Limiting factors for the enzymatic accessibility of soybean protein

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Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. M.J. Kropff
in het openbaar te verdedigen
op maandag 23 oktober 2006
des namiddags te half twee in de Aula

The research described in this thesis was performed at the Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands and in the laboratories of Novozymes A/S, Denmark.

Fischer, Morten

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Ph.D. Thesis, Wageningen University

ISBN 90-8504-496-0

Preface

I would like to express my sincere gratitude to those who contributed, directly and indirectly, to accomplishing all the laborious work presented in this thesis. Many more people should probably have been mentioned. To the ones forgotten, please forgive me.

I would like to thank Lene Venke Kofod, my supervisor from Novozymes, my scientific inspirator in the early days, and former department manager of Feed Applications. Lene, since 1996 when I met you for the very first time in the present Department of Food Functionality you have been a source of inspiration with your enthusiasm for science, your magic brains that seem to recall every little detail of scientific matters, and your capability as a manager to make people sweat while smiling and being joyful at the same time.

Very special thanks to all the present and former Feed Applications staff and to my former and present directors Lars Dalgaard Andersen and Anders Østergaard for always being so supportive, to Lone for the gentle push and your support to get this thesis completed, and to Pia for her constant support and encouragement. I am also grateful to Per Munk Nielsen and Gerda Jensen for their support along the way.
Thanks to all of you for your incredibly enthusiastic working style - without all of you going to work would have been much less inspiring and fun.

Sincere appreciation should go to my promoters Harry Gruppen and Fons Voragen from the Laboratory of Food Chemistry at Wageningen University for their supervision, assistance, suggestions and criticism to my daily research activities and to the manuscripts that form the basis of this thesis. Thanks to Henk Schols who played an important and committed role in the early phase of the project. Furthermore, I would, in particular like to mention Sander Piersma for his supportive personality and for his ‘un-nerdy’ approach to science and Jolan de Groot for her extraordinary efforts to resolve technical problems, strong advices and for always being there when help was urgently needed. Thanks to Jan Cozijnsen for help with GC analysis. ‘Afstudeervak’ students Geerten de Rooij and Bas Kuipers, now Masters of Soy, are thanked for performing experiments that helped support this work. To my department room mates Stephanie, Laurice, Mir-

jam, Jolanda and all the other present and former colleagues at the Laboratory of Food Chemistry and within the department of Agrotechnology and Food Sciences I would like to express my thankfulness for providing a pleasant working atmosphere.

Thanks to all my 'Dutch' friends - in particular to Harold, Carmen, Gabriela, Vesna, Francesca and Stefano, and Bjarne for always being there 'for good, – and for bad',– when the grey, rainy sky was hanging low above the dike-embraced Dutch country.

I extend my gratitude to Novozymes A/S and Academy of Technical Sciences (ATV, Denmark) for their financial support to this study and to Professor Mogens Jakobsen from Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Family and friends should not go unmentioned for their constant encouragement and for patiently listening to my lengthy monologues about 'exciting' science when luck was on my side – and long hours of complaints when motivation was out. Finally, kisses to my beautiful wife and life companion, Ditte Louise for giving me the unique and special chance of experiencing The Netherlands with you by my side. This added a completely new dimension to my life – which I shall always carry in my heart.

In memory of my fantastic mother
– January 2006

Abstract

Fischer, Morten	Limiting factors for the enzymatic accessibility of soybean protein
Ph.D. Thesis	Wageningen University, The Netherlands, 2006
Key words:	*Glycine max*, cell wall, carbohydrate, protein, protease, carbohydrase, peptide, aggregation, solubility

