both enzymes were used. Three hundred milligrams of WUS was weighed into 10 ml Kimax tubes with screw caps. Three milliliters of 0.05 M sodium acetate buffer (pH 5.0) containing the enzymes was added. The enzyme addition was standardized to 750μg of protein for the separate incubations with Neutrase and Energex and 2 x 375μg of protein for the combined incubation with these enzymes. The tubes were rotated at 37°C for 0, 15, 60, and 240 min and 24 h, then quickly cooled to 0°C and centrifuged (10 min, 3000g) at 4°C. For the nitrogen determination 0.8 ml of the supernatant was pipetted in Kjeldahl tubes. For the molecular weight distribution of the carbohydrate fraction and for anion exchange chromatography analysis 0.8 ml supernatant was transferred to Eppendorf cups, which already contained 0.4 ml of 20% trichloroacetic acid (TCA). After precipitation of the proteins (20 min at room temperature), and centrifugation (10 min 3000g), 1 ml of supernatant was transferred from these cups to HPLC vials and subjected to analysis. Half of a milliliter of the supernatant was transferred to Eppendorf cups, heated for 10 min at 100°C and centrifuged (10 min, 3000g) after which time the supernatant was used for determination of the neutral sugar and uronic acid content. The residue was washed four times with excess water (0°C), freeze-dried,

weighed, and ground. The residue was analyzed for sugar composition and subjected to SDS-PAGE analysis in to study protein breakdown.

Molecular weight distribution

The molecular weight distribution of solubilized cell wall polysaccharide fragments was studied using high performance size-exclusion chromatography (HPSEC), which was performed on a SP8800 HPLC (Spectra Physics, San Jose, California, USA) equipped with three Bio-Gel TSK columns (each 300 x 7.5 mm) in series (40XL, 30XL, and 20XL; Bio-Rad Labs, Hercules, CA) in combination with a TSK XL guard column (40 x 6 mm) and eluted at 30°C with 0.4 M acetic acid/sodium acetate (pH 3.0) at a flow rate of 0.8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector (Showa Denko K.K., Tokyo, Japan). The system was calibrated with dextran standards (10 to 500 kDa).

High Performance Anion-Exchange Chromatography (HPAEC)

Chromatographic analysis of the mono- and oligomeric fragments in the samples was performed using a Dionex Bio-LC system (Sunnyvale, California, USA) equipped with a CarboPac PA-1 column (250 x 4 mm) in combination with a CarboPac PA quard column (25 x 3 mm). Samples were injected using a Spectra Physics SP 8800 autosampler, and chromatograms were recorded with a Spectra Physics Winner system. The effluent was monitored using a pulsed electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode[14]. To elute monosaccharides and oligosaccharides, the following 0.1 M NaOH gradient was used: 0-26 min, 15 mM; 26-30 min, 15-100 mM; 30-59 min, 100-75 mM; 59-59.1 min, 75-0 mM; 59.1-64 min, 0 mM; 64-64.1 min, 100 mM; 64.1-70 min, 100 mM; 70-80 min, 100-15 mM. The simultaneous gradient of 1 M NaOAc in 0.1M NaOH was as follows: 0-30 min, 0 mM; 30-59 min, 0-250 mM; 59-59.1 min, 250-1000 mM; 59.1-64 min, 1000 mM; 64-64.1 min, 1000-0 mM; 64.1-80 min, 0 mM. Samples (20 μL) were injected at 80 min.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Protean™ II electrophoresis system from Bio Rad. To reduce the protein, in order to obtain subunits, disulfide bonds were cleaved by β-mercaptoethanol. In general, the method of Laemmli[15] was followed with or modifications. From the residue an amount of sample corresponding to 2 mg of protein was dissolved in 1 ml buffer solution (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 10% glycerol, 0.00125% bromophenol blue and 2.5% β-mercaptoethanol). Reduction and solubilization of the proteins were obtained after 3 h of mixing head over tail in Eppendorf cups at 37°C. Every 90 min, the samples were treated in an ultrasonification bath at 60°C for 15 min. Runs were performed in homogeneous slab gels (T=12.5%, C=2.6%). Gel slabs were fixed and stained in a solution of 40% methanol, 10% acetic acid, and 50% water containing 0.1% Coomassie Brilliant Blue R-250. The staining solution was filtered just before use. The excess of Coomassie Brilliant Blue was removed by diffusion in a destaining solution containing 40% methanol, 10% acetic acid, and 50% water.

Thermal behavior studies

Differential scanning calorimetry (DSC) was used to study the thermal behavior of the main proteins in USBM, TSBM and ExUSBM as well as the WUS obtained from these materials. Samples of 10% dispersions in water were sealed in high-pressure aluminum pans. A pan filled with distilled water was used as reference. The samples were analyzed in a Setaram Model Micro Disc III calorimeter. Heating was performed from 20 to 40°C at a rate of 3°C/min, followed by 30 min stabilization at 40°C, and then a second heating from 40 to 120°C at a rate of 0.5°C/min.

Sugar composition analysis

NSP were analyzed for neutral sugar composition according to the method of Englyst and Cummings[16] using inositol as internal standard. After pretreatment with 12 M H_2SO_4 for 1 h at 30°C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C, the monosaccharides were reduced to alditols with $NaBH_4$, and converted into their corresponding alditol acetates using 1-methylimidazole and acetic anhydride. The alditol acetates were separated on a glass column (3 m x 2 mm i.d.), packed with Chrom WAW 80-100 mesh coated with 3% OV275 in a Carlo Erba Fractovap 2300 GC operated at 200°C and equipped with a FID detector set at 270°C.

Protein determination

Total nitrogen in the supernatants after enzyme incubation and in the starting materials was determined by a semiautomated micro-Kjeldahl method using a Cu/Ti catalyst. Protein content was estimated as total nitrogen x 6.25.

Neutral sugars

Fragments of neutral cell wall polysaccharides released during enzyme incubations and total neutral sugars in the cell wall polysaccharides were analyzed, as neutral sugars, with the automated orcinol method[17] using D-glucose and D-galactose (50:50 w/w) as standards.

Uronic acids

Fragments of acidic cell wall polysaccharides released during enzyme incubations and total uronic acid content in the cell wall polysaccharides were analyzed, as uronic acids, with the automated meta-hydroxydiphenyl assay according to the method of Thibault[18]. Sodium tetraborate (0.0125 M) was added to the 96% (w/w) H_2SO_4 to quantify glucoronic acid as well as galacturonic acid residues.

RESULTS AND DISCUSSION

Composition and yield of WUS

Table 1 shows the yield and composition of the WUS obtained from USBM, TSBM, and ExUSBM, respectively. Total yields of WUS were higher after toasting and extrusion of SBM compared with the native meal. Moreover, protein content in WUS from the heat treated samples was considerably higher compared with USBM. Previous studies have revealed that heat treatment of USBM sharply decreased nitrogen solubility in USBM[6]. Protein insolubility due to denaturation could also be seen from DSC analysis (Figure 1). The area under the peak is directly proportional to the enthalpic change, and its direction indicates whether the thermal event is endothermic or exothermic. Denaturation is an endothermic process. At high protein concentrations denaturation is mostly followed by aggregation, which is generally considered as an exothermic process[19]. Energies involved in aggregation are low[20]. If one neglects the energies involved in aggregation, a rough approach of the degree of denaturation can be made by calculating the surfaces of the peaks and with the assumption that proteins in USBM are native. Compared with the peaks in USBM, in TSBM about 90% of β-conglycinin and about 50% of glycicin were denatured during toasting, while after extrusion both proteins were completely denatured. A complete denaturation of the proteins in soy protein isolate after extrusion was also found by Kitabake and Doi[21].

TABLE 1
Yield and composition of WUS (percent weight) isolated from USBM, TSBM, and ExUSBM

Material	USBM	TSBM	ExUSBM
Yield[a]	40.0	67.4	70.7
Moisture	5.5	5.7	5.5
Protein	44.4	63.6	69.0
Non starch polysaccharides	43.0	22.8	18.5
rhamnose	1.3	0.6	0.5
fucose	0.6	0.3	0.2
arabinose	5.0	2.6	2.1
xylose	1.2	0.7	0.6
mannose	1.5	1.0	0.9
galactose	12.1	6.0	5.1
glucose	11.2	6.5	5.1
uronic acid	10.1	5.1	4.0
Analyzed	92.9	92.1	93.0

[a] Expressed as weight percentage (as is basis) of USBM, TSBM and ExUSBM

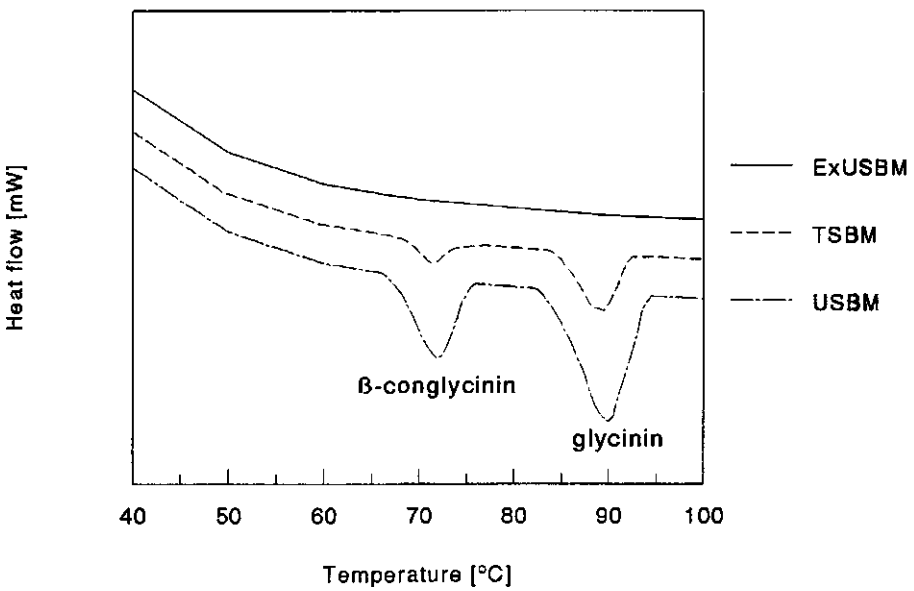

FIGURE 1
Thermal behavior, using DSC analysis, of β-conglycinin and glycinin in USBM, TSBM, and ExUSBM.

The main sugars in the cell wall polysaccharides in WUS are galactose, glucose and arabinose. Also, considerable amounts of uronic acids were found. Despite the lower NSP content in the heat treated samples, the composition among the different SBM samples shows only or differences. The relative mannose and glucose contents are higher in the WUS after heat treatment compared with USBM, while the uronic acid content has the tendency to decrease.

Effect of different enzymes on the solubilization of soybean carbohydrates and proteins

In Table 2 the proportions of solubilized proteins, neutral sugars, and uronic acids after incubation of WUS from USBM and TSBM with seven commercial enzyme preparations are given. The results were corrected for the blanks, which did not exceed

TABLE 2

Amounts of solubilized protein, neutral sugar (NS), and uronic acid (AUA) [percent] after incubation of WUS from USBM and TSBM with different commercial enzyme preparations

	USBM			TSBM		
	protein	NS	AUA	protein	NS	AUA
Esperase (pH 9.0)						
0.025%	46.6	0.4	<0.1	40.8	0.9	<0.1
0.25%	59.2	4.5	<0.1	59.7	5.6	<0.1
Esperase (pH 5.0)						
0.025%	16.7	2.1	<0.1	17.0	7.4	<0.1
0.25%	29.6	4.3	0.2	29.6	10.2	0.1
Neutrase						
0.025%	19.1	9.2	0.9	18.5	14.0	1.1
0.25%	32.4	16.6	1.1	30.7	23.3	2.0
Bio-Feed Pro						
0.025%	18.4	4.3	0.2	10.4	8.8	<0.1
0.25%	29.5	6.1	0.8	32.4	11.6	2.6
Bio-Feed Plus						
0.025%	3.1	2.5	0.1	2.6	17.2	2.3
0.25%	10.1	16.2	2.3	10.8	43.7	7.3
SP-249						
0.025%	9.3	11.5	8.6	4.7	12.6	8.7
0.25%	22.0	55.3	21.6	13.6	47.4	34.4
Energex						
0.025%	8.2	9.0	7.8	3.8	12.1	8.4
0.25%	20.0	50.6	30.3	8.3	48.9	32.9
Driselase						
0.025%	13.5	13.3	8.5	11.3	22.3	5.9
0.25%	29.3	52.4	21.6	24.6	49.8	22.1

5% for any given component, except for the protein blank of WUS from USBM at pH 9.0, which was 13.4% (no further results shown). It can be concluded that Neutrase, Bio-Feed Pro and especially Esperase were all three powerful proteolytic enzyme preparations, while Energex, SP-246 and Driselase were the most active cell wall degrading enzymes. To study the differences between toasting and extrusion for their *in vitro* accessibility towards enzymic activity in more detail, Neutrase (as protease) was chosen, because the high pH optimum of Esperase limits its industrial application. From the cell wall degrading enzymes Energex was chosen, because it showed the most favorable ratio between a high ability in solubilizing neutral sugars and uronic acids and relatively low activity in solubilizing proteins compared with SP-246 and Driselase®. In previous research, both enzyme preparations were also used for an *in vivo* experiment with broiler chickens[22].

Proportion of solubilized proteins

The amounts of protein released by Neutrase, Energex and the combination of both enzymes are given in figure 2A. The solubility was corrected for the soluble protein in the blank, which did not exceed 5%. It can be seen that Neutrase, as expected, was very effective in solubilizing proteins. In USBM and TSBM about equal amounts of protein were solubilized, which was in line with the results from the previous experiment (Table 2). However, the amount of protein released by Neutrase sharply increased after extrusion. Energex was also able to solubilize up to 20% of the proteins, but limited effects were found among the different SBM samples. Work from others[23] also showed that typical cell wall degrading enzyme preparations, e.g. Viscozyme™ and Multifect™ were able to solubilize 35-70% of the nitrogen in whole SBM after 25 h of incubation at 40°C. In Energex, a considerable amount of protease activity, as determined with the azocaseine assay (results not shown), may explain why from all materials proteins were solubilized (Figure 2A). If the combination of both enzymes was applied, it was shown that about equal amounts of protein were solubilized compared with the separate Neutrase addition, despite the fact that Neutrase was added at only half the concentration compared to the separate incubation with Neutrase. This suggests a slight synergistic effect between Neutrase and Energex.

Process conditions can explain the differences in protein solubility obtained after extrusion and toasting. During extrusion, at intense shear forces and an excess of specific mechanical energy (SME), non covalent interactions as well as covalent disulfide bonds between proteins are easily broken compared with toasting[24]. Upon cooling the denatured proteins will aggregate rather than renature, because it is statistically unlikely that after extrusion the original bonds will be re-formed. After toasting, however, most disulfide bonds remain unaffected, which means that upon cooling the protein can return to its native conformation[25]. Therefore, the intensity of the thermal treatment may explain the increased proportion of solubilized proteins after enzyme incubation in ExUSBM compared with TSBM.

Proportion of solubilized neutral sugars and uronic acids

The effects of incubation with Neutrase, Energex and the combination of both enzymes on neutral sugar and uronic acid solubility are given in part B and C of Figure 2,

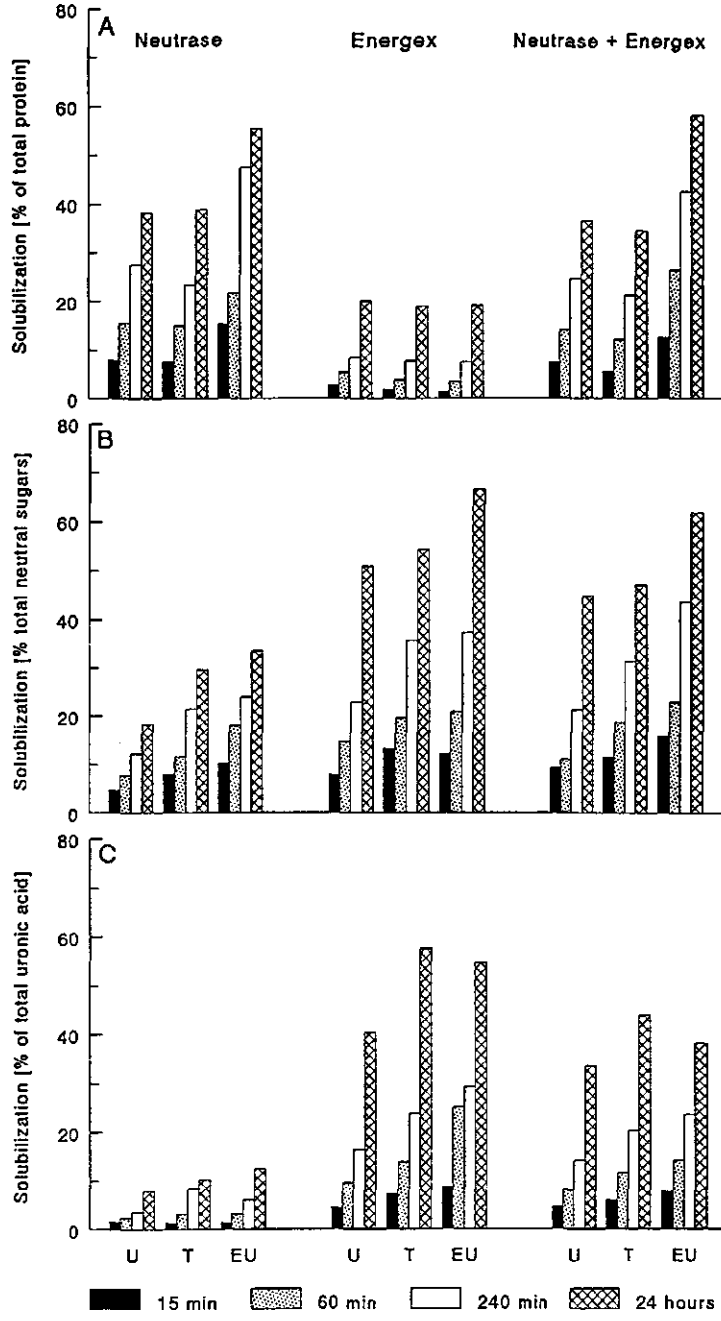

FIGURE 2

Proportion of solubilized (A) proteins, (B) neutral sugars (NS) and, (C) uronic acids (AUA) (percent of total protein, neutral sugars, and uronic acids, respectively) after incubation of USBM (U), TSBM (T), and ExUSBM (EU) with Neutrase, Energex, and the enzyme combination at different times.

respectively. From Figure 2B it can be seen that Energex was able to solubilize large amounts of neutral sugars, up to 67% of all neutral sugars present from ExUSBM. Extrusion significantly increased the proportion of solubilized neutral sugars for all enzyme treatments compared with TSBM and USBM, while in the latter the lowest release of sugars was noticed. Treatment with the combination of both enzymes showed that the proportion of solubilized neutral sugars was lower compared with the separate Energex incubation. For the uronic acids, it appeared that after incubation with Energex the proportions of solubilized uronic acids in both TSBM en ExUSBM were increased compared with USBM (Figure 2C). In this case the solubility in the extruded samples after 24 h had the tendency to decrease again. Also, uronic acid solubility was lower after treatment with the combination of both enzymes compared with the separate incubation with Energex (Figure 2C).