The research described in this thesis deals with the efficacy of enzymatic extraction of protein and carbohydrates from soybean meals subjected to different heat treatments.
The meals were extracted by a repeated hydrolysis procedure using excessive concentrations of different combinations of commercial protease and carbohydrase preparations. For all soybean meals enzymatic treatment extracted most of the protein (89 - 94%). The use of carbohydrase preparations did not improve protein extraction. High humidity heat treatment led to an effective enzymatic extraction, which seemed to correlate to the extent of protein denaturation. A protein solubilisation model was developed and tested. Experiments indicated that the efficacy of the hydrolysis is to some extent negatively affected by the degradation products of the proteins. The ratio of the endo/exo proteases in mixture was found to be an important parameter, which could affect both protein solubilisation and degree of hydrolysis in both negative and positive direction. Results indicated that a combined objective of high protein solubilisation and high degree of hydrolysis in a single hydrolysis product is conflicting.
Analysis of the enzyme-unextractable proteinaceous material indicated that a large proportion of the enzyme-unextractable material was composed of aggregated peptides of low molecular weight. The largest aggregates were observed for meals heat-treated at high humidity. Following solubilization, aggregates were fully degraded upon additional proteolytic treatment. Similar results were found for the insoluble components of a digesta sample obtained from pigs fed a feed consisting of only soybean meal.

The results of this thesis show that the importance of peptide aggregation upon enzymatic degradation of proteins as a limiting factor for enzymatic accessibility is not limited to *in vitro* laboratory situations, but is also occurring in the digestive system of animals.

Contents

CHAPTER 1

GENERAL INTRODUCTION

Background and Perspective

This thesis deals with the effects of proteases and carbohydrases on the composition of the enzyme-extractable and the enzyme-unextractable fractions of soybean meal (SBM) following enzymatic treatment. The focus is primarily on the soy proteins, but the composition of the cell wall polysaccharides in different types of samples is also given some attention.

Little is known about the composition of enzyme-resistant soy material in general. Knowledge on this material is a prerequisite for selection of appropriate enzymes to optimise and increase the utilisation of soybean meal (derived) products. Mechanistic understanding is also valuable for the discovery of novel enzymes and for development of tailor-made proteases and carbohydrases. Therefore, this research project is focussed on gaining understanding of enzymatic hydrolysis of SBM with emphasis on proteins and identification of barriers to enzyme activities.

The Soybean and its Application in Foods and Feed

Soybean (*Glycine max*) is the most commonly grown of all oil crops throughout the world. It belongs to the legume seeds (*Leguminosae*) family. The crop has a history, which spans 4000-5000 years. The bean originated in Asia where it is widely grown due to the favorable climate. The soybean was brought to Europe and was introduced in the United States early on in the nineteenth century where large scale production started during the 1850's (*1*, *2*).

Soy is a good source of protein for humans and animals even though soy protein is limiting in methionine (*3*). Among cereals and other legume species the soybean has the highest protein content (above 40%). Other legumes, e.g. canola, have protein contents between 20% and 30%, whereas cereals have protein contents in the range of 8–15%. Other valuable components found in soybeans include phospholipids, vitamins, and isoflavones.

Soy is applied in a variety of products including bread, cakes and snacks. In spite of its widespread use in foods, only a small percentage of global soy protein production goes into such products. Today soybeans are grown primarily for the production of vegetable oil for human consumption but, as a by-product, soybean meal (SBM) is becoming increasingly important. On a global scale, soy is dominating the market for protein meals due to its high protein content and good availability. This makes soy an excellent ingredient in feed formulations and soybean accounts for 70-80% of all protein rich meals fed to livestock in the US. The EU is one of the leading markets for import of SBM. A large part of this SBM goes into feed for the production of monogastric animals. Soy is particularly important for poultry production, constituting approximately 40% of a standard soy/maize diet since broilers and layers require a high proportion of protein in their diets. For ruminants protein-rich diets are less important because these animals primarily derive protein in an indirect manner through rumen fermentation of roughage.

A number of studies have shown that exogenous enzymes can be highly effective in enhancing animal performance (*4*, *5*, *6*, *7*, *8*). The digestive tract must break down the feed so that animals can absorb and utilize nutrients. The main benefits of supplementing feed with enzymes are better feed utilization (feed conversion ratio), faster growth of the animal, more standardised production, better health status and improved environmental impact of production (*8*). The nutritional value of several main feed ingredients can be improved using exogenous enzymes. Pigs and poultry benefit significantly from enzymes in feed because their digestive systems cannot break down plant cell walls (*9*, *10*). The addition of commercial enzymes to a corn/SBM broiler diet significantly improved weight gain and feed conversion ratio (*4*, *11*). The increase in performance is related to an increase in ileal digestibility of crude protein (*12*), starch and fat (*4*), as well as to an improvement in ileal digestibility of non-starch polysaccharides (NSP) (*7*, *13*, *14*). Disruption of the cell wall matrix released assumingly entrapped protein and led to a more effective degradation of diet proteins by the digestive proteases (*5*, *8*). Enzyme effects depend on the activities present in the enzyme preparation and also on the inclusion level of enzyme in the diet (*6*).