Neutrase, a well-known proteolytic enzyme preparation, was also able to release considerable amounts of neutral cell wall polysaccharide fragments, measured as neutral sugars (Figure 2B). After extrusion, slightly more neutral sugars were analyzed compared with TSBM. Especially in the heat-treated samples, breakdown of protein aggregates may lead to a release in cell wall polysaccharide fragments. However, a nonstandardized amount of cellulase activity, present in Neutrase, may also explain the presence of soluble cell wall polysaccharide fragments. Only small amounts of acidic cell wall polysaccharide fragments, measured as uronic acids, were found after incubation with Neutrase (Figure 2C). Limited differences were found between toasting and extrusion.

When the results from Figure 2 are combined, it can be concluded that, to solubilize considerable amounts of both protein as well as cell wall components, it is more effective to add a mixture of a protease and a cell wall degrading enzyme preparation in lower concentrations rather than to add each of the preparations separately in higher, e.g. double, concentrations.

Molecular weight distribution and mono/oligosaccharide ratio

HPSEC was used to study the molecular weight distribution of the solubilized cell wall polysaccharide fragments after enzyme incubation. It appeared that for all enzyme incubations the same HPSEC pattern were obtained. A typical example is given in Figure 3 for TSBM incubated with the combination of Neutrase and Energex. From the dextran standards used it can be calculated that the large peak at 36 min corresponds with a monosaccharide, while at 33 and 34 min small oligomers up to a degree of polymerization of 5 were eluted from the column. Therefore, it can be concluded that if cell wall components were solubilized, they also were degraded to small oligomers and monomers.

The degradation to small oligomers and monomers was also confirmed by HPAEC. Despite the fact that several oligomer standards were used, it was not possible to identify the various oligomers, because a high number of different oligomers resulted in much overlap between peaks. If it is assumed that monomers and oligomers have similar PAD response, the ratio between monomers and oligomers in the soluble fractions after the different enzyme incubations can be calculated. The results are given in Figure 4. After incubation with Energex for 15 min, 50-55% of the solubilized sugars were monomers, but after longer incubations relatively more oligomers, up to 55-70%, were released. After treatment with Neutrase, the relative amount of oligomers represented more

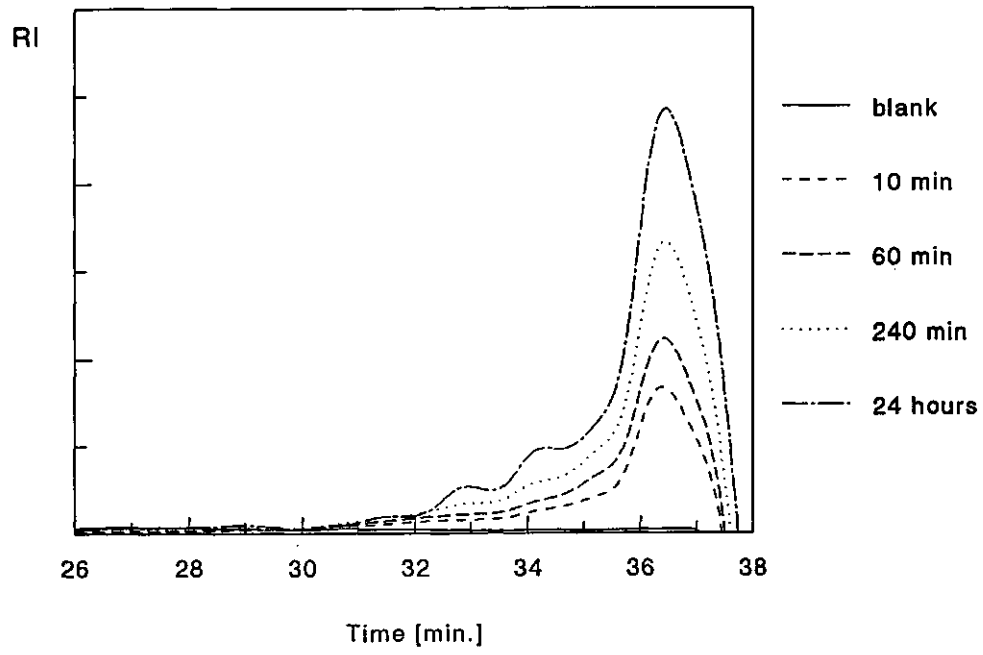

FIGURE 3
Molecular weight distribution of the soluble carbohydrate fraction of TSBM after incubation with the combination of Neutrase and Energex.

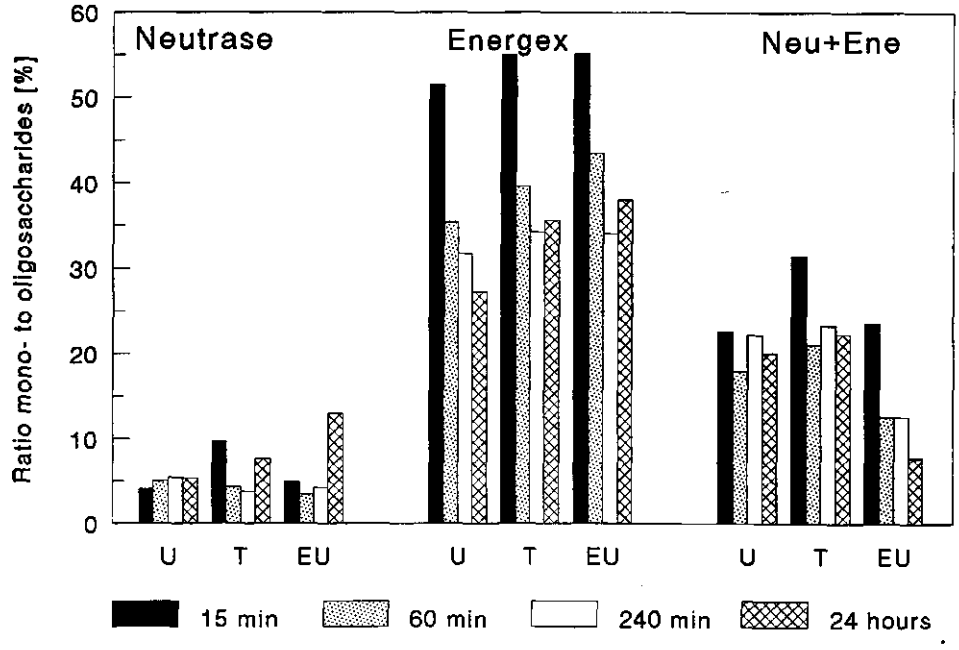

FIGURE 4
Monosaccharide/oligosaccharide ratio (percent of the soluble fraction) of USBM (U), TSBM (T), and ExUSBM (EU) after incubation with Neutrase, Energex, and the enzyme combination at different times.

than 90% of the total solubilized cell wall constituents, indicating the lack of proper enzyme activities in Neutrase to degrade these oligomers to monomers as was seen for Energex. While the total amount of neutral sugars released by the combination of Neutrase and Energex was slightly lower than the amount released by Energex alone (Figure 2B), the ratio between monomers and oligomers was much lower for the enzyme combination (Figure 4). This indicates that enzyme activities present in Energex can further degrade fragments released by Neutrase. Only small differences in monomer / oligomer ratios between the native, toasted, and extruded SBM were found.

Breakdown of cell wall constituents to small oligomers and monomers was also found by Schols et al[26], who incubated a deproteinated untreated SBM WUS with several crude enzyme preparations. Although monomers were released by some enzyme preparations, the question is if these amounts would be sufficient to increase the metabolizable energy value (ME) of the feed[9]. If the amount of monomers obtained after HPAEC analysis were expressed on WUS basis, it appeared that after 24 h incubation with Neutrase, Energex and the enzyme combination, 2-5, 12-22, and 5-10%, respectively, of the total cell wall components in the WUS were degraded to monomers. An estimation of the ME increase after extrusion and incubation with Energex is made, using the following assumptions: 22% sugar release in the WUS, a yield of WUS of 70%, all sugars regarded as glucose and fully absorbed (17 kJ/g) and SBM for 30% incorporated in the diet. The increase in ME can be calculated as 790 kJ/kg. Normally, the ME of a chickens diet is about 13000 kJ/kg, which means that the ME may increase by 6%.

Sugar composition

The molar sugar composition of the soluble sugar fraction after 24 h of incubation with Neutrase, Energex and the enzyme combination are given in Table 3. Despite the fact that after extrusion enzyme activity resulted in a higher amount of solubilized neutral sugars and uronic acids compared with TSBM (Figure 2B), limited differences in the molar sugar composition were found between toasting and extrusion (Table 3). After incubation with Energex, 80-85% of the soluble carbohydrate fraction consisted of galactose, arabinose, and uronic acids, while limited amounts of glucose, mannose, and xylose were found (Table 3). Neutrase was able to solubilize significant amounts of glucose, most likely due to the cellulase activity in Neutrase. Up to 25% of the soluble carbohydrates consisted of glucose and 60-65% of galactose, arabinose, and uronic acids, while also small amounts of xylose and mannose were found. The composition of the soluble fractions after incubation with both Neutrase and Energex showed a composition which was about the average of the separate incubations with these enzymes.

In chickens, glucose and galactose are known to be the best absorbed and utilized, while xylose, arabinose, and uronic acids can be absorbed but are utilized less efficiently compared with glucose[23,27]. It can be questioned if the energy obtained after absorption and utilization of some monomeric sugars is high enough to justify the use of cell wall degrading enzymes. Energy obtained after fermentation of cell wall components by the hindgut flora should also be considered[9]. In this study, Neutrase seems to be the best choice because it is able to break down high amounts of protein, especially after extrusion, but also due to its ability to release substantial amounts of glucose components.

Also, the combination of enzymes will result in a high release of both proteins and carbohydrates, but the feed quality of the released cell wall fragments may be lower, due to the different nutritional values of the released sugars: galactose, uronic acid, and arabinose, instead of glucose.

TABLE 3

Molar sugar composition of the soluble sugar fraction obtained after 24 h of incubation of WUS from USBM, TSBM, and ExUSBM with Neutrase (Neu), Energex (Ene) and Neu+Ene (combination of both enzymes)

	USBM			TSBM			ExUSBM		
Sugar	Neu	Ene	Neu+ Ene	Neu	Ene	Neu+ Ene	Neu	Ene	Neu+ Ene
Rhamnose	4.7	5.5	4.4	4.9	5.5	4.8	3.6	5.3	3.7
Fucose	0.7	0.9	0.9	1.0	1.2	0.8	1.0	1.1	0.6
Arabinose	13.0	17.8	17.2	15.7	23.1	19.2	14.4	23.4	17.4
Xylose	0.5	<0.1	0.5	<0.1	<0.1	<0.1	1.6	<0.1	0.6
Mannose	4.7	1.4	3.8	4.6	<0.1	4.1	4.8	<0.1	3.8
Galactose	26.2	38.5	33.7	31.0	49.6	38.3	32.9	49.5	35.6
Glucose	25.0	10.1	17.0	21.5	2.2	13.4	21.7	<0.1	17.4
Uronic acid	25.2	25.7	22.5	21.2	18.3	19.3	20.0	20.6	20.9

Protein breakdown

To study the effect of the different enzymes on protein breakdown in the native and heat-treated SBM, SDS-PAGE was performed on the residues. The results are given in Figures 5-7 for USBM, TSBM, and ExUSBM, respectively. In these figures, lane 1 shows the starting material (WUS), followed by the results obtained after incubation with Neutrase (lanes 2-4), Energex (lanes 5-7) and the combination of both enzymes (lanes 8-10) after 15 and 60 min and 24 h of incubation, respectively. The main attention was focused on the two main storage proteins β-conglycinin and glycinin. β-conglycinin shows three components, the α, α', and β subunit, while glycinin consists of the acidic (A) and basic (B) polypeptides[28].

In the residue from USBM and TSBM (Figures 5 and 6) it can be seen that after 15 min (lanes 2, 5, and 8) and 60 min (lanes 3, 6, and 9) no substantial protein breakdown could be noticed for all enzyme incubations. After 24 h, Neutrase and the enzyme combination were able to degrade all of the subunits from β-conglycinin (lanes 4 and 10, respectively). Energex could also degrade the α, and α' subunits but failed to degrade the β subunit (lane 7). New bands were noticed in the area of the β subunit (MW $\pm$ 60 kDa) and just above the B polypeptide (MW $\pm$ 25 kDa). The A and B polypeptides from glycinin could resist enzymic breakdown, even after 24 h.

After extrusion, degradation of protein by the different enzymes was significantly increased when compared with toasting (figure 7). After 15 min of incubation with Neutrase (lane 2) or the combination of both enzymes (lane 8), the three subunits from β-

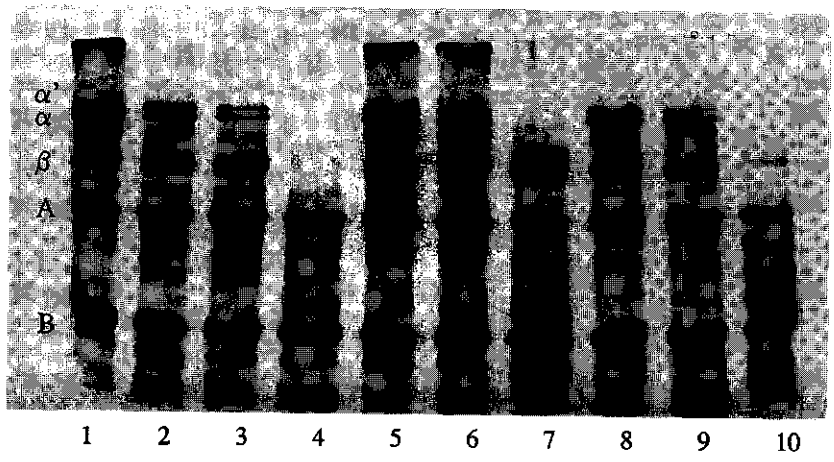

FIGURE 5

SDS-PAGE analysis of the residue of USBM after enzyme incubation: (lane 1) WUS; (lanes 2-4) incubation with Neutrase; (lanes 5-7) incubation with Energex; (lanes 8-10) incubation with the enzyme combination after 15 and 60 min and 24 h of incubation, respectively.

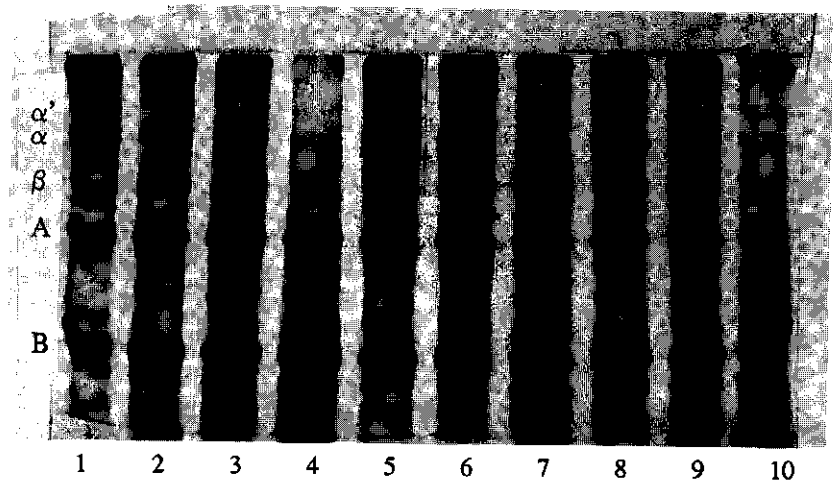

FIGURE 6

SDS-PAGE analysis of the residue of TSBM after enzyme incubation. (lane 1) WUS; (lanes 2-4) incubation with Neutrase; (lanes 5-7) incubation with Energex; (lanes 8-10) incubation with the enzyme combination after 15 and 60 min and 24 h of incubation, respectively.

conglycinin and the A polypeptide from glycinin were fully degraded. Only small residual amounts of the B polypeptide could be noticed, but the B polypeptide was further degraded after longer incubations. A part of the degraded proteins appeared in a large band just below the B subunit (figure 7). However, no protein breakdown was noticed after 15 and 60 min of incubation with Energex. After 24 h, Neutrase and Neutrase combined with Energex showed a complete breakdown of β-conglycinin and glycinin,

while Energex was only able to degrade β-conglycinin and the A polypeptide from glycinin. It is concluded that after extrusion both β-conglycinin and glycinin were effectively degraded by all enzymes compared with toasting, after which only the β-conglycinin was (partly) degraded. These results correspond with the overall protein solubility as shown in Figure 2A.