Processing of Soybeans

To obtain SBM and oil a commonly used method of soybean processing is shown in Figure 1 (*15*). The hulls are separated from the cotyledons by seed cracking. After dehulling a preconditioning step (65 - 70°C; 10-30 min) is used to make the beans less rigid during flaking (*16*). Flaking increases the speed and effectiveness of oil extraction. A solvent, usually hexane, is commonly used to extract the soybean oil from the meal (*17*). Toasting or desolventization is performed at a temperature of 70–80°C for about 20 min (*16*, *18*, *19*). Heat treatment is generally considered the most critical stage in the processing of soybeans. Control of processing parameters such as temperature, moisture content, pressure, and processing time is essential to maintain a high solubility of the final soybean meal product (*20*). The last step includes grinding and classification of the final products to meet specific standards for products such as soy grits and defatted SBM.

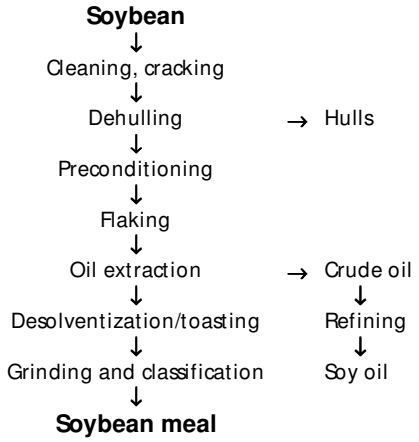

Figure 1. Processing of soybeans.

Composition of Soybean Meal

The chemical and nutritional characteristics of SBM are subject to large variation depending to a large degree on genetic variation and growing conditions which have large effect on the composition of carbohydrates and protein (*3*, *21*, *22*). On dry weight basis soybean meal is composed of 30-40% carbohydrates, 45-55% protein, less than 1 % fat, and 6% ash. An average composition of SBM is given in Table 1.

Table 1. Approximate composition of soybean meal

Constituent	% on dry weight basis	
Carbohydrates	30 - 40	
Saccharose		6 – 9
Raffinose		1 - 1.5
Stachyose		5 – 8
Starch		0 – 5
Non-starch polysaccharides		16 – 22
Protein	45 - 55	
Glycinin + β-conglycinin		~80
Antinutritional factors (e.g. KSTI, BBI, lectin)		~5
Other proteins		~15
Fat	< 1	
Ash	6	

Compiled from (3, 22, 23, 24, 25, 26).

Proteins

Traditionally, soy proteins have been classified using a sequential extraction by a solvent series (*27*). Based on their solubility patterns, legume seed proteins are divided into water soluble albumins and salt solution soluble globulins. Approximately 80% of the protein present in soybean meal is in the form of storage globulins. Most soy proteins are insoluble in water at their iso-electric point, but are solubilised in the presence of salt. Studies of soy proteins by analytical ultracentrifugation in a 35 mM sodium phosphate buffer of pH 7.6 containing 0.4M NaCl (I=0.5) and 0.01M β-mercaptoethanol, revealed the presence of 4 fractions with Svedberg coefficients of 2S, 7S, 11S and 15S (*28*). These fractions are not homogeneous but contain a mixture of proteins.

The 2S fraction has been reported to contain from 8 to 22% of the phosphate buffer (pH 7.6) extractable soybean protein and consists of a number of enzymes and small proteins (*29*, *30*). The fraction is composed of a number of proteins with a variety of molecular weights, with an average molecular weight of 26 kDa (*31*). Also the protease inhibitors are included in this fraction (see later). The 15S protein fraction comprises about 5% of the total extractable protein. It is poorly characterized and is primarily composed of polymers of the other soy proteins (*30*, *32*).

The 11S fraction comprises approx. 30 – 50% of the soluble soy proteins (*33*, *34*). **Glycinin** is the major globulin and composes about 60 – 80% of the soy proteins (*33*). Glycinin consists of an acidic polypeptide, A (~40.000 Da) and a basic polypeptide, B (~20.000 Da), linked by a single disulfide bridge and thereby forming an individual AB subunit (*35*, *36*). At least six acidic (A_{1a}, A_{1b}, $A_2 – A_5$) polypeptides and five basic (B_{1a}, B_{1b}, $B_2 – B_{4)}$ polypeptides have been identified (*37*). The acidic polypeptides have isoelectric points varying from 4.75 to 5.4. The basic polypeptides can be separated into three groups having isoelectric points of 8.0, 8.25 and 8.5, respectively (*38*). At neutral pH and ionic strengths of above 0.35 glycinin has an hexameric structure (320.000 – 375.000 Da) consisting of a heterogeneous population of subunits (*39*). Glycinin has on average 2 –SH and 18-20 S-S bonds per hexamer (*27*). An overview of the constituents of glycinin and β-conglycinin is given in Table 2.

Table 2. Physicochemical properties of glycinin and β-conglycinin

Property	Glycinin	β-Conglycinin
Molecular weight (Da) of subunits/polypeptides	A: 37.000-45.000 B: 22.500	α: 57.000-72.000 α': 57.000-68.000 β: 42.000-52.000
Glycosylation (%)	0	~5
SH-groups	0-2 / hexameric molecule	0
S-S bonds	18-20 / hexameric molecule	2 / trimeric molecule
Isolelectric pH (average)	4.9 (hexameric molecule)	4.6 (trimeric molecule)

Compiled from (37, 38, 40, 41).

The 7S fraction of soy protein comprises about 35% of the soluble protein. This fraction contains enzymes, a number of hemagglutinins (SBA) and predominantly a protein fraction known as 7S globulins. About 85% of the 7S fraction is made up of **β-conglycinin** (*33*, *37*, *42*). This heterogeneous protein has trimeric quaternary structure and is glycosylated for about 5%. β-Conglycinin is composed of seven different combinations of three subunits. The subunits consist of three subunit proteins labelled α, α' and β. The α' and α subunits have molecular weights of 57.000–72.000 Da. The β subunit has a molecular weight of 42.000-52.000 Da. The seven combinations B_0-B_6 are, βββ, ββα', ββα, βαα', βαα, ααα', ααα, respectively (*27*). The subunits are non-covalently associated by hydrophobic and hydrogen bonding (*41*). At low ionic strengths β-conglycinin

exists as a trimer having molecular weights ranging from 140.000 to about 170.000 Da. β-conglycinin has no free –SH groups and on average two disulfide bonds per trimeric molecule. At high ionic strengths, the β-conglycinin forms oligomers with a molecular weight of about 280.000 to 350.000 Da and a sedimentation coefficient of 9S. The isoelectric point of the trimeric β-conglycinin is 4.64 (*38*).

Protease Inhibitors

The nutritive value of unprocessed SBM is negatively affected by the presence of antinutritional factors (ANF) (*3*, *43*). The best characterised ANF are protease inhibitors (*44*, *45*), lectins (*46*, *47*, *48*), phytate (*44*, *49*, *50*), and phenolic compounds (*51*, *52*). In addition, oligosaccharides (*22*, *53*) and allergenic epitopes of storage proteins (*54*) are also considered among the antinutritional factors of soybean meal. Protease inhibitors in soybean include trypsin and chymotrypsin inhibitors. Trypsin inhibitors (TI) are proteins with the ability to inhibit most serine proteases (*45*, *55*). Two families are known: the Kunitz soy trypsin inhibitor (KSTI) and the Bowman Birk (BBI) trypsin inhibitor.