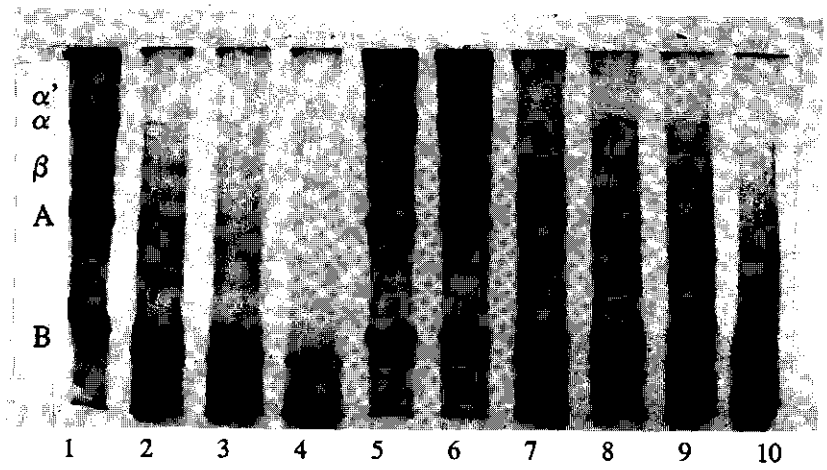

FIGURE 7

SDS-PAGE analysis of the residue of ExUSBM after enzyme incubation. (lane 1) WUS; (lanes 2-4) incubation with Neutrase; (lanes 5-7) incubation with Energex; (lanes 8-10) incubation with the enzyme combination after 15 and 60 min and 24 h of incubation, respectively.

In general, it can be concluded that β-conglycinin is easy to degrade, whereas glycinin showed more resistance against proteolytic activity. These findings are in agreement with work from others[29]. Within the glycinin fraction, it appeared that the B polypeptide was degraded much more slowly than the A polypeptide. These results were also found by Romagnolo et al[28], who incubated SBM with ruminal fluid. Proteins in USBM showed an extremely high resistance towards proteolytic activity, which can be explained by its compact native structure[30]. In a previous study, it was concluded that mainly non covalent interactions were broken during the toasting process and that disulfide bonds remained more or less intact (presented as chapter 7 of this thesis). In β-conglycinin only two S-S bonds were available, whereas glycinin contains 18-20 S-S bonds[31]. This explains why only β-conglycinin could be degraded in TSBM. Both non covalent interactions and disulfide bonds were broken during extrusion (chapter 7 of this thesis), which means that β-conglycinin as well as glycinin were degraded in ExUSBM. The relatively high resistance of the B polypeptide against proteolytic activity compared with the A polypeptide can also be explained by the fact these polypeptides have the tendency to form large insoluble complexes, which make them less susceptible to enzyme hydrolysis[32].

ACKNOWLEDGEMENTS

The authors wish to thank Nutreco Agribusiness R&D, Boxmeer, The Netherlands for financially support of this project.

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chapter 7

The effect of toasting and extrusion at different shear levels on soy protein interactions

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Journal of Agricultural and Food Chemistry, submitted

ABSTRACT

The effect of toasting and extrusion at different shear levels on protein interactions in soybean meal (SBM) was studied by extraction methods using buffers containing urea and dithiothreitol (DTT). Non covalent interactions were the main forces in protein structure formation during the toasting process but are less important during extrusion. After extrusion and upon cooling after the process, both non covalent interactions and disulfide bonds were involved during low shear extrusion. At higher shear levels, also other covalent cross linking reactions may occur.

After extrusion, mainly polypeptides of glycinin were found in the protein fractions obtained after extraction with DTT, especially the acidic polypeptide. In combination with *in vitro* protein digestibility results, it was concluded that glycinin is less digestible compared with β-conglycinin.

It appeared that after toasting and especially after extrusion an increasing amount of still active trypsin inhibitors could be detected after extraction with DTT, urea and both urea and DTT, respectively. This suggests that trypsin inhibitors were embedded in the protein matrix and were thereby protected against heat inactivation.

Keywords: soy protein, extrusion, toasting, non covalent interaction, disulfide bond, trypsin inhibitor, digestibility

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chapter 7

INTRODUCTION

While primarily recognized as an oil crop, soybeans are also a rich source of protein for feeding both animals and man. The two most abundant soybean proteins are glycinin and β-conglycinin. Glycinin is made up of six subunits, each consisting of a basic polypeptide (B) and an acidic polypeptide (A) which are connected by a single disulfide bond. The heterogeneity of the glycinin molecule is expressed in its molecular size which ranges from 320 to 375 kDa. At least six different acidic and five basic polypeptides were detected in glycinin[1] and it has 0-2 SH groups and 18-20 S-S bonds per molecule[2]. β-Conglycinin is a trimeric glycoprotein composed of at least seven different combinations of three subunits, α, α', and β associated via hydrophobic interactions having a molecular weight of 141 to 204 kDa[1]. Two S-S bonds, but no SH groups are present in the β-conglycinin molecule[2].

In general, the nutritional potential of soybean protein is attained if a certain amount of heat is applied. Heat is necessary to inactivate heat-labile antinutritional factors (ANFs) e.g. protease inhibitors, lectins and antivitamins[3], but also in order to denature the storage proteins to increase their digestibility[4]. Some studies reported that heat treatment is not always suitable to achieve complete denaturation of soy proteins, especially the glycinin fraction which can resist certain loads of heat[5,6]. During extrusion cooking of soybean meal (SBM), some unique processing features are present, because the SBM is subjected to high pressure in combination with severe shear forces and high temperatures.

The types of interactions between proteins during extrusion have been examined by extraction of the extruded materials with different solvents. In general, it is assumed that non covalent interactions are broken by solvents like urea and sodium dodecyl sulfate, solubilizing those proteins which were made insoluble by hydrogen bonding or hydrophobic interactions during extrusion or upon cooling after the process. Dithiothreitol (DTT) and sodium sulfite are known to cleave disulfide bonds and are, therefore, able to solubilize large aggregates held together by disulfide bonds after extrusion[7,8]. However, it is possible that insoluble protein may be solubilized by breaking non covalent interactions, but also by cleaving disulfide bonds. Hager[7] showed that disulfide bond formation was the most important interaction during extrusion of SBM concentrates, while Ning and Villota[9] concluded that non covalent interactions appeared to be the driving force in protein structure formation. The formation of covalent bonds, e.g. isopeptide bonds and Maillard products, was suggested by Burgess and Stanley[10], Stanley[11] and Horváth and Czukor[12]. However, most reports showed that both disulfide bonds and non covalent interactions were important in protein structure formation during extrusion and upon cooling[13-16].

The discrepancy in interpretation of protein interaction mechanisms may be attributed to differences in process temperatures. Next, it should also be mentioned that extrusion trials were performed with different raw materials and different types of extruders. While temperature is one important process parameter, the amount of shear forces developed during extrusion should also be considered. It has been shown that development of a certain amount of shear forces can increase the *in vitro* accessibility of SBM proteins towards hydrolytic enzymes[17].

In this research, untreated SBM is toasted and extruded at different shear levels. The types of interactions involved in the insoluble protein fractions, after extraction with

a phosphate buffer, are studied using buffers containing urea, DTT or a combination of both solvents. The different protein fractions obtained were also analyzed for trypsin inhibitor activity and for *in vitro* protein digestibility.

MATERIALS AND METHODS

Materials

Commercial solvent-extracted and toasted (85°C, 20 min) soybean meal (TSBM) with a protein content (N x 6.25) of 51% was supplied by Cargill, Amsterdam. A part of the solvent-extracted meal was not toasted, but air dried yielding untoasted soybean meal (USBM). The Protein Dispersibility Index (PDI) of TSBM and USBM were 21.2 and 90.8%, respectively.

Extrusion

An Almex Battenfeld single-screw extruder was used. USBM with an initial moisture content of 25% was extruded using torpedo elements with different lengths in order to vary the shear level during extrusion[17]. These torpedo elements, assembled at the end of the screw, were equipped with 0, 4, and 8 rows of flights and were encoded Ex-0, Ex-4, and Ex-8, respectively. The die diameter was 7 mm and the screw speed 100 rpm. The product temperature at the die was measured manually using a thermocouple and adjusted at 120°C. Moisturization was performed with a Sunther-Papenmeier mixer. The other process conditions during extrusion are described elsewhere[17]. After extrusion the extrudates were dried for two days at 45°C and ground to pass a 0.2 mm screen.

Extraction procedure

USBM, TSBM and extruded SBM samples (Ex-0, Ex-4, and Ex-8) were used for two sequential extraction procedures using different solvents. A simplified extraction scheme is given in Figure 1. Two batches of 30 g of USBM, TSBM, Ex-0, Ex-4 and Ex-8 were extracted with 400 ml 0.05 M sodium phosphate buffer (pH 7.0) for two h at 20°C. The extract was centrifuged (20 min, 11000g, 10°C). The residue was re-suspended in 200 ml buffer solution and centrifuged. The last step was repeated and the supernatants of each batch were collected. Part of the supernatant (Figure 1, left side) was freeze-dried directly resulting in the buffer extractable, non dialyzed fraction (BE). The supernatant from the other batch (Figure 1, right side) was dialyzed extensively against demineralized water. The retentate, the part of material retained after dialysis of the buffer extractable fraction, was freeze-dried yielding the buffer retentate fraction (BR). The fraction BE-BR is considered to contain SBM components, removed by dialysis.

The residues obtained after buffer extraction were extracted with 400 ml of the same buffer containing either 8 M urea or 0.01 M DTT, as shown in Figure 1. After extraction for 2 h at 20°C, the extracts were centrifuged (20 min 11000g, 10°C). The residues obtained were re-extracted with 200 ml of the same solvent. The last step was repeated once. The supernatants of each treatment were combined, dialyzed against demineralized water and freeze-dried yielding the U (urea) fraction and D (DTT) fraction, respectively.

The residues obtained after urea or DTT extraction were extracted with 400 ml buffer containing a mixture of 8 M urea and 0.01 M DTT. After extraction and centrifugation, the residues were re-extracted with the same solvent. The last step was repeated once. The supernatants were combined, dialyzed against demineralized water and freeze-dried giving the UD (urea, DTT) and DU (DTT, urea) fractions, respectively. The final residues were dialyzed against demineralized water and freeze-dried to yield the UD_{res} and DU_{res} fractions.

Preparation of the blanks

Blanks were prepared by adding each extraction solvent used directly to USBM, TSBM, Ex-0, Ex-4 and Ex-8. After 2 h stirring at 20°C, the complete reaction mixtures were dialyzed extensively against demineralized water and freeze-dried.

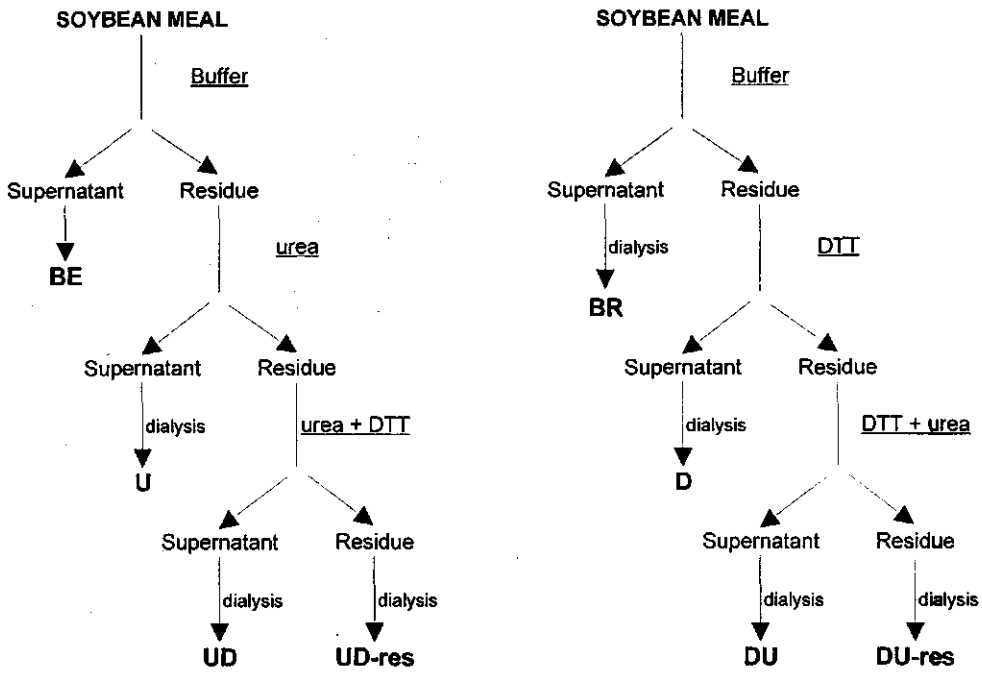

FIGURE 1
The extraction procedure used

Nitrogen and protein content

The nitrogen determination of all the fractions was performed by a semi-automated micro-Kjeldahl method. Protein content was estimated using a conversion factor of N x 6.25.

***In vitro* protein digestibility**

For determination of the *in vitro* digestibility of the proteins in the obtained fractions, the pH-STAT method was used. The pH-STAT method is described by Pederson and Eggum[18] and used with some modifications as described elsewhere[17].

Cysteine content

The amino acid determination was performed according to Rudemo et al[20] with some modifications. After oxidation of 10 mg protein with 1 ml oxidation reagent (3% H_2O_2, 80%

formic acid and 10 mg phenol/ml) for 16 h at 0°C, the excess of performic acid and hydrogen peroxide was degraded by adding 168 mg $Na_2S_2O_5$. After adding 4 ml 7.5 M HCl, containing 25 mg phenol, hydrolysis was performed for 21 h at 110°C under N_2. The samples were cooled on ice and brought to pH 2.2 with 4.25 ml 7.5 M NaOH, where the temperature did not exceed 40°C. After adding internal standard (norleucine) the sample was diluted to 15 ml with the loading buffer (sodium citrate, pH 2.2). After centrifugation, the samples were analyzed by a Biochrom 20 amino acid analyzer (Pharmacia) using a sodium citrate buffer system (pH 3.2-10).

Trypsin inhibitor activity (TIA)

TIA was determined using a modified Kakade's method according to Smith et al[19]. Benzoyl-DL-arginine-p-nitroanilide hydrochloride was used as the substrate for trypsin. The trypsin inhibitor activity (TIA) is expressed as mg inhibited trypsin per gram of protein.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Protean™ II electrophoresis system (Biorad) after reduction of the proteins by β-mercaptoethanol. The method of Laemmli[21] was followed with or modifications. A sample, corresponding with 3 mg protein, was dissolved in 1 ml buffer solution (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 10% glycerol, 0.00125% bromophenol blue and 5% β-mercaptoethanol). Reduction and solubilization of the proteins were obtained after 3 h of mixing head over tail in Eppendorf cups at 37°C. Every 90 min the samples were treated in a ultrasonification bath at 60°C for 15 min. Runs were performed in homogeneous slab gels with a monomer concentration of 12.5% and a cross-linking concentration of 2.6%. Gel slabs were fixed and stained in a solution of methanol, acetic acid, water and Coomassie Brilliant Blue R-250.

Free sulfhydryl groups

Free sulfhydryl content in the protein of USBM, TSBM, Ex-0, Ex-4 and Ex-8 was studied by using the method of Ellman[22], with some slight modifications. Proteins were suspended in a phosphate buffer (20mM, pH 8.0) containing 0.5% SDS[23]. After 2 h stirring, 0.01 M DTNB (buffered with 20mM phosphate buffer, pH 8.0) was added and the reaction was allowed to proceed 30 min at room temperature. After centrifugation (10 min, 10000g at room temperature), the extinction was measured at 412 nm. For calculation of the content of free sulfhydryl groups, an extinction coefficient of 13,600 M^{-1} cm^{-1} was used.

RESULTS AND DISCUSSION

Yields and protein content

Yields and protein contents of the fractions obtained are given in Table 1. The recovery for the different fractions ranged from 96 to 100%. After extraction with urea, DTT and a combination of both reagents, mainly proteins have been extracted, especially in the heat treated samples (69 to 89%). The high amount of dialyzable material from each starting material (7 to 20%) is the result of the high content of saccharose, stachyose and raffinose in SBM. In the residues, the protein content varied from 4% in USBM to values exceeding 50% in Ex-8. In these fractions also cell wall components like pectin, cellulose and hemicellulose will be present[24].

TABLE 1

Yields (g) and protein content (%) of the fractions obtained after sequential extraction with buffer, urea, DTT, urea and DTT.

Fraction	USBM	TSBM	Ex-0	Ex-4	Ex-8
Starting material	30.0[1]/51[2]	30.0/51	30.0/51	30.0/51	30.0/51
Dialyzable material	7.7/20	7.5/ 7	6.9/ 8	7.2/10	6.9/ 8
BR	8.5/85	2.7/53	1.5/42	1.6/28	2.0/30
U	6.9/76	8.1/89	5.2/85	3.4/77	2.4/72
UD	1.8/51	4.3/81	8.1/84	9.7/86	8.6/87
UD_{res}	4.2/ 4	8.3/30	8.3/29	7.6/34	10.0/50
Total	29.1	29.8	30.0	29.5	29.9
Dialyzable material	7.7/20	7.5/ 7	6.9/ 8	7.2/10	6.9/ 8
BR	8.5/85	2.7/53	1.5/42	1.6/28	2.0/30
D	4.5/77	5.0/82	2.1/72	2.3/75	1.7/69
DU	4.2/65	6.3/84	9.8/83	9.5/85	7.4/84
DU_{res}	5.9/ 4	8.4/31	8.6/35	9.1/47	12.2/58
Total	30.8	29.9	28.9	29.7	30.2

[1] The yield is expressed on as is basis.

[2] The protein content is expressed as weight percentage (as is basis) of each fraction

Buffer extractable nitrogen

In Figures 2 and 3, the nitrogen distributions among the BR, U, UD and UD_{res} fractions and the BR, D, DU and DU_{res} fractions are given, respectively. For a good comparison, the BR fraction is presented in both figures.

The difference in the amount of nitrogen between the BE and BR fraction, representing the part of nitrogen which is removed by dialysis, is considered as small non protein nitrogen (NPN) constituents, amino acids and small peptides. For USBM, TSBM, Ex-0, Ex-4 and Ex-8, their contribution was, calculated as BE-BR, 10, 3, 4, 5 and 4% of the total nitrogen in the meals, respectively (no further results shown). The NPN content in the heat treated samples was lower compared with USBM. Most likely during heat treatment, NPN constituents are enclosed by or attached to the protein-protein or protein-cell wall matrix, preventing their removal by dialysis. In USBM, also endogenic protease activity should be considered. However, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of USBM and USBM treated with the buffer, the buffer blank, showed similar pattern of the main subunits in glycinin and β-conglycinin (no further results shown). In addition, no breakdown of azo-casein was found after incubation with untreated SBM extract for 2 h at room temperature (no further results shown). In the following paragraphs nitrogen distribution is considered to be the same as protein distribution.

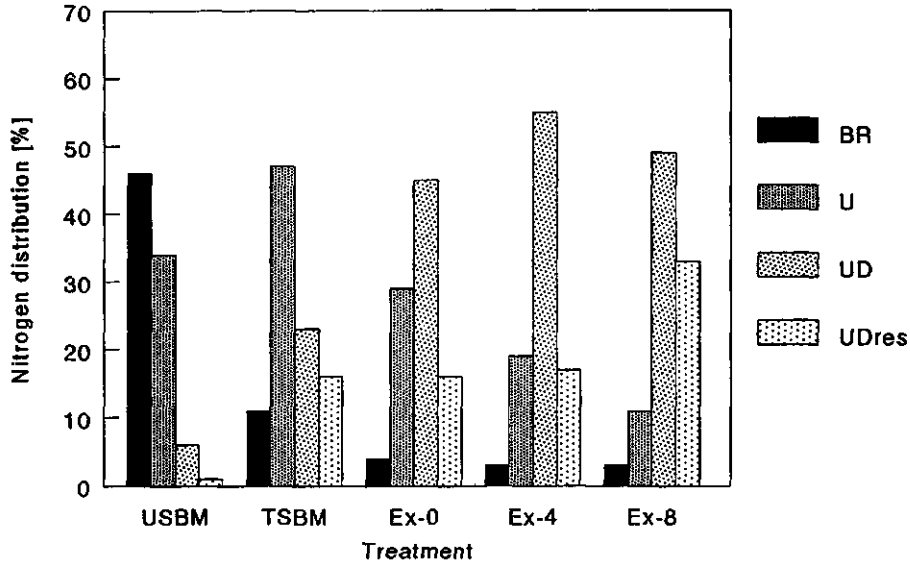

FIGURE 2
Nitrogen distribution [% of total nitrogen] after extraction with urea and both urea and DTT.

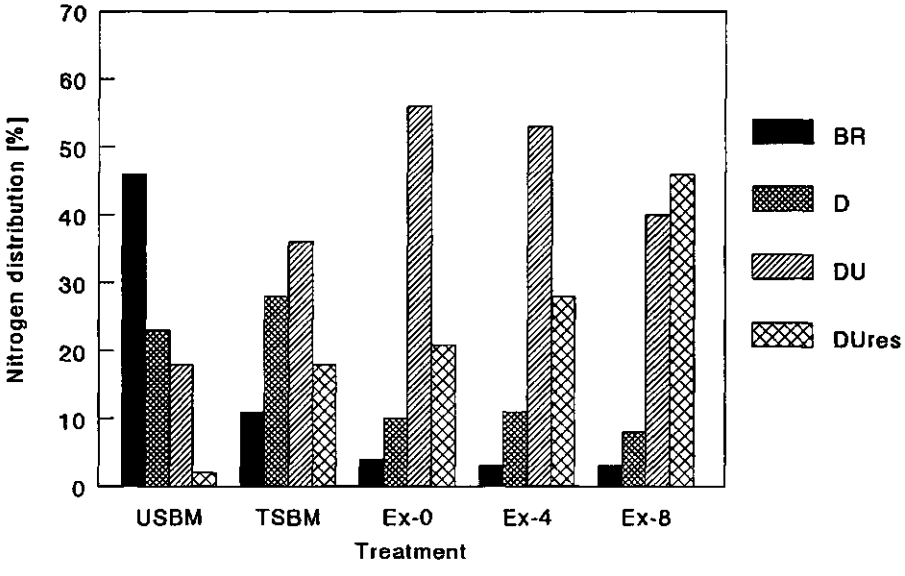

FIGURE 3
Nitrogen distribution [% of total nitrogen] after extraction with DTT and both DTT and urea.

Heat treatment had a marked effect on the protein solubility in the BR fractions. In USBM, 46% of the nitrogen could be solubilized with the buffer, but this amount decreased to 11% after toasting, while after extrusion even lower levels were found (Figure 2). Among the extruded samples, increasing shear levels did not change the amount of protein found in the BR fractions. The sharp decrease in buffer soluble nitrogen as a result of toasting or extrusion is due to denaturation and aggregation of the native proteins in USBM, whereby extrusion had more impact on nitrogen solubility compared with toasting, which was also shown in a previous research by the differences in NSI in potassium hydroxide and PDI values between these meals[17].

SDS-PAGE of the obtained BR fractions of USBM and TSBM showed typical soybean protein pattern, whereas in the extruded samples hardly any protein could be detected (no further results shown). This means that still buffer soluble proteins were extracted with USBM and TSBM but that nitrogen constituents in the BR from the extruded samples were most likely small non protein constituents which were running through the gels.

Urea extractable proteins

By breaking non covalent bonds between the proteins in the residues after buffer extraction, 34% of the proteins in USBM could be solubilized. This means that 90% (10% NPN + 46% BR + 34% U) of the nitrogen in USBM could be extracted by a sodium phosphate buffer containing urea, thus by breaking non covalent interactions. Also, in TSBM a relative high amount of the proteins (47%) could be extracted by the urea buffer, but extrusion at increasing shear levels showed a constant decrease in protein yields from 29 to 11% (Figure 2). This suggests that non covalent interactions are the main forces in protein structure formation during the toasting process but become less important during extrusion at increasing shear levels. The relatively high protein solubility in urea of TSBM protein may be due to the presence of small aggregates held together by mainly non covalent interactions and less by disulfide bonds. It is known that in aqueous solutions of glycinin and β-conglycinin upon heating first soluble aggregates are formed, stabilized by hydrophobic interactions, followed by the formation of larger insoluble aggregates in which disulfide bonds are involved[25].

SDS-PAGE was performed in order to study the protein composition. In Figure 4, the results for the U fraction of USBM, TSBM, Ex-0, Ex-4 and Ex-8 are given in lane 1-5, respectively. The α, α', and β subunits of β-conglycinin and the basic (B) and acidic (A) polypeptides of glycinin were detected in every fraction, with the exception of USBM (lane 1), of which most of these proteins were extracted with the buffer. While the total amount of protein decreased as a result of extrusion at increasing shear levels (Figure 2), only limited shifts in the ratio's between subunits of β-conglycinin and glycinin were found among the U fractions (Figure 4, lane 3-5). This means that β-conglycinin as well as glycinin were extracted by breaking non covalent interactions.

DTT extractable proteins

Extraction of the buffer residue with the buffer containing DTT (D fractions) showed that, in general, less protein was extracted compared with the urea extraction (Figure 3). For USBM, 79% of all the nitrogen was extracted with a buffer containing DTT (10% NPN + 46% BR + 23% D). In TSBM, 28% of the proteins could be

solubilized with the DTT reagent and this amount decreased to 10% after extrusion. There was only a limited shear level effect (Figure 3).

Hager[7] defined four *states* of protein. 1) Protein soluble in "simple" buffers, 2) Protein insoluble due to non covalent forces, 3) Protein insoluble due to disulfide covalent bonds and 4) Protein insoluble due to a combination of both disulfide bonds and non covalent interactions. In this scheme, it was not taken in account that some aggregated proteins may be solubilized by breaking non covalent interactions, but also by cleaving disulfide bonds. This means that the same proteins may be found in *state* 2 and 3, resulting in double counts. In this research, with these defined *states* of protein, for USBM double counts were calculated. If in USBM, 56% of the nitrogen is extracted by the sodium phosphate buffer, 34% by a buffer containing 8 M urea and 23% by a buffer containing 0.01M DTT, it can be calculated that with a nitrogen yield of 113% at least 13% of the nitrogen in USBM could be solubilized by breaking non covalent interactions as well as cleaving disulfide bonds. This number can be slightly higher or lower considering analysis errors. Because of the low yields of the buffer extractable fractions, it is not possible to calculate double counts in TSBM and the extruded samples, but most likely they will also occur in these materials.

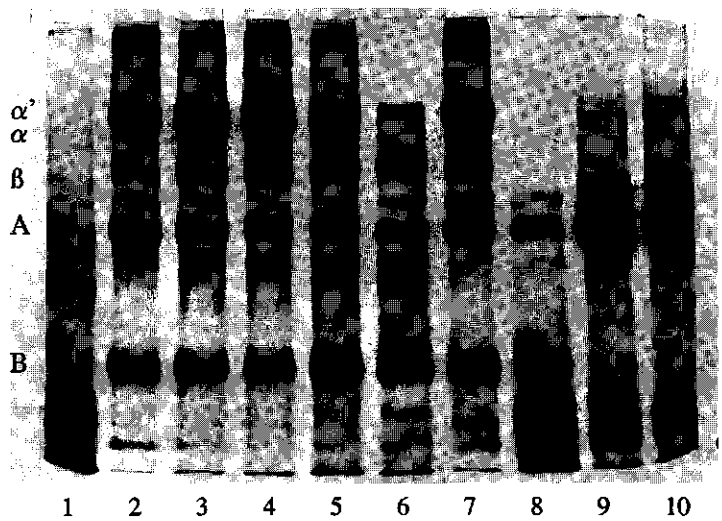

FIGURE 4

Soy protein fractions obtained after urea and DTT extraction. Lane 1-5, USBM, TSBM, Ex-0, Ex-4 and Ex-8 extracted with urea. Lane 6-10, USBM, TSBM, Ex-0, Ex-4 and Ex-8 extracted with DTT.

The cysteine content of each fraction (%) is given in Figure 5. A slight increase in cysteine was found in the D fractions after toasting compared with USBM. However, cysteine contribution tripled after extrusion compared with USBM. This suggests that an increasing part of the proteins in these fractions were obtained by breaking disulfide bridges rather than breaking non covalent interactions. The amount of free sulfhydryl (SH) groups decreased from 4.0 nmol/mg protein in USBM to 1.8, 1.7, 1.7 and 1.1 nmol/mg protein in TSBM, Ex-0, Ex-4 and Ex-8, respectively (no further results shown). This also indicates that formation of extra disulfide bonds may be formed upon cooling after heat treatment. These results are not in line with the results of Hager[7], who reported an increase in free SH groups after extrusion of soy concentrate, but also stated that intermolecular disulfide bonding is an important factor contributing to extrudate structure at low-temperature extrusion.

SDS-PAGE analysis (Figure 4) showed that the main part of the protein fraction in the extruded samples consists of the A polypeptide from glycinin, while the B polypeptide and the subunits of β-conglycinin could hardly be found (lane 8-10). However, in USBM and TSBM all the subunits of β-conglycinin and the A and B polypeptides of glycinin were found (lane 6 and 7, respectively).

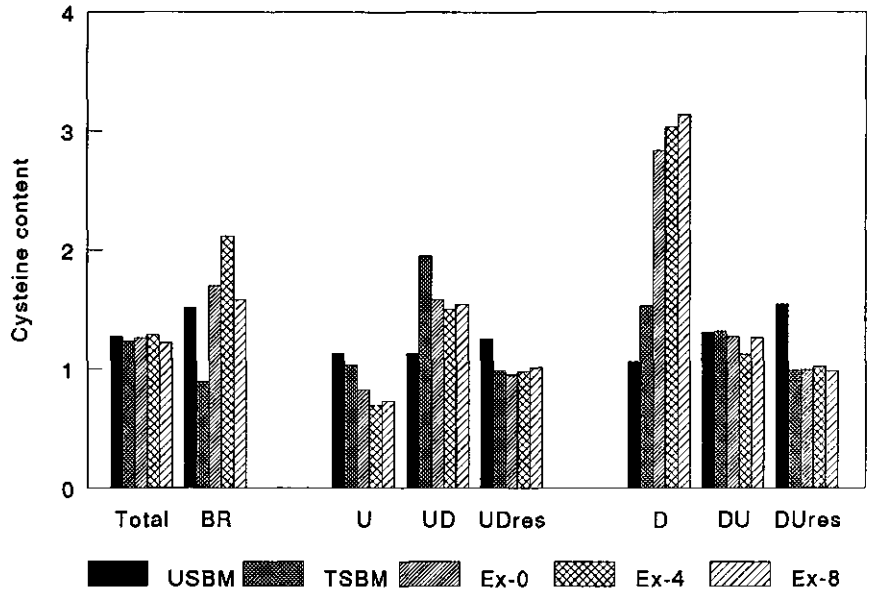

FIGURE 5

Cysteine content [% of protein in the fraction] after extraction with DTT, urea and a combination of urea and DTT.

In general, it is stated that after heating (80°C) of β-conglycinin and glycinin in buffered solutions, the B polypeptides and β subunits are located in the precipitate as a B-

β complex[26], in which both non covalent and disulfide bonds were important in maintaining network structures[27]. The existence of this complex also depends on the ionic strength of the buffer used[28]. The A polypeptide and α, α' subunits are found in the supernatant[29]. However, most of these studies were performed with protein concentrations up to 12% (w/v). In this research, extrusion was performed at an initial moisture content of 25% at relatively high shear forces, which mean that dissociation and aggregation occurred under different environmental conditions. Nevertheless, the high content of the A polypeptide in the DTT extracts of the extruded meals (Figure 4, lane 8-10) suggests a complex consisting of only A polypeptides held together by disulfide bonds (AA complex). The presence of A polypeptides coincides with the higher total cysteine content found in these D fractions (Figure 5). It is known that two-thirds of the disulfide bonds of glycinin are contributed by the A polypeptides and the residual part by B polypeptides [29]. The fact that DTT alone was not able to solubilize complexes in which also non covalent interactions are involved, may explain that only limited amounts of the B polypeptide and β subunits were found in these fractions. While it has been suggested that at low protein concentrations, α and α' subunits may form a complex with the AA complex[25], in this study after extrusion, limited amounts of the α' and α subunits were found in the D fractions (lane 8-10). Probably, at high shear levels also α, α' subunits are participating in the B-β complex and remain insoluble after DTT extraction.

Urea plus DTT extractable protein and the remaining residue

The amounts of protein in the UD and DU fractions are shown in Figures 2 and 3, respectively. In USBM, almost all of the urea and DTT insoluble proteins were extracted (Figures 2 and 3, respectively). In TSBM also relatively high amounts of protein were extracted, but the highest protein yields (45 to 55%) were obtained in Ex-0 and Ex-4. However, at high shear levels (Ex-8) protein yields started to decrease again. It can also be seen that especially, for TSBM and Ex-0, more proteins were extracted from the residue after DTT extraction (Figure 3) compared with the amount of proteins extracted from the residue after urea extraction (Figure 2). This confirms the hypothesis that DTT alone was only able to cleave disulfide bonds on the outface of the protein aggregates. Protein composition, as studied by SDS-PAGE, showed for the UD and DU fractions of TSBM, Ex-0, Ex-4 and Ex-8 typical soybean protein patterns. However, it should be noted that results were overshadowed by a high amount of insoluble complexes which remained in the stacking gel even if SDS-PAGE with the presence of urea in the gels was used (results not shown). The decrease in urea and DTT extractable proteins at high shear forces was accompanied by an increase in yields of proteins remaining insoluble after urea and DTT extraction (UD_{res} and DU_{res} in Figure 2 and 3, respectively). It was expected that the yield of UD_{res} should be similar to that of DU_{res}. It is not known why this is not the case in this study.