The **BBI** molecule consists of 71 amino acids and has a molecular weight of 7.8 kDa. The protein can form dimers and trimers in solution which explains its association with the other 2S proteins (*31*). The BBI molecule is highly symmetrical and composed of a number of rings held together by the presence of 7 disulfide bonds. While many soy proteins are low in sulfur-containing amino acids, this inhibitor has 14 of its 71 amino acids composed of cysteine. The trypsin inhibiting site is the bond between lysine-16 and serine-17. While trypsin would normally cleave a Lys-Ser bond, it appears that the rigid ring structure in which this bond exists often prevents cleavage. Although trypsin binds to this protein, it cannot cleave the bond and is not readily released to cleave other molecules. At the opposite end of the molecule, the bond between leucine-43 and serine-44 interacts with chymotrypsin. This band is also not cleaved. While named the Bowman-Birk trypsin inhibitor, the protein can also inhibit the activity of chymotrypsin depending on the experimental conditions (*44*).

The **KSTI** molecule is composed of 181 amino acids with a molecular weight of approximately 21.5 kDa (*44*). It has two disulfide bridges making it a less rigid molecule than the Bowman-Birk inhibitor. Arginine-63 and isoleucine-64 form the bond at the

active site of the inhibitor. Trypsin cleaves this bond, but the enzyme is not released from the inhibitor once contact has been made (*45*, *56*, *57*, *58*).

Carbohydrates

The carbohydrates of soybean meal can be divided into water-extractable and water-unextractable carbohydrates (*59*). The main **water-extractable carbohydrates** of defatted soy flour consist of oligosaccharides, saccharose (~8% w/w), stachyose (~5%), raffinose (~1%), maltose (~0.5%), and verbascose (trace), together with the monosaccharides, glucose (0.3%), arabinose (0.1%), and ribose (0.1%) (*60*, *61*). Oligosaccharides are responsible for the flatulence problems that are often associated with consumption of soy products. However, most of the soluble carbohydrates are removed during the manufacture of more refined soy products such as protein concentrates (>70% protein) or isolates (>85% protein). The proportion of water-extractable NSP in soy is negligible (<5%) (*59*). The water- and enzyme-unextractable plant cell wall polysaccharides are the subject of interest of this thesis.

The **water-unextractable carbohydrates** comprise ~50% of total saccharides of soy and are present as cell wall polysaccharides (*25*, *59*, *62*, *63*). Compared to soy proteins, characterisation of the cell wall polysaccharides is more difficult because the polymers form a complex matrix of pectic substances, hemi-celluloses, celluloses and structural proteins. Direct comparison between individual studies is difficult due to different methods of extraction, separation, fractionation, and analysis. The analyses are based on chemical or enzymatic hydrolysis of sugar linkages to release both monomeric and oligomeric degradation products. The monomers are then identified by chromatographic techniques, the oligomers are purified by chromatographic techniques and their fine chemical structure established by e.g. NMR and MS (*24*).

The monosaccharide compositions of NSP of soybean meal and the water-unextractable, de-proteinized solids (WUS) of soybean meal obtained in a study by Huisman and co-workers (*59*) are presented in Table 3.

Table 3. Carbohydrate composition of non-starch polysaccharides of soybean meal and water-unextractable solids (WUS) from soybean

	Carbohydrate composition (mole %)								
Fraction	rha	fuc	ara	xyl	man	gal	glc	galA	carb. content[a]
Soybean meal[b]	2	3	19	8	3	28	21	18	14.5
WUS[c]	2	3	19	8	2	29	21	17	89.3

[a] *Carbohydrate content. Expressed as % (w/w). Rhamnose, rha; fucose, fuc; arabinose, ara; xylose, xyl; mannose, man; galactose, gal; glucose, glc; galacturonic acid, galA.*
[b] *Excluding oligosaccharides and after enzymatic removal of starch. Adapted from Huisman et al, 1998 (59).*
[c] *Water-unextractable solids, produced by repeated extraction with distilled water and subsequent removal of proteins by sodium dodecylsulphate (1.5% w/v) in water containing 10 mM 1,4-dithiothreitol (59).*

The polysaccharides in the soybean meal and in the WUS fraction consists mainly of galactose, glucose (mainly cellulose), arabinose and galacturonic acids, which is an indication of considerable amount of pectins (*59*). The cellulose contents of soybean meal and the WUS fraction are about 2.3 and 13.7% (w/w), respectively. The remainder of glucose is present in xyloglucans, corresponding roughly to 1.6% xyloglucan in the soybean meal and 8.8% in the WUS (*64*). The composition of the WUS fraction is very similar to that of the soybean meal and the high recovery of galacturonic acids in the WUS shows a low water solubility of the pectins (*59*).