From the high yields of U, UD, D and DU after extrusion, it is concluded that both disulfide bonds and non covalent interactions were involved during low shear extrusion (Ex-0) as found by Jeunink and Cheftel[13] and Mitchell and Arêas[14]. However, at higher shear levels (Ex-4 and Ex-8) the high yields of UD_{res} and DU_{res} suggest that also covalent cross linking reactions become more important. The latter was also found by Horváth and Czukor[12] after extrusion of full fat soy flours at temperatures exceeding 140°C.

***In vitro* protein digestibility of the blanks**

The protein digestibility of the different fractions will depend on the characteristics of the proteins present, but are also influenced by the effect exerted by the extractant on the protein structure. Also, the presence of residual TIA's in the fractions obtained have to be considered. In order to study the effect of the extractant on the *in vitro* protein digestibility and TIA, blanks were prepared by adding each extractant used directly to USBM, TSBM, Ex-0, Ex-4 and Ex-8. The results of the TIA and *in vitro* protein digestibility are given in Figures 6 and 7.

In USBM, the TIA decreased from 57 mg/g protein in the buffer treated fraction to 37, 20 and 11 mg/g protein after treatment with urea, DTT and the combination of both reagents, respectively (Figure 6). Much lower TIAs were found in the buffer treated blanks of the toasted and extruded SBM samples. However, when treated with urea, DTT and the combination of both reagents an increase in TIA could be noticed, especially after extrusion (Figure 6). Two possible reasons may explain this increase: 1) The extraction procedure in the TIA determination procedure was not effective in extracting all trypsin inhibitors. Both, breaking non covalent interactions as well as cleaving disulfide bonds were necessary to extract higher amounts of trypsin inhibitor; 2) Thermo-mechanical treatments, like toasting and extrusion can effectively decrease TIA levels, but at the same time it is possible that still active trypsin inhibitors can be embedded in or bound to the protein matrix or other components and thereby be protected against inactivation. This result was also seen by others. If dehulled SBM was boiled for 30 min, this heating process reduced the TIA from 53 to 1 mg/g. When these boiled SBMs were incubated with crude enzyme preparations of R. *oligosporus*, the TIA increased significantly (up to 35 mg/g). An increase in TIA was not seen if raw SBM was incubated with these enzyme preparations[30]. Also, Delobez et al.[31] reported the release of trypsin inhibitors from heat treated soybeans by peptic digestion of by acid treatment indicating that these bound trypsin inhibitors will be set free by gastric digestion.

The *in vitro* protein digestibility in the USBM blank showed a sharp increase from 12% in the buffer fraction to 70% after treatment with both urea and DTT. TSBM treated with urea, DTT and both reagents showed a clear increase in digestibility values, while also in the extruded samples pH-STAT values were slightly increased after treatment with urea and a combination of both solvents (Figure 7). From the results of Figure 6 and 7 it can be concluded that the increase in digestibility in the USBM blank may partially be explained by the sharp decrease in TIA. However, it should be considered that also native glycinin and β-conglycinin will be denatured by urea and DTT, which make them more accessible for enzyme attack. In the absence of trypsin inhibitors, Rothenbuhler and Kinsella[32] also found sharp increasing digestibility values after treatment of the native glycinin with urea and DTT.

The increase in TIA in the heat treated samples (Figure 6), did obviously not lower the corresponding *in vitro* protein digestibilities. This should be ascribed to a further denaturation of the storage proteins, especially in TSBM, resulting in a net result of an increasing *in vitro* protein digestibility. This means that also a solvent effect should be taken in account when determining the *in vitro* protein digestibility in the fractions obtained.

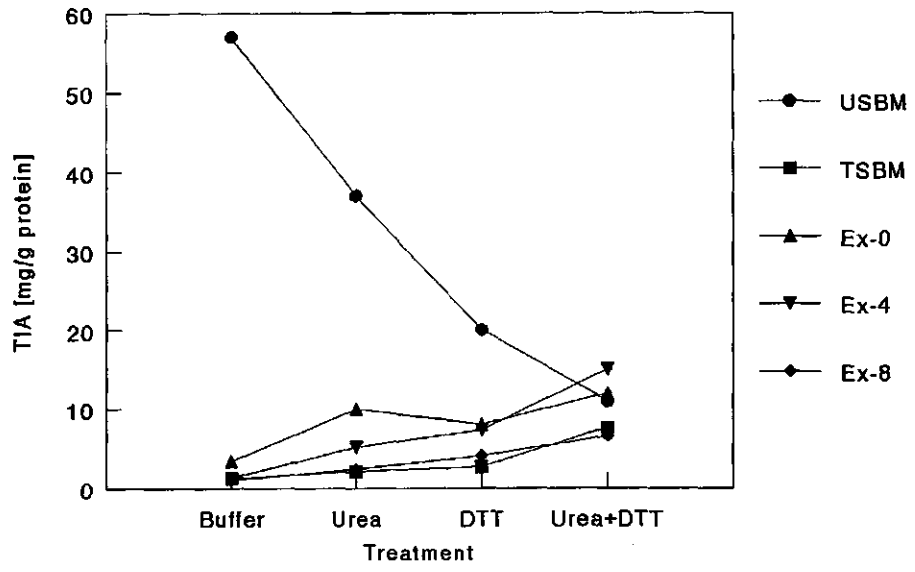

FIGURE 6

Trypsin inhibitor activity [mg/g protein] in the USBM, TSBM, Ex-0, Ex-4 and Ex-8 blanks after treatment with buffer, urea, DTT and a combination of urea and DTT.

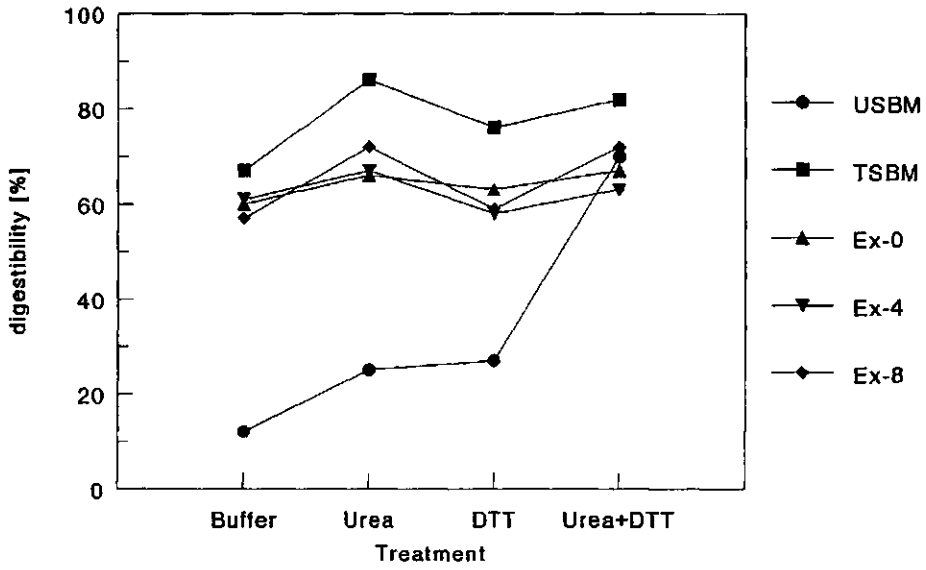

FIGURE 7

In vitro protein digestibility [%] in the USBM, TSBM, Ex-0, Ex-4 and Ex-8 blanks after treatment with buffer, urea, DTT and a combination of urea and DTT.

***In vitro* protein digestibility of the fractions obtained**

The TIA and *in vitro* protein digestibility of the fractions obtained are given in Tables 2 and 3, respectively. As mentioned in the previous paragraph, the nutritional value is the net result of protein characteristics, TIA and solvent effect. If the protein digestibility of the different fractions obtained are correlated with the corresponding TIA values for these fractions, no correlation between both parameters (R^2=0.37, N=38) was found. For example, within a TIA range of 45-50 mg/g protein the *in vitro* protein digestibility varied from 8-70% (no further results shown). In this research, only very high TIA values seem to lower the *in vitro* protein digestibility. At these high TIA levels, all the trypsin added to these fractions in the pH-STAT method were inhibited by trypsin inhibitors. This suggests that protein characteristics may be more important than TIA content. However, among the different SBM fractions within the U or D fractions, the *in vitro* protein digestibility seems negatively correlated with the TIA, suggesting that the TIA should be considered when interpreting the *in vitro* protein digestibility results. It should be mentioned that TIA levels in the fractions obtained were much higher than was expected from the levels found in the starting materials (Table 2) and the yields of the fractions obtained after extraction with the different solvents (Table 1). This should be explained by the release of active trypsin inhibitors which were embedded in the protein matrix during processing.

From Table 3 it can be seen that the *in vitro* protein digestibility of the total TSBM was lower compared with the values of the total extruded samples, while in the different TSBM fractions relatively high digestibility values were obtained if compared with the extruded samples. This can mainly be ascribed to an extraction solvent effect, which was much greater for TSBM compared with extruded samples (Figure 7).

TABLE 2

Trypsin inhibitor activity (TIA) [mg/g protein] in the fractions obtained after sequential extraction with buffer (BR), urea (U), DTT (D), urea and DTT (UD, DU) and the residues (UD_{res}, DU_{res}).

Fraction	USBM	TSBM	Ex-0	Ex-4	Ex-8
Starting material	54	12	7	3	2
BR	138	14	50	8	5
U	7	7	48	22	10
UD	t[a]	47	46	39	13
UD_{res}	-[b]	14	2	2	1
D	2	24	91	56	54
DU	1	8	13	6	5
DU_{res}	-[b]	17	2	2	1

a: traces < 1 mg/g protein; b: not determined

The solvent effect and the high level of TIA in some of the fractions makes it complicated to draw any conclusions from the results obtained from the *in vitro* protein digestibility of the fractions. For that reason, also the relative *in vitro* protein

digestibilities are given in Table 3. In this case the digestibility values were compared with the corresponding blanks. A number lower than 100% suggests a fraction which has a lower digestibility than the corresponding blank, while a number higher than 100% suggests a fraction which has a digestibility higher than the corresponding blank.

The low relative protein digestibilities in the buffer extractable fractions of the extruded samples compared with the buffer extractable fraction from TSBM (Table 3) can be explained by much higher non protein contents in the extracts of the extruded samples. By taking 1 mg nitrogen per milliliter a higher percentage of the nitrogen will originate from non protein compounds resulting in an under-estimating of the protein digestibility.

TABLE 3

In vitro protein digestibility[1] [%] and relative *in vitro* protein digestibility[2] [% of the corresponding blank] in the fractions obtained after sequential extraction with buffer (BR), urea (U), DTT (D), urea and DTT (UD, DU) and the residues (UD_{res}, DU_{res}).

Fraction	USBM	TSBM	Ex-0	Ex-4	Ex-8
BR	5[1]/42[2]	52/78	8/13	17/28	20/35
U	72/35	80/93	54/82	72/107	89/124
UD	44/63	69/84	40/60	56/89	74/103
UD_{res}	-[a]	75/91	58/87	63/100	72/100
D	30/90	40/53	5/8	30/51	26/44
DU	60/86	80/98	74/110	90/143	90/125
DU_{res}	-[a]	76/93	60/90	58/92	73/101

1: the measured *in vitro* protein digestibility, 2: the measured *in vitro* protein digestibility as a percentage of the correponding blank, a: not determined

In the DTT extracted fractions of the heat treated samples, low relative protein digestibilities were found compared with the corresponding blanks, while extraction with urea showed the opposite effect and resulted in protein fractions with higher relative digestibilities compared with the corresponding blanks. From these results, it can be concluded that proteins extractable by breaking non covalent interactions are better accessible for enzyme attack compared with proteins which are extractable after cleaving disulfide bonds. More evidence for this conclusion was found by the fact that if the urea and DTT insoluble proteins were extracted with a combination of both urea and DTT, proteins extracted from the DTT insoluble residue were more digestible compared with proteins extracted from the urea insoluble residue. SDS-PAGE showed that after extrusion mainly subunits of glycinin were found in the DTT fractions. These polypeptides, especially the A polypeptide, showed a high resistant against the proteases used in the pH-STAT method. This can be explained by the high level of disulfide bonds, but also by the two free sulfhydryl groups in glycinin. Sulfhydryl-disulfide intrachange in the subunits or sulfhydryl-disulfide interchange among subunits may occur during processing, resulting in a complex protein aggregate, where also non covalent interactions and other covalent cross linking reactions are playing a role. From these results, it is stated that glycinin is

less digestible compared with β-conglycinin. The same conclusion was drawn by other workers[33,34] and in a previous research where the *in vitro* accessibility of heat treated SBM towards hydrolytic enzyme preparations was studied[35].

The relative *in vitro* protein digestibility of the residue fractions showed values which were slightly lower or equal compared with the corresponding blanks. The absolute pH-STAT values showed numbers which were laying between the values obtained in the U and D fraction. In this research, proteins which could not be solubilized by breaking non covalent interactions and disulfide bonds, thus suggesting all kind of other cross linking reactions, could not be associated with a decreasing nutritional value as measured with the pH-STAT method.

ACKNOWLEDGMENT

The authors wish to thank Mrs M.W.J. Bredius from the Wageningen Agricultural University, the Netherlands and Mrs A.P. Moreira from the Instituto Superior de Agronomia, Lisbon, Portugal for their assistance with the extraction procedure and chemical analysis. This project was financially supported by Nutreco Agribusiness R&D, Boxmeer, The Netherlands.

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General discussion

Aim of this research

When the project started in 1991, the choice of the raw material was soybean meal (SBM), because of its high protein content, availability, attractive price, but also as a representative of feed raw materials containing antinutritional factors (ANFs), which need to be inactivated by processing. Because of the 1992 GATT agreement, there was also some interest in processing rapeseed meal (RSM). However, preliminary extrusion trials with toasted RSM showed disappointing results. From a scientific point of view it was also better to start with untoasted RSM. Since, this material was not available at that time, we limited ourselves to SBM.

The extrusion process is mostly considered as a 'black box'. Control of the process was and is mostly based on experience and 'trial and error'. In order to affirm our presumption that shear forces during extrusion could be responsible for an additional effect in improving the nutritional value of extruded SBM, preliminary extrusion trials were performed. It could be affirmed that, indeed, development of shear forces during extrusion cooking plays an important role in increasing the nutritional value of SBM. Special screw tips were developed in order to prepare extruded SBM's exposed to different shear levels.

Another aim of this thesis was to study if some monitoring parameters, which are frequently used in other thermo-mechanical treatments e.g. toasting and expansion, were also useful in monitoring the extrusion process and, therefore, protein quality.

Valorization of the processed SBM by adding hydrolytic enzymes was another topic in this thesis. Different enzyme preparations were used to measure the *in vitro* and *in vivo* accessibility of untreated, toasted and extruded SBM for proteases and carbohydrases.

Finally, research was focused on the effect of shear forces on protein interactions during SBM extrusion. Results are compared with the results obtained after toasting of SBM and untreated SBM.

The results of all studies, as presented in chapters 2-7, are combined and the main conclusions are given in this chapter.

Shear forces during extrusion

Many process parameters can be changed during extrusion cooking. It was not possible and also not the intention of this research to study the complete set of process variables. A preliminary research was performed in order to affirm our presumption that the development of shear forces during extrusion could be an interesting process variable which could be related to the nutritional value of extruded SBM. This experiment is described in chapter 2.

Untoasted soybean meal (USBM) and toasted soybean meal (TSBM) were extruded on a single-screw extruder. Temperature, screw speed and initial moisture content were varied. Results showed that extrusion of USBM at increasing temperatures resulted in an increasing *in vitro* protein digestibility. This was mainly due to inactivation of trypsin inhibitors, but also denaturation of the main storage proteins in USBM should be considered. A negative correlation between trypsin inhibitor activity (TIA) and nutritional value was often seen in SBM extrusion[1-3]. In our study, however, a slight further increase in nutritional value was noticed at increasing temperatures and a constant low TIA level. This was seen after extrusion of USBM, but was even more obvious after extrusion of TSBM. Clearly, other factors than TIA should be also considered.

Most striking was the fact that the highest *in vitro* protein digestibility was found at high screw speeds in combination with low moisture contents. This was remarkable, because a high screw speed implies a short residence time, while a low moisture content is known to increase the denaturation temperatures of β-conglycinin and glycinin[4]. It was concluded that the development of shear forces, which are quite unique for the extrusion process, could (partly) be responsible for the increase in nutritional value. Therefore, we decided to focus our further research on the effect of shear forces during single-screw extrusion on the nutritional value of SBM's.

Monitoring of the extrusion process

Several target parameters were used to monitor the chemical, physical and physiological changes as a result of extrusion cooking. Some of them were useful but others (e.g. protein dispersibility index (PDI) and urease activity (UA)) were of less importance and are not discussed in this paragraph.

The Nitrogen Solubility Index in potassium hydroxide (NSI)

The NSI in potassium hydroxide proved to be a powerful target parameter in monitoring heat treatments including extrusion cooking. The NSI in potassium hydroxide for USBM was 98%, while after toasting still a NSI of 73% was found (chapter 2). This leaves a wide margin for a further decline at more severe process conditions (chapters 2-4). However, the NSI never became lower than 25%, not even after severe extrusion conditions (chapter 3).

We correlated the NSI in potassium hydroxide with the *in vitro* protein digestibility, growth performance and apparent ileal nutrient digestibility of broiler chickens as measured in several studies. In literature, it was seen that correlation of NSI in potassium hydroxide with the nutritional value of processed SBM strongly depends on the raw material and the thermo-mechanical treatment used[5-7]. The use of 0, 1 and 2 twin lead slotted screws (TLSS) during single-screw extrusion of TSBM decreased the NSI from 73% to 50-55%. The *in vitro* protein digestibility, as measured with the pH-STAT method, showed a strong increase from 61 to 80% as a result of extrusion, while a slight further increase was obtained at higher shear levels (chapter 3). This means that a NSI of 73% is still too high for optimal *in vitro* protein digestibility. This was confirmed in a growth experiment with broiler chickens (chapter 4), where it was shown that in TSBM

with a NSI level of 73% gave a feed conversion ratio (FCR) of 1.71 g/g compared with a FCR of 1.65 g/g at NSI levels of 50-55% after extrusion of SBM. These findings are conflicting with results from literature. After autoclaving of SBM, Araba and Dale[8] concluded that, related to a growth experiment with broiler chickens, it was found that the NSI in potassium hydroxide in excess of 85% or less than 70% indicate under- or overprocessing of SBM, respectively. Our results showed that after extrusion a lower NSI in potassium hydroxide is associated with optimal *in vitro* and *in vivo* digestibilities. In our studies, there was no indication that SBM was overprocessed.

From the results of chapter 3, in which USBM was extruded with torpedo elements with different lengths, it was concluded that an optimal *in vitro* protein digestibility was obtained at a NSI level of 52%, while higher NSI values were associated with underprocessing and lower NSI values indicate overprocessing. A growth experiment with broiler chickens (chapter 5) showed that a significant improvement in FCR between TSBM with a NSI of 73% and extruded USBM (ExUSBM) with a NSI of 52% (1.56 vs 1.62 g/g).

At the different shear levels, the effects on growth performance were less obvious. At a $P < 0.10$ level, the performance of birds fed a diet containing USBM extruded at the highest shear level showed a deteriorating FCR in broiler chickens (chapter 5) indicating overprocessing (chapter 5). The NSI of this material was 37% (chapter 3), which mean that NSI values lower than 40% may be associated with overprocessing of SBM. Nevertheless, it was shown that the NSI in potassium hydroxide, which is a cheap, easy and reproducible method, is an useful target parameter estimating the *in vitro* protein digestibility and growth performance of chickens after thermo-mechanical treatment of SBM.

The nutritional value

It is clear that the nutritional value should be the best parameter for monitoring the quality of processed materials. In this study we used the pH-STAT protein digestibility method. An advantage of this method is that results can be obtained within 30 min. Because of the short incubation time of 10 min, it is better to define this method as the *in vitro* accessibility of processed SBM for proteases. A disadvantage of the pH-STAT method is the reproducibility which was less reliable compared with the NSI in potassium hydroxide. The method is also more expensive when compared with the NSI in potassium hydroxide.

In several studies, it appeared that the pH-STAT protein digestibility showed an increase after extrusion of USBM and TSBM when compared with TSBM (chapters 3-5). Also, the FCR in broiler chickens fed a diet containing the corresponding extruded SBM showed a significant increase (chapters 4 and 5). Both target parameters showed the similar trends for the different shear levels during extrusion with various lenghts of torpedo elements. An optimum in *in vitro* protein digestibility and the *in vivo* apparent ileal protein digestibility ($P < 0.1$) was obtained after extrusion at medium shear levels. These results showed that the pH-STAT method, despite some questions about what the method is really measuring, is an useful tool in screening processed materials on their *in vitro* accessibility towards proteases.

The trypsin inhibitor activity

In general, toasting or extrusion at relatively mild conditions was sufficient enough to decimate the TIA compared with the initial level in USBM (chapter 2). It was concluded that the TIA in extruded TSBM was a less important factor explaining the increase in nutritional value, because while no further decrease in TIA was noticed, the *in vitro* protein digestibility continued to improve with higher extrusion temperatures (chapter 2).

Increasing shear forces resulted in an additional decrease in TIA (chapters 3 and 4). This was in contradiction with Van den Hout[9], who developed a trypsin inhibitor inactivation model and stated that deformation of trypsin inhibitor molecules during extrusion at various shear forces, developed by different die holes, is not likely to take place and is, therefore, not a relevant factor. It appeared that the relative deformation of molecules obtained after the use of different die holes was lower[9] compared with the relative deformation obtained in the torpedo section in our studies (see later). The sharp increase in nutritional value of USBM extruded at a low shear level may partly be explained by heat inactivation of trypsin inhibitors. That also other factors than changes in TIA levels should be considered appeared from results obtained after extrusion of USBM at high shear levels. Under these circumstances, the TIA was still decreasing, while also the *in vitro* protein digestibility started to decrease again after reaching a maximum digestibility at medium shear levels (chapter 3). Also in broiler chickens, the FCR started to deteriorate under these conditions (chapter 5).

However, in chapter 7 it appeared that in the blanks, after treatment of TSBM and ExUSBM with 0.05 M sodium phosphate buffer (pH 7.0) containing urea, DTT or a combination of both reagents, the TIA was much higher compared with the levels which were found after the buffer treatment alone. After treatment with urea and DTT, TIA levels in the extruded SBM were higher compared with toasting. The highest TIA was found after treatment with both urea and DTT in USBM extruded at the lowest shear level. The extraction procedure in the Kakade[10] method could be questioned. However, also with affinity chromatography, the method of Roozen and De Groot[11], the similar results were obtained (no further results shown). A possible explanation may be that still active trypsin inhibitors are embedded in or bound to the protein matrix or other components, which may protect them against inactivation. After treatment with urea and DTT, these still active trypsin inhibitors were released and may decrease the nutritional value of SBM. However, it should be mentioned that it is also expected that, once released, trypsin inhibitors will also be partly inactivated by urea and DTT as was the case in USBM. Therefore, it is most likely that the TIA values found in this experiment are underestimated. The fact that after extrusion more TIA was released compared with toasting can be explained by the process conditions. Extrusion is a high temperature short time process, whereas toasting is performed at 85°C for 20 min.

Extrusion cooking vs toasting

In several chapters presented in this thesis, toasting, which is considered as a non shear process, is compared with extrusion cooking. Toasting is a process in which the

SBM is heat treated at 80-85°C for about 20 min, while during extrusion cooking the SBM is exposed to shear forces at a temperature of about 120°C for only 30 seconds. The results obtained from different studies are summarized in Table 1.

Some of the results have already been discussed in the previous paragraph. The UA, PDI and NSI in potassium hydroxide gave lower values after extrusion compared with toasting. The TIA after extrusion was slightly higher than after toasting, but in comparison with the content in USBM of 24 mg/g, it is still a considerable decrease in TIA. It should also be mentioned that extrusion at more severe conditions gave lower TIA values when compared with the value found after toasting (see later). Probably, starting with USBM, the short time at 120°C at low shear conditions is not as effective as toasting. The lectin content, which in general follows the inactivation of trypsin inhibitors, decreased to lower levels by extrusion compared with toasting.

After extrusion, it appeared that the SBM was more accessible for proteolytic enzymes, as showed by the *in vitro* protein digestibility measured with the pH-STAT method, when compared with TSBM.

TABLE 1

Comparison of chemical, physical and physiological target parameters of untoasted, toasted and extruded SBM[1] (USBM, TSBM and ExUSBM, respectively)

parameter		USBM	TSBM	ExSBM	Chapter
UA	[dpH]	1.70	0.25	0.00	2
PDI	[%]	90.8	21.2	8.0	3
NSI in potassium hydroxide	[%]	±95	72.5	63.0	2/3
TIA	[mg/g]	±24	2.9	3.5	2/5
Lectin	[mg/g]	3.3	0.3	<0.1	4
In vitro protein digestibility	[%]	±16	60.7	75.0	2/3
Weight gain chickens	[g]	n.d.[2]	1319	1344	5
Feed intake chickens	[g]	n.d.	2145	2098	5
Feed conversion ratio	[g/g]	n.d.	1.62	1.56	5
DC_{CP}	[%]	n.d.	82.2	87.5	5
DC_{Fat}	[%]	n.d.	77.0	76.6	5
DC_{NSP}	[%]	n.d.	11.4	26.7	5

[1] Extrusion of USBM without the use of additional shear developing screws tips
[2] not determined.

Extrusion increased the nutritional value of USBM more than toasting of USBM. A growth experiment with broiler chickens fed with diets containing ExUSBM and TSBM showed a significant improvement in FCR after extrusion when compared with TSBM. Especially the DC_{CP} increased significantly after extrusion which was also shown by the *in vitro* protein digestibility. The significant increase in DC_{NSP} after extrusion compared with TSBM should be interpreted with care, because there was an interaction effect between thermal processing and enzyme treatment (chapter 5). However, when the

apparent ileal digestibilities of NSP after enzyme treatment are studied in more detail, a considerable higher DC_{NSP} could be found after extrusion compared with toasting.

Non covalent interactions appeared to be the main forces in protein structure formation as a result of the toasting process. These are less important in protein structure formation as a result of extrusion. During extrusion, without the use of special screw tips, both non covalent interactions and disulfide bonds were involved (chapter 7).

Effect of shear forces during extrusion: a theoretical approach

Before drawing any conclusions of the effect of different shear forces on the nutritional value of SBM, it needs to be evaluated, theoretically, if shear forces during extrusion cooking are sufficient enough to achieve deformation of the protein molecules in SBM. Only at these conditions protein denaturation will occur.

In the extruder, shear could be developed at four places:

1 between channel and barrel wall
2 between the flight of the screw the and barrel wall
3 between the torpedo flight of the screw and the barrel wall
4 in the die

Using a method developed by Van den Hout[9], the relative deformation of the proteins at various positions in the extruder can be calculated. However, he did not use special screw tips but different die holes in order to vary shear forces. It was assumed that a relative deformation of 20-30%[9] will be necessary to denature the protein molecule.

In Table 2 some extruder and raw materials characteristics are given. Most extruder characteristics can be measured or were already available. Data concerning the SBM characteristics were obtained from Van den Hout[9], Van Zuilichem et al.[12] and Wallapapan et al.[13].

TABLE 2
Extruder and raw material characteristics

$d_{extr.}$	$= 48.8 \times 10^{-3}$	diameter extruder	[m]
d_{die}	$= 7.0 \times 10^{-3}$	diameter die	[m]
h	$= 4.175 \times 10^{-3}$	height of channel	[m]
δ	$= 8.5 \times 10^{-4}$	height of flight	[m]
$\delta_{torp.}$	$= 3.0 \times 10^{-5}$	height flight torpedo section	[m]
N	$= 1.667$	screw speed	$[s^{-1}]$
ρ	$= 600$	density of extrudate	$[kg/m^3]$
Q_v	$= 2.50 \times 10^{-6}$	flow of extrudate	$[m^3/s]$
ρ	$= 1200$	density	$[kg/m^3]$
μ	$= 600$	viscosity	[Pa.s]
n	$= 0.26$	flow behavior index	[-]
E	$= 10^6 - 10^7$	Elasticity modulus	[Pa]

The relative deformation (ϵ) of a molecule can be calculated by the law of Hooke:

$$\epsilon = \sigma/E \qquad [-]$$

where, σ is the shear stress [Pa].

The shear stress during extrusion cooking is approximately equal to:

$$\sigma = \mu \, . \, \gamma \qquad [Pa]$$

γ is the shear rate at the different positions in the extruder. The shear rate at the die wall can be calculated with:

$$\gamma_{die} = \frac{3n + 1}{4n} \, . \, \frac{32 \, Q_v}{\pi d_{die}^3} \qquad [s^{-1}]$$

where n is the flow behavior index, Q_v the flow of the extrudate and d_{die} the diameter of the die. The shear rate in the screw channel, flight clearance and torpedo flight clearance can be calculated with:

$$\gamma = \pi . N . D_{extr.}/X \qquad [s^{-1}]$$

where N is the screw speed, $D_{extr.}$ the diameter of the extruder and X the height of the screw channel (h) or flight clearance (δ) or torpedo flight clearance ($\delta_{torp.}$).

The different shear rates and relative deformations can now be calculated and are given in Table 3.

TABLE 3

Calculated shear rate and the relative deformation of SBM at different positions in the extruder

Position	shear rate [s^{-1}]	relative deformation [%]
channel	61	4
flight clearance	301	18
torpedo flight clearance	8520	511
die wall	127	8

From Table 3 it can be seen that in the torpedo flight clearance a relative deformation of 511% was achieved. In the other sections of the extruder, the relative deformation was less than 18%. Even if the elasticity modulus would be 10^7 in stead of

10^6 Pa, as was suggested by Van den Hout[9], the relative deformation of the molecules in the torpedo flight clearance of the extruder remains more than 50%, which means that storage proteins may denature and that also trypsin inhibitors will be inactivated by shear forces in this section of the extruder. It is obvious that more rows of flights on the torpedo screw will lead to a longer exposure of SBM to high shear rates and, therefore, to a higher deformation rate of the protein molecules.

Effect of different shear forces during single-screw extrusion

Special designed screw tip elements were developed in order to vary the amount of shear forces during single-screw extrusion. Twin lead slotted screws and torpedo elements were used. The results are described in chapters 3-6. Because most of the research was focused on the influence of torpedo elements during USBM extrusion, the results of these experiments are summarized in Table 4.

TABLE 4

The influence of different shear forces during extrusion cooking of USBM on physical, chemical and physiological target parameters

parameter		Ex-0[1]	Ex-4[2]	Ex-8[3]	Chapter
PDI	[%]	8.0	7.6	6.6	3
NSI in potassium hydroxide	[%]	63.0	53.4	37.2	3
Specific mechanical energy	[kJ/kg]	900	1350	1700	3
TIA	[mg/g]	3.5	1.7	1.1	5
In vitro protein digestibility	[%]	75.0	83.4	76.5	3
Weight gain chickens	[g]	1384	1357	1330	5
Feed intake chickens	[g]	2167	2111	2138	5
Feed conversion ratio	[g/g]	1.57	1.56	1.61[4]	5
apparent DC_{CP}	[%]	84.9	86.6[5]	85.6	5
apparent DC_{Fat}	[%]	74.0	71.9	76.4	5
apparent DC_{NSP}	[%]	24.2	24.4	21.5	5
Soluble NSP	[%]	4.3	7.7	13.4	5
Chyme viscosity	[cP]	3.20	3.8	5.16	5
Water holding capacity	[g/g]	5.65	6.12	6.45	5

[1-3] Torpedo screw with 0 (Ex-0), 4 (Ex-4) and 8 (Ex-8) rows of flights on the screw, respectively

[4] Deteriorating FCR at a $P < 0.1$ level

[5] Increased DC_{CP} at a $P < 0.1$ level

From this table it is clear that the PDI was not a suitable parameter to differentiate between different shear and mixing levels during extrusion. The NSI in potassium hydroxide proved to be a much better indicator in monitoring the extrusion process. It should be mentioned that the NSI in USBM showed a complete other pattern compared

with extrusion of TSBM (chapter 3). In the latter, an optimum NSI was found after extrusion with 4 rows of flights on the screw. The high SME during extrusion of USBM compared with extrusion of TSBM may explain the difference between solubility behavior of both extrudates (chapter 3). Also, the fact that native proteins in USBM may react completely different under shear extrusion compared with proteins in TSBM, which were already partly denatured.

When the level of shear forces was increased during extrusion, the *in vitro* protein digestibility also showed an increase compared with extrusion without the use of shear tip elements. However, at high shear forces the *in vitro* protein digestibility started to decrease again. This was seen for both the extrusion of USBM (Table 4) and TSBM (chapter 3). The increase in the *in vitro* protein digestibility after extrusion can partly be explained by a further decrease in TIA and, more likely, by a complete denaturation of the proteins. In the previous paragraphs it was shown that more shear forces can lead to more deformation of molecules, resulting in protein denaturation. The decrease in nutritional value of ExUSBM at high shear forces can be explained by the high SME, which suggests overprocessing. Under conditions of excess energy, all kind of modifications of proteins can occur e.g. formation of new covalent bonds.

An increase in shear forces had no effect on the growth performance of broiler chickens. The FCR did not change at a $P < 0.05$ level. However, for chickens fed a diet containing SBM extruded at the highest shear level, the FCR started to deteriorate at the $P < 0.1$ level. There was no correlation between *in vitro* protein digestibility and growth performance of broiler chickens fed with SBM containing diets exposed to various shear forces. Moreover, also the apparent DC_{CP} did not change as a result of increasing shear forces ($P < 0.05$). It should be mentioned that the highest apparent DC_{CP} values were found after extrusion with 4 rows of flights on the screw. This effect was significant at a $P<0.1$ level. Probably, if the trial had been continued for a few days longer, a more significant worsening FCR at the highest shear level would have been found and one might also speculate that an optimal DC_{CP} at medium shear levels would occur. This means that a remarkable correlation between *in vitro* protein digestibility and some *in vivo* results would be obtained.

The deterioration of FCR found for SBM containing diets treated at the highest shear level may be explained by a significant increase in the solubilization of NSP and water holding capacity of the chyme when compared with extrusion at the lowest shear level. As a result of that, the chyme viscosity also showed a significant increase and this may have resulted in a reduced nutrient absorption in the gastro intestinal tract of the chicken. High shear extrusion is resulting in an additional release of soluble NSP. It is known that a high concentration of soluble NSP is related with an increasing viscosity in the gastro intestinal tract[14,15].