The Plant Cell Wall - Simplified

Cell walls are a major component of plant material. A recent model of cell wall architecture suggests that cell walls of dicotyls are constructed from at least two independent networks, a cellulose/xyloglucan network and a pectin network, with a third network of structural protein in some cells (Figure 2) (*65*). According to the model the cell wall is a network of cellulose microfibrils, which are interlaced with xyloglycan polymers. The cellulose-xyloglucan framework is embedded in a pectin network. The structural proteins of the cell wall are called extensins and are rich in hydroxyproline (*66*) (not shown in Figure 2).

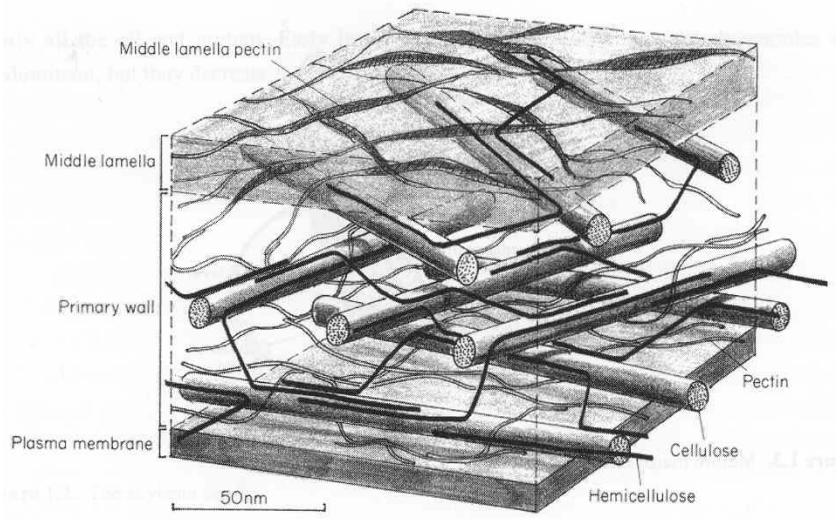

Figure 2. Simplified structural model of the primary cell wall (*65*).

*The Cellulose/Xyloglucan Network***:** Cellulose ((1,4)-linked β-D-glucan) is the major component of the primary cell wall. The cellulose chains are associated into microfibrils by intermolecular hydrogen-bonding. The microfibrils are coated with hemicelluloses to prevent them from aggregating. The principal hemicelluloses are xyloglucans, which have also been identified in the cell walls of soy (*59*). Xyloglucan is thought to form a tightly bound molecular monolayer on the surface of cellulose of which part can interact with cellulose microfibrils via multiple hydrogen bonds thereby cross-linking the microfibrils (*67*).

*The Pectin Network***:** The second polysaccharide network is composed of pectic polysaccharides, of which some of the known structures are very abundant in soy (*59*). *Homogalacturonan* is the most well known part of pectic substances consisting of (1,4)-linked α-galacturonic acids residues but in soy this polymer is not a common structural element (*68*). *Xylogalacturonan* is a relatively recently discovered sub-unit of soy pectic substances (*69*, *70*). The backbone consists of (1,4)-linked α-D-galacturonic residues. Xylose residues are β-(1,3)-linked to part of the galacturonic acid residues which can be partly methyl esterified. Xylogalacturonan is probably associated with rhamnogalacturonan regions (*70*, *71*).

Rhamnogalacturonan (RG) is another major type of pectic polysaccharide. Polymers containing this backbone are present in most if not all higher plant cell walls. Two types of rhamnogalacturonan exist, namely rhamnogalacturonan I and II. In recent literature

RG II is termed highly branched galacturonan (*70*). Type I is by far the most abundant (*70*). It consists of a long chain of alternating α-(1,4)-linked galacturonosyl, and α-(1,2)-linked rhamnose units called rhamnogalacturonan. Next to rhamnose and galacturonic acid residues in the backbone, RG I is composed of arabinofuranosyl-, galactopyranosyl-, and minor quantities of fucopyranosyl residues (*70*, *72*).