At low shear extrusion, both non covalent interactions and disulfide bonds were involved in protein structure formation. At increasing shear forces, also other covalent cross linking reactions may occur (chapter 7). Proteins which could not be solubilized by breaking non covalent interactions and disulfide bonds could also not be associated with a reduced nutritional value as measured with the pH-STAT method (chapter 7). This means that for a good accessibility towards enzymes, the substrate does not have to be completely soluble.

chapter 8

Application of hydrolytic enzymes

USBM, TSBM and ExUSBM were treated with different protease and carbohydrase preparations and the accessibility of proteins and polysaccharides in these SBM preparations were measured by *in vivo* (chapters 4 and 5) and *in vitro* (chapter 6) assays. The aim was to study effects of commercially available enzyme preparations with a broad activity spectrum. The results obtained are summarized in Table 5 for an overall enzyme effect and in Table 6 for the effects among the different enzyme preparations.

No effects on body weight gain, feed intake and FCR in broiler chickens were found (Table 5). Also, no interaction was found between enzyme treatment and thermal processing. It can be concluded, therefore, that addition of these enzyme preparations, in the concentrations used, to diets containing TSBM or ExUSBM did not result in a better growth performance of the chickens.

Surprisingly, the DC_{CP} and DC_{NSP} showed a significant increase as a result of enzyme addition. However, these improvements could not be translated to a significant improved in growth performance.

In another study (chapter 4), TSBM, in stead of USBM, was extruded with different screw tip elements. After extrusion, a mixture of Energex and Neutrase was added. In a growth experiment with broiler chickens it was shown that extrusion significantly improved the FCR compared with TSBM, but that the addition of the enzyme preparation to a diet containing ExTSBM did not result in additional improvement of the FCR when compared with chickens fed a diet containing the corresponding ExTSBM without the addition of enzymes.

TABLE 5

Growth performance, apparent ileal nutrient digestibility and chyme characteristics in broiler chicks fed with diets containing TSBM and ExUSBM treated with or without enzyme preparations

parameter		No enzyme	Enzyme	Chapter
Weight gain chickens	[g]	1321	1355	5
Feed intake chickens	[g]	2104	2125	5
Feed conversion ratio	[g/g]	1.59	1.59	5
DC_{CP}	[%]	83.7	85.2	5
DC_{Fat}	[%]	75.0	77.4	5
DC_{NSP}	[%]	14.5	20.6	5
Soluble NSP	[%]	9.1	10.5	5
Chyme viscosity	[cP]	3.53	3.44	5

Among the enzymes, growth performance, DC_{CP} and DC_{Fat} were almost the same for Neutrase and Energex (Table 6). The FCR after addition of a combination of both enzymes had the tendency to improve, but failed to be significant. It might be that a combination of both enzymes may lead to additional effects. The DC_{NSP} was significantly increased after addition of Energex.

Water unextractable solids (WUS) isolated from TSBM and ExUSBM were

incubated with Energex, Neutrase and a combination of both enzymes (chapter 6). It was shown that, after treatment of WUS from ExUSBM with Neutrase, the amount of solubilized proteins was sharply increased. With Neutrase, 55% of the total WUS ExUSBM protein was released compared with 39% protein release in the WUS of TSBM. On the other hand, Energex could release more cell wall polysaccharides, measured as total neutral sugars, of WUS from ExUSBM compared with WUS from TSBM, but the amount of total uronic acids released from TSBM and ExUSBM did not change as a result of Energex treatment. After 24 h incubation, Energex was able to solubilize 67% of the total cell wall polysaccharides in the WUS from ExUSBM compared with 54% of the total cell wall polysaccharides in WUS from TSBM.

Calculation revealed that the monosaccharides released by Energex could give an increase in the metabolizable energy (ME) of 790 kJ/kg (chapter 6). This is an increase of about 6% compared to a normal chicken diet. If Neutrase is also added, also the released protein may contribute to a higher ME.

Energex was also able to solubilize up to 20% of the proteins. This was mainly due to a proteolytic activity as indicated by the azocaseine assay. Therefore, it was not possible to determine if proteins were released by breaking the polysaccharide network of the cell wall. Neutrase is known to contain a variable amount of glucanase activity. This activity enables Neutrase to solubilize some cell wall material. This glucanase activity was well reflected in the sugar composition of the solubilized cell wall components. While Energex released mainly galactose, arabinose and uronic acids, Neutrase released in addition also considerable amounts of glucose.

TABLE 6

Growth performance, apparent ileal nutrient digestibility and chyme characteristics in broiler chicks fed with diets containing TSBM and ExUSBM treated with Neutrase, Energex or a combination of both enzyme preparations

parameter		Neutrase	Energex	Both enzymes	Chapter
Weight gain chickens	[g]	1316	1327	1362	5
Feed intake chickens	[g]	2106	2125	2145	5
Feed conversion ratio	[g/g]	1.60	1.60	1.57	5
DC_{CP}	[%]	85.6	85.7	84.5	5
DC_{Fat}	[%]	79.8	77.8	74.7	5
DC_{NSP}	[%]	18.3	23.6	19.8	5
Soluble NSP	[%]	12.6	9.3	9.6	5
Chyme viscosity	[cP]	3.35	3.58	5.41	5

From the results presented in chapter 6, it could be concluded that in order to solubilize considerable amounts of both proteins and cell wall polysaccharides from heat treated SBM, it is more effective to add a mixture of both enzymes preparations in lower concentrations rather than adding each of the preparations separately in double concentrations. A possible synergistic action between both enzymes could also be seen from size-exclusion chromatography analysis (chapter 6). It appeared that Energex was

not only able to solubilize cell wall polysaccharides, but was also able to degrade them to smaller fragments. Neutrase alone was not able to do this.

SDS-PAGE analysis showed that after enzyme addition to WUS from ExUSBM, proteins were more rapidly and more completely degraded compared with enzyme addition to TSBM and USBM. Neutrase appeared to degrade both β-conglycinin and glycinin. Energex could only, partly, degrade β-conglycinin. The basic polypeptide from glycinin showed the highest resistance against proteolytic activity (chapter 6).

These results from the *in vitro* accessibility study (chapter 6) were not reflected in the *in vivo* results (chapters 4 and 5). Probably, the environmental conditions in the *in vitro* study were more favorable than the environmental conditions in the gastro intestinal tract of the chicken e.g. pH, viscosity, enzyme concentration and nutrient absorption.

Concluding remarks

In this thesis the main aim was to compare extrusion cooking of SBM at different shear levels with the toasting process. This was done be measuring several monitoring parameters.

The NSI in potassium hydroxide, pH-STAT *in vitro* protein digestibility, TIA, SME and DSC analysis are monitoring parameters which can be used in evaluating the extrusion process. The PDI and UA are of no use for this purpose.

During toasting, which is a heat treatment at 85°C for 20 min, proteins were only partly denatured as was shown by DSC analysis. The decreasing protein solubility is mainly the result of breaking non covalent interactions. By leaving most of the disulfide bonds intact the denaturation process may still be reversible after the toasting process. This may lead to a partially renaturation of the protein, where the protein may re-form to its original globular structure, which is known to be resistant against enzymic breakdown. Due to the relative long residence time during toasting, trypsin inhibitors are effectively inactivated.

During extrusion, which is considered to be a high-temperature-short time process, DSC analysis showed that proteins were fully denatured. It is shown, both theoretically and experimentally, that an increase in shear forces has an additional effect on protein denaturation. Both non covalent as well as disulfide bonds were broken during the process. The latter will result in irreversible protein denaturation, which make these proteins better accessible towards enzyme actions compared with TSBM. It was shown that after extrusion, both β-conglycinin as well as glycinin were much better degraded by proteases when compared with toasting. Significant increases in FCR, some apparent ileal nutrient digestibility parameters and *in vitro* protein digestibility were obtained when compared with toasting. If the level of shear forces during extrusion is increased, the SME started to increase significantly and under these high energetic conditions overshearing resulted in a decreasing nutritional value of the SBM. Under these circumstances, all kind of cross linking reactions may occur. At normal extrusion conditions the trypsin inhibitors are effectively inactivated. However, during extrusion of USBM, it was found that still active trypsin inhibitor molecules were embedded in or bound to the matrix and were, therefore, protected against inactivation.

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Summary

The aim of the research described in this thesis was 1) to study the chemical, physical and nutritional changes in soybean meal (SBM) exposed to different shear forces during single-screw extrusion, 2) to elucidate the usefulness of new monitoring target parameters in SBM extrusion technology and 3) to compare the chemical, physical and nutritional changes in SBM as a result of single-screw extrusion with changes obtained after toasting of SBM.

In chapter 1 background information is given about SBM and its composition. The process of single-screw extrusion was explained by describing terms like process, system and target parameters. Also, the main differences between extrusion and toasting were discussed in this chapter. Some important monitoring parameters were summarized and it was concluded that some experiments would be necessary in order to study the usefulness of additional of these monitoring parameters in extrusion technology. Finally, protein denaturation and extrusion variables which can influence protein denaturation are discussed.

A preliminary research was performed in order to affirm the presumption that shear forces during extrusion could be responsible for an additional effect in improving the nutritional value of extruded SBM (chapter 2). Untoasted and toasted soybean meal (USBM and TSBM, respectively) were extruded at a wide range of temperatures, screw speeds and moisture contents. Trypsin inhibitors were effectively inactivated when temperatures exceeded 110°C during extrusion of USBM and this resulted in a sharp increase in nutritional value, as measured with the pH-STAT *in vitro* protein digestibility. However, under more severe extrusion conditions, a further increase in nutritional value was obtained, while the trypsin inhibitor activity (TIA) remained at a constant low level. Therefore, also other factors than TIA which are related with the nutritional value of the extruded SBM and should be considered. The highest nutritional value was obtained after extrusion at high screw speeds in combination with relative low moisture contents. These results were considered as a further evidence that the development of shear forces, which are unique for the extrusion process, are responsible for the increase in nutritional value. At this point, it was decided that the main attention of the research would be focused on the effect of shear forces during single-screw extrusion.

In chapter 3, USBM, TSBM and toasted rapeseed meal (TRSM) were extruded in a single-screw extruder at various shear and mixing levels. The usefulness of some monitoring target parameters for evaluating the effect of the extrusion process was also studied in this research. Two different single-screw extruders, both provided with special screw tips in order to vary shear forces, were used. None, 1 and 2 twin lead slotted screws (TLSS) were used on a Wenger X-20 single-extruder and torpedo elements with 0, 2, 4, 6 and 8 rows of flights on the screw on a Almex Battenfeld single-screw extruder. It appeared that the protein dispersibility index (PDI) and the urease activity (UA) were not suitable monitoring parameters to differentiate between extrusion conditions. The nitrogen solubility index (NSI) in potassium hydroxide was a much better indicator to evaluate the effect on protein solubility after extrusion at various shear levels. The NSI in potassium hydroxide for USBM was almost 100%, while after toasting a NSI of 73% was found. This leaves a considerable margin for a further decline at more severe extrusion conditions e.g. increasing shear forces. Extrusion without the use of any special screw tip increased the nutritional value of all extruded samples when compared with toasting. By increasing the number of TLSS, a further increase in *in vitro* protein digestibility was obtained during extrusion of TSBM. There was no indication that the TSBM was overprocessed at the highest shear level. However, the nutritional value of both USBM and TSBM extruded with the torpedo elements showed a clear optimum in *in vitro* protein digestibility after extrusion at medium shear levels. At higher shear levels, the nutritional value started to decrease. This effect was more clear in ExUSBM than in ExTSBM. This may be explained by the specific mechanical energy (SME),

which was much higher during USBM extrusion than extrusion of TSBM, suggesting that all kind of other reactions might occur.

A growth experiment with broiler chickens, fed diets containing SBM extruded at different shear levels, is described in chapter 4. It was investigated whether the increase in *in vitro* protein digestibility observed in the previous chapter could also be measured as a better growth performance in chickens. TSBM extruded with 0, 1 and 2 TLLS were incorporated in a diet and fed to broiler chickens. In addition, also the effect of a mixture of a protease (Neutrase) and a hemicellulase (Energex) preparation, added before and after extrusion of TSBM extruded with 1 TLSS, was studied. The *in vitro* protein digestibility increased from 60.7% in TSBM to 78.3% in ExTSBM after extrusion with 0 TLSS. Also, a significant improvement in feed conversion ratio (FCR) was found after extrusion with 0 TLSS compared with TSBM (1.65 vs 1.71 g/g). No significant differences in growth performance between the different shear levels was obtained, despite the fact that the *in vitro* protein digestibility showed a further increase to 81.1% after extrusion with 2 TLSS. The addition of enzymes before and after extrusion improved the FCR compared with TSBM. However, no differences in growth performance was found between extruded TSBM and the extruded TSBM supplied with a enzyme preparation.

A second experiment with broiler chickens was performed (chapter 5). In this study, USBM extruded with torpedo elements with 0, 4 and 8 rows of flights on the screw was compared with TSBM. Growth performance, apparent ileal nutrient digestibilities and chyme characteristics were studied in broiler chicks fed diets with SBM as the main protein source. The effect of addition of Neutrase, Energex and a combination of both enzyme preparations to TSBM and ExUSBM was also studied. Compared with TSBM, extrusion significantly improved the FCR (1.56 vs 1.62 g/g) and increased the apparent ileal digestibilities of crude protein (CP) and non starch polysaccharides (NSP) (87.5 vs 82.2% and 26.7 vs 11.4%, respectively). Enzyme treatment resulted in a higher apparent ileal digestibility of CP and NSP compared with no enzyme treatment (85.2 vs 83.7% and 20.6 vs 14.5%, respectively). Among the different shear levels, the FCR deteriorated at a $P < 0.1$ level after extrusion at the highest shear level. A significant increase in solubilization of NSP, water holding capacity and viscosity in the chyme may explain this. The apparent ileal digestibility of CP showed an optimum at medium shear level, also at a $P < 0.1$ level. Most likely, these effects would be significant at a $P < 0.05$ level if the experiment would have been continued for some days. Among the enzyme treatments, no differences were found in growth performance and apparent ileal nutrient digestibilities. Only the apparent ileal digestibility of NSP after addition of Energex showed a significant increase compared with the other enzyme treatments.

In chapter 6, the *in vitro* accessibility of USBM, TSBM and ExUSBM for hydrolytic enzyme preparations was studied in more detail, using the same protease (Neutrase) and hemicellulase (Energex) preparations as with the growth experiments with broiler chickens (chapters 4 and 5). Water unextractable solids (WUS) were prepared from USBM, TSBM and ExUSBM and were incubated with different enzyme preparations. Neutrase incubation of WUS from ExUSBM yielded considerably more solubilized protein compared with incubation of TSBM WUS (55 vs 39%, respectively) after 24 h incubation, but also after 15 or 60 min more proteins were solubilized in the WUS from ExUSBM when compared with the corresponding TSBM samples. Energex could release more cell wall polysaccharides from ExUSBM when compared with TSBM. A rough calculation showed that the metabolizable energy (ME) of an average diet was increased with 6% due to the release of monosaccharides. It was also shown that cell wall polysaccharides released by Energex were predominantly present as monomers and small oligosaccharides. SDS-PAGE analysis showed that proteins in the ExUSBM were more rapidly and completely degraded when compared with TSBM. Neutrase was able to degrade both β-conglycinin and glycinin, while Energex could only, partly, degrade β-conglycinin. The basic polypeptide from glycinin showed the highest resistance against enzymic breakdown. In this study it was also concluded from DSC analysis that both β-conglycinin and glycinin were only partly

TSBM, while a complete denaturation of these proteins was obtained after extrusion.

Finally, we tried to establish which types of interactions in proteins were involved during toasting and extrusion at different shear levels (chapter 7). Information about the types of interactions was obtained by carrying out extraction experiments using buffers containing urea and DTT. Urea is able to break non covalent interactions, while DTT is able to cleave disulfide bonds. Shifts in yields of extractable protein from differently processed SBM were measured by using two sequential extraction procedures. During toasting, mainly non covalent interactions were involved in the protein structure formation. At low shear extrusion, both non covalent as well as disulfide bonds were involved, while at increasing shear forces during extrusion also other cross linking reactions should be considered. Deterioration of the nutritional value of the fractions obtained showed that proteins extracted with DTT were less digestible compared with proteins obtained after urea extraction. Mainly, acidic polypeptides from glycinin were extracted with DTT. Sulfhydryl-disulfide intrachange in the subunit and interchange among subunits from glycinin may explain the low nutritional value of this fraction. After toasting and extrusion, an increasing amount of TIA was noticed after treatment with DTT and urea when compared with TIA levels after buffer treatment alone. It is suggested that active trypsin inhibitors are embedded in the protein matrix during processing and are thereby protected against inactivation. This effect was more obvious after extrusion than after toasting.