Bound to the backbone at the rhamnose unit are sidechains of arabinan, galactan, and highly branched arabinogalactans. They look like hairs and highly substituted rhamnogalacturonans are called the hairy regions (*66*). Pectic L-arabinans consist of (1,5)-linked α-L-arabinose residues, which are substituted with mainly monomeric arabinose but also oligomeric arabinose chains mainly at O-3 but also at O-2. Pectic D-galactans contain primarily β-(1,4)- linked galactose units, with little or no additional saccharide material present in the molecule. Generally, the galactan sidechains are longer than arabinan and less branched (*70*, *73*).

Arabinogalactans are divided into two types, type I and II. Pectin mostly contains type I (*74*). Type I arabinogalactan has a backbone of β-(1,4)-linked galactans with branches of α-(1,5)-linked arabinose or galactose chains bound α-(1,6) to the galactan backbone (*66*). The ratio of arabinose and galactose in arabinogalactan, and the branching of arabinose show considerable variation within different plant species (*70*).

An approximate composition of pectin in soybean meal is presented in Table 4.

Table 4. Occurrence and proportion of the various structural elements of pectin in soybean meal

	Soybean meal
Total polysaccharide (w/w % of dry matter)	16
Pectic substances (w/w % of total polysaccharides)	59
Structural element (% of pectic substances)	
Homogalacturonan	0
Xylogalacturonan	21
Rhamnogalacturonan II	4
Rhamnogalacturonan I backbone	15
Rhamnogalacturonan I side chain	
Arabinan + arabinogalactan I	60
arabinogalactan II	0

Adapted from Whitaker et al, 2003 (70).

The pectic substances of soy are unique compared to pectic substances of cell wall from other plants in the absence of homogalacturonan and arabinogalactan II (*68*), the presence of fucose residues in the xylogalacturonan, and two uncommon structural features of the pectic arabinogalactan sidechains, namely the presence of internal (1,5)-linked arabinofuranose and terminal arabinopyranose (*75*).

Cell Wall Degrading Enzymes

Due to the complexity of the soy cell wall a number of enzyme activities are needed for complete degradation (*69*, *76*). The general practice is to divide the plant cell wall degrading enzymes into pectinases, hemicellulases and cellulases. Commercial carbohydrase preparations, e.g. pectinases from fungi, contain a wide range of different enzyme activities relevant for soy degradation (*77*). A simplified overview is given in the following paragraphs with focus on the enzymes applicable for soy.

A complex mixture of cellulolytic enzymes are required for complete degradation of cellulose, including endo-(1,4)-β-D-glucanase (cellulase), exo-(1,4)-β-D-glucanase (cellobiohydrolase), β-D-glucosidase (cellobiase). The enzymatic degradation of cellulose is rather complex and is not yet fully understood.

Hemicellulases are divided into groups which designate the type of hemicellulose they are capable of degrading. Typical hemicellulases are endo-β-(1,4)-D-xylanases, endo-β-(1,4)-D-mannanases, and endo-β-(1,3)-(1,4)-glucanases (*70*, *78*). More relevant for soy degradation are the galactan and arabinan degrading enzymes endo-α-(1,5)-L-arabinanases, endo-β-(1,4)- and -β-(1,3)/(1,6)-D-galactanases are categorized both as hemicellulases and as pectin degrading enzymes (*70*). With respect to chemical structure there is no sharp distinction between hemicelluloses and the side chains of the pectic substances (*70*, *77*). They differ in solubility and hemicelluloses are only soluble in alkaline conditions (*70*). The activity of these endo-enzymes is generally enhanced by the presence of glycosidases like β-xylosidase, β-galactosidase, and α-arabinofuranosidases, particularly by removing side chains.

The homogalacturonan degrading enzymes include xylogalacturonan degrading endo and exo-polygalacturonases (*70*), pectin lyases, pectate lyase (*77*), endo-xylogalacturonase, and the group of esterases, which split of substituents ester-linked to pectins, like pectin methyl esterases, pectin acetyl esterases and feruloyl esterases (*70*). The rhamnogalacturonan degrading enzymes include endo-rhamnogalacturonase, endo-rhamnogalacturonan lyase, rhamnogalacturonan rhamnohydrolyase, rhamnogalacturonan galacturonohydrolyase (*25*, *79*), and since arabinogalactans type I and arabinans are side chains of the rhamnogalacturonan backbone, also arabinanases, α-arabinofuranosidases, galactanases and galactosidases can be classified into this group (*70*, *80*). A rhamnogalacturonan acetyl esterases specific for rhamnogalacturonan structures has also been identified (*80*).