Finally, the results obtained are summarized and discussed in chapter 8. It was tried to correlate the NSI in potassium hydroxide with the *in vitro* protein digestibility and some *in vivo* parameters. A NSI of more than 70% is associated with underprocessing of SBM, while NSI values lower than 40% are indicating overprocessing. The pH-STAT *in vitro* protein digestibility showed similar trends as some *in vivo* results. If TSBM is compared with ExUSBM, both the pH-STAT digestibility and the FCR of broiler chickens showed a significant improvement after extrusion. An optimum in *in vitro* and *in vivo* protein digestibility ($P < 0.1$) was found after extrusion of USBM at medium shear levels. At high shear levels, the pH-STAT digestibility and the FCR ($P < 0.1$) both started to deteriorate. Extremely high SME values and, therefore, the occurrence of all kinds of cross linking reactions may explain this decrease in nutritional value. It is obvious that shear forces may lead to a better nutritional value of the extruded SBM, but that at severe conditions 'overshearing' may occur.

A theoretical approach was performed in order to study the effect of shear forces during extrusion. By calculation the relative deformation of molecules, it was concluded that in some sections of the extruder, especially in the torpedo section, shear forces may be responsible for an additional denaturation effect in β-conglycinin, glycinin and trypsin inhibitors. This was also seen after some experimental extrusion trials.

Samenvatting

Het doel van het onderzoek zoals beschreven in dit proefschrift was 1) het bestuderen van chemische, fysische en nutritionele veranderingen in sojameel dat tijdens extrusie blootgesteld is aan verschillende afschuifkrachten, 2) het identificeren van enkele monitoring parameters welke geschikt zouden kunnen zijn voor het evalueren van het sojameel extrusie proces en 3) het vergelijken van chemische, fysische en nutritionele veranderingen in sojameel als een gevolg van enkelschroefs extrusie met de veranderingen die optreden na het toasten van sojameel.

In hoofdstuk 1 wordt achtergrond informatie gegeven over sojameel en de samenstelling van sojameel. Het proces van enkelschroefs extrusie is uitgelegd aan de hand van termen als proces-, systeem- en doelparameters. Ook de belangrijkste verschillen tussen extrusie en toasten zijn genoemd in dit hoofdstuk. Enkele belangrijke monitoring parameters zijn beschreven en geëvalueerd en er is geconcludeerd dat experimenten nodig zijn om te onderzoeken of sommige daarvan geschikt zijn om het extrusieproces te monitoren. Als laatste is aandacht besteedt aan eiwitdenaturatie en de proces parameters tijdens extrusie die dit denaturatieproces kunnen beïnvloeden.

Een inleidend onderzoek is uitgevoerd om het vermoeden te bevestigen dat afschuifkrachten tijdens extrusie verantwoordelijk zouden kunnen zijn voor een verdere verbetering van de nutritionele waarde van geëxtrudeerde sojameel (hoofdstuk 2). Ongetoaste en getoaste sojameel (resp. USBM en TSBM) zijn geëxtrudeerd bij zeer uiteenlopende temperaturen, schroefsnelheden en vochtgehaltes. Door extrusie van USBM bleek dat de trypsineremmers doeltreffend werden geïnactiveerd als de temperatuur boven de 110°C kwam. Dit resulteerde in een scherpe toename van de nutritionele waarde van het sojameel, zoals gemeten met de pH-STAT *in vitro* eiwit verteerbaarheids methode. Echter, bij een toenemende intensiteit van het extrusieproces bleek dat de nutritionele waarde verder toenam, terwijl trypsineremmer activiteit niet meer verder daalde. Hieruit blijkt dat ook andere factoren dan trypsineremmers overwogen moeten worden om de verbetering van de nutritionele waarde van het sojameel door extrusie te verklaren. De hoogste voedingswaarde werd bereikt na extrusie bij hoge schroefsnelheden in combinatie met relatief lage vochtgehaltes. Er werden aanwijzingen verkregen dat de ontwikkeling van afschuifkrachten, die uniek zijn voor het extrusieproces, verantwoordelijk zijn voor de verhoging van de nutritionele waarde. Er werd besloten dat verder onderzoek gericht zou worden op het effect van afschuifkrachten tijdens enkelschroefs extrusie.

In hoofdstuk 3 worden de resultaten beschreven verkregen na het extruderen van USBM, TSBM en getoast raapzaadmeel in een enkelschroefs extruder, waarbij de afschuifkrachten gevarieerd zijn. Ook de geschiktheid van enkele monitoring parameters om het extrusieproces te evalueren zijn onderzocht in dit onderzoek. Twee verschillende enkelschroefs extruders zijn hiervoor gebruikt, elk voorzien met een speciale schroefconfiguratie om op die manier de hoeveelheid afschuifkrachten te kunnen variëren. Op een Wenger X-20 enkelschroefs extruder zijn resp. 0, 1 en 2 twin lead slotted schroeven (TLSS) gebruikt. Op de andere extruder, een Almex Battenfeld enkelschroefs extruder, zijn torpedo elementen met resp. 0, 2, 4, 6 en 8 rijen met flights gebruikt. Het bleek dat de eiwit dispergeerbaarheid (PDI) en de urease activiteit (UA) niet geschikt zijn als monitoring parameters om tussen extrusie condities verschillen te meten. De stikstof oplosbaarheids index (NSI) in kaliumhydroxide was een veel betere indicator om tijdens extrusie het effect tussen verschillende niveaus van afschuifkrachten te meten. De NSI in kaliumhydroxide van USBM bedroeg bijna 100%, terwijl na toasten een stikstof oplosbaarheid van 73% werd aangetoond. Met deze getallen is er een aanzienlijke marge beschikbaar om effecten, als bijvoorbeeld het verhogen van afschuifkrachten, te meten. Extrusie zonder gebruik van speciale schroefconfiguraties verhoogde de nutritionele waarde van alle geëxtrudeerde monsters in vergelijking met TSBM. Bij gebruik van 1 of 2 TLSS's bleek dat de *in vitro* eiwit verteerbaarheid

tijdens extrusie van TSBM verder toenam. Er was geen enkele indicatie dat bij de hoogste afschuifkrachten overprocessing had plaatsgevonden. Echter, na extrusie van zowel USBM als TSBM met behulp van de torpedo elementen bleek dat de nutritionele waarde een duidelijk optimum vertoonde in de *in vitro* eiwit verteerbaarheid bij gemiddelde afschuifkrachten (4 rijen met flights). Als de hoeveelheid afschuifkrachten verder werd verhoogd bleek dat de nutritionele waarde weer begon te dalen. Dit effect was in ExUSBM veel duidelijker waar te nemen dan in ExTSBM. Dit kan verklaard worden door de specifieke mechanische energie (SME) die veel hoger was tijdens het extruderen van USBM in vergelijking met extrusie van TSBM. Verondersteld wordt dat dan allerlei andere reacties kunnen optreden.

Een groei experiment met kippen, gevoerd met dieten die sojameel bevatten die geëxtrudeerd zijn bij verschillende afschuifkrachten, is beschreven in hoofdstuk 4. Er is onderzocht of de verhoogde *in vitro* eiwit verteerbaarheid uit het vorige hoofdstuk zich ook manifesteerde in een betere groeiperformance van kippen. TSBM geëxtrudeerd met 0, 1 en 2 TLLS's zijn verwerkt in een dieet en gevoerd aan kippen. Verder is in dit onderzoek ook gekeken naar het effect van het toevoegen van een mengsel van een protease (Neutrase) en een carbohydrase (Energex) preparaat voor en na het extruderen van TSBM geëxtrudeerd met 1 TLSS. De *in vitro* eiwit verteerbaarheid steeg van 60.7% in TSBM tot 78.3% in ExTSBM na extrusie met 0 TLSS. Ook een significante verbetering in de voederconversie ratio (FCR) werd aangetoond na extrusie met 0 TLSS in vergelijking met TSBM (1,65 vs 1,71 g/g). Tussen de verschillende afschuifkrachten werd geen significant effect in de groeiperformance van de kippen geconstateerd, ondanks het feit dat de *in vitro* eiwit verteerbaarheid wel verder toenam tot 81,1% na extrusie met 2 TLSS's. Het toevoegen van enzymen voor en na extrusie verbeterde de FCR in vergelijking met TSBM. Echter, geen verschillen in groeiperformance werden aangetoond tussen ExTSBM en ExTSBM voorzien met een enzym preparaat.

Hoofdstuk 5 beschrijft een tweede groei experiment met kippen. USBM geëxtrudeerd met torpedo elementen, voorzien met 0, 4 en 8 rijen met flights op de schroef, is vergeleken met TSBM (hoofdstuk 5). Groeiperformance, schijnbare ileale nutriënt verteerbaarheid en darminhoud karakteristieken zijn bestudeerd van kippen die gevoerd zijn met een dieet waarin hitte behandelde sojameel de belangrijkste eiwitleverancier was. Het effect van het toevoegen van Neutrase, Energex en een combinatie van beide enzym preparaten aan TSBM en ExUSBM is eveneens onderzocht. In vergelijking met TSBM bleek dat extrusie significant de FCR verbeterde (1,56 vs 1,62 g/g) en dat ook de schijnbare ileale verteerbaarheid van eiwit (CP) en niet-zetmeel polysacchariden (NSP) hoger was (resp. 87,5 vs 82,2% en 26,7 vs 11,4%). Enzym behandeling resulteerde in een hogere schijnbare ileale verteerbaarheid van CP en NSP in vergelijking met geen enzym behandeling (resp. 85,2 vs 83,7% and 20,6 vs 14,5%). Tussen de verschillende afschuifkrachten bleek de FCR te verslechteren ($P < 0.1$) na extrusie met de hoogste afschuifkrachten. Een significante stijging in de oplosbare NSP, water vasthoudend vermogen en viscositeit van de darminhoud zou dit kunnen verklaren. De schijnbare ileale CP verteerbaarheid liet een optimum zien bij gemiddelde afschuifkrachten (opnieuw op een $P < 0.1$ niveau). Het is waarschijnlijk dat bovenstaande effecten op een $P < 0.05$ niveau significant waren geweest als de proef enkele dagen was voortgezet. Tussen de enzym behandelingen werden geen verschillen aangetoond in groeiperformance en in schijnbare ileale nutriënt verteerbaarheden. Alleen de schijnbare ileale NSP verteerbaarheid na het toevoegen van Energex liet een significante stijging zien in vergelijking met de overige enzym behandelingen.

In hoofdstuk 6 wordt een onderzoek beschreven naar de *in vitro* toegankelijkheid van USBM, TSBM en ExUSBM voor hydrolytische enzymen. Dezelfde protease (Neutrase) en carbohydrase (Energex) preparaten zijn gebruikt als tijdens de groei experimenten met kippen (hoofdstukken 4 en 5). Niet-water-extraheerbare componenten (WUS) van USBM, TSBM en ExUSBM werden geïncubeerd met verschillende enzympreparaten. Na extrusie bleek dat 24 uur incubatie van ExUSBM WUS met Neutrase resulteerde in een hogere opbrengst van opgelost eiwit dan na incubatie van TSBM WUS (55 vs 39%), maar ook na 15 of 60 minuten bleken in

ExUSBM WUS meer eiwitten opgelost te worden dan de overeenkomende TSBM monsters. Energex was in staat meer celwandpolysacchariden vrij te maken van ExUSBM dan van TSBM. Een ruwe berekening laat zien dat de metabole energie van een gemiddeld dieet met 6% was gestegen door het vrijmaken van monosacchariden. Het bleek dat als celwandpolysacchariden vrij gemaakt werden door Energex deze ook afgebroken werden tot monomeren en kleine oligosacchariden. Electroforese (SDS-PAGE) analyse liet zien dat eiwitten van ExUSBM sneller en meer compleet werden afgebroken dan eiwitten in TSBM. Neutrase was in staat om zowel β-conglycinine als glycinine af te breken, terwijl Energex alleen β-conglycinine, gedeeltelijk, kon afbreken. Het basische polypeptide van glycinine was het meest resistent tegen enzymatische afbraak. In dit onderzoek is ook middels DSC analyse geconcludeerd dat β-conglycinine and glycinine in TSBM maar gedeeltelijk gedenatureerd zijn, terwijl een volledige denaturatie van deze eiwitten werd bereikt na extrusie.

Als laatste werd getracht om te bepalen welke typen interacties tussen eiwitten betrokken zijn tijdens toasten en extrusie bij verschillende afschuifkrachten (hoofdstuk 7). Informatie over de typen interacties tussen eiwitten werd verkregen met extractie experimenten uitgevoerd met buffers die ureum en DTT bevatten. Ureum is in staat om non covalente interactie te verbreken, terwijl DTT zwavelbruggen kan splitsen. Verschuivingen in de opbrengsten van extraheerbaar eiwit is gemeten door gebruik te maken van twee extractie procedures. Tijdens toasten waren voornamelijk non covalente interacties betrokken bij de structuur formatie van de eiwitten. Bij extrusie met lage afschuifkrachten bleek dat zowel non covalente als zwavelbruggen optraden, terwijl als de hoeveelheid afschuifkrachten toeneemt ook andere cross linking reacties kunnen optreden. Eiwitfracties verkregen na DTT extractie hadden een lagere nutritionele waarde dan eiwitten verkregen na extractie met ureum. Na extractie met DTT werden voornamelijk de zure polypeptides van glycinine verkregen. De lage nutritionele waarde van deze fractie kan verklaard worden door zwavelwaterstof-zwavelbrug uitwisselingen in een subunit en tussen subunits van glycinine. Na toasten en extrusie bleek dat, in de fracties verkregen na behandeling met DTT en ureum, de trypsineremmer activiteit toenam in vergelijking met het behandelen van deze fractie met alleen de buffer. Er is gesuggereerd dat actieve trypsineremmers tijdens het proces van toasten en extrusie zich inbedden in de matrix en op deze manier beschermd worden tegen inactivatie. Dit effect was duidelijker waarneembaar na extrusie dan na toasten.

Als laatste zijn, in hoofdstuk 8, alle resultaten samengevat en bediscussieerd. Er is getracht de NSI in kaliumhydroxide te correleren met de *in vitro* eiwit verteerbaarheid en enkele *in vivo* parameters. Een NSI van meer dan 70% is geassocieerd met te milde proces omstandigheden, terwijl NSI waarden lager dan 40% duiden op te extreme proces condities. De pH-STAT *in vitro* eiwit verteerbaarheid vertoonde dezelfde trend als sommige *in vivo* resultaten. Als TSBM vergeleken wordt met ExUSBM bleek dat zowel de pH-STAT verteerbaarheid als de FCR significant verbeterden. Een optimum in de *in vitro* en *in vivo* eiwit verteerbaarheid ($P < 0.1$) werd aangetoond na extrusie van USBM bij gemiddelde afschuifkrachten. Bij hoge afschuifkrachten bleek dat zowel de pH-STAT verteerbaarheid als de FCR ($P < 0.1$) beide begonnen te verslechteren. Dit laatste zou verklaard kunnen worden door de extreem hoge SME waarden met als gevolg het mogelijk optreden van allerlei cross linking reacties. Het is duidelijk dat afschuifkrachten kunnen leiden tot een hogere nutritionele waarde van geëxtrudeerde sojameel, maar dat een te extreem proces, bijvoorbeeld te hoge afschuifkrachten, kan leiden tot een verminderde nutritionele waarde van het sojameel.

Een theoretische benadering is toegepast om het effect van afschuifkrachten tijdens extrusie te bestuderen. Door het berekenen van de relatieve deformatie van de moleculen bleek dat in sommige secties van de extruder, vooral in de torpedo sectie, afschuifkrachten verantwoordelijk gesteld kunnen worden voor een additioneel denaturatie effect van β-conglycinine, glycinine en de trypsineremmers. Dit was ook al aangetoond uit eerder genoemde extrusie experimenten.

Curriculum vitae

Gerrit Jan Pieter (Gerard) Marsman werd geboren op 21 augustus 1963 te Ommen. In 1981 behaalde hij het MAVO diploma aan de gereformeerde school voor MAVO te Hardenberg en in 1983 het HAVO diploma aan de gereformeerde scholengemeenschap prof. dr. S. Greijdanus te Zwolle. In datzelfde jaar is hij aan het werk gegaan bij een ambachtelijke slager te Ommen. Tegelijkertijd werd één dag in de week in Zwolle een slagersopleiding gevolgd, waarbij in 1984 het slagersgezel diploma werd behaald. Vervolgens pakte hij toch weer een volledige dagstudie op en ging hij naar de rijks middelbare school voor levensmiddelen technologie te Bolsward. Na twee jaar middelbaar onderwijs volgde één jaar hogere levensmiddelen technologie te Bolsward. Deze studie kreeg zijn vervolg in september 1987 toen de overstap naar de Landbouwuniversiteit te Wageningen werd gemaakt. De doctoraalfase omvatte het hoofdvak Levensmiddelenchemie. Een stage periode werd doorgebracht bij AVEBE te Foxhol. In oktober 1991 studeerde hij af. Inmiddels was hij al begonnen als assistent-in-opleiding bij de sectie Levensmiddelenchemie en Microbiologie van de Landbouwuniversiteit te Wageningen. In samenwerking met de vakgroep Veevoeding en de sectie Proceskunde werd een project gestart, waarvan de resultaten beschreven zijn in dit proefschrift. De begeleiding van dit project werd verzorgd door prof. dr. ir. A.G.J. Voragen, prof. dr. ir. M.W.A Verstegen, dr. ir. H. Gruppen en ir. A.J. Mul. Het praktische gedeelte van het onderzoek werd begin 1996 afgerond. In mei 1996 startte hij als produkt technoloog babyvoeding bij Coberco Isoco te Lochem. Ten gevolge van de fusie tussen Coberco en Friesland Dairy Foods in december 1997 werd in februari 1998 de overstap gemaakt naar de R&D afdeling van Friesland Specialities te Leeuwarden.