An extensive degradation of the soy cell wall polysaccharides is known to demand several enzyme activities (*70*). Multicomponent enzyme preparations from e.g. *Aspergillus aculeatus* (used in Chapter 2) are known to be effective for degradation of soy cell walls (*77*).

Heat Treatment

Feeding of unheated soy proteins to animals has been demonstrated to have a detrimental affect on growth due to the inhibitory effects of trypsin inhibitors (*81*). Therefore, as

shown in Figure 1, the most common processing technology applied to the soybean is heat treatment, which has proven effective for reducing levels of trypsin inhibitors and soybean lectin. Commercial heat treatment is carefully controlled: underheating often results in adequate inactivation of ANF while overheating can reduce availability of important meal components (e.g. lysine) through the occurrence of Maillard reactions (*81*, *82*, *83*, *84*).

Heat treatment of soybean meals affects the solubility of the carbohydrates. High temperature and low water content enhance Maillard reactions. The Maillard reaction is not a clearly defined single reaction but encompasses a family of reaction pathways that have a common first step: the condensation of an amino group with a reducing sugar. Following the initial condensation, the product undergoes rearrangement, fragmentation, degradation, dehydration, and other reactions resulting in a large number of compounds that eventually impart coloration to the soybean meal. Numerous factors determine the outcome of the Maillard reaction including the abundance of carbonyl and amino groups, pH, temperature, water activity, and reaction time. The extent to which Maillard reactions occur during heating of soybean meals can be estimated by measuring decrease in total soluble sugar content (*85*). The above mentioned reactions negatively affect the solubility and accessibility of the carbohydrates and proteins by carbohydrases and proteases (*14*, *84*, *86*, *87*, *88*, *89*).

KSTI and BBI in purified preparations respond differently to thermal treatment then when they are embedded in the soybean meal matrix (*45*). Purified protease inhibitors (PI) are less susceptible to heat treatment indicating that the higher sensitivity seen in soybean meal is caused by the presence of other constituents of the soybean such as free thiol agents (*90*) or cell wall polysaccharides that somehow promote inactivation of the inhibitors (*45*). Literature generally agrees that antinutritional factors (ANF) such as lectins and protease inhibitors are more rapidly inactivated at high water activities than at lower water activities. For intact soybeans, residual PI activity was only 3% of original after only 20 minutes of cooking in water (*91*). Comparable results were found for raw defatted soybean meal subjected to autoclaving at 121°C for 10 min (*45*).

Heating of β-conglycinin causes dissociation of subunits which can then unfold, reassociate and aggregate to form precipitates making them less accessible for enzymatic degradation than in their native condition**.** At concentrations below 7% protein and low

ionic strength, β-conglycinin is reported as being relatively heat stable. The proteins are stable toward heat treatments at temperatures in excess of 100°C (*41*, *92*).

Heating of glycinin at 100°C at around neutral pH rapidly converted approx. 50% of the protein into a buffer soluble aggregate (*93*). With continued heating, the soluble aggregates increased in size and precipitated. The precipitated fraction consisted of the basic polypeptides, whereas the acidic polypeptides remained soluble. However, when soybean meal is heated at comparable conditions, the basic polypeptides of glycinin remains soluble and no precipitate was observed (*94*).

Accessibility of Soybean Meal Constituents

When soybean meal is used industrially, a certain proportion of the protein remains inaccessible or insoluble or indigestible to the animals. From both a financial and environmental perspective this material represents a loss of an important and valuable nutrient. For many years it has been anticipated that a major reason for the observed inaccessibility of the protein was the presence of plant cell wall polymers, which supposedly trapped or shielded the proteins from enzymatic degradation (*66*). This has led to investigations with extensive use of plant cell wall degrading enzymes for improved protein release and digestibility (*8*). In these studies it was, however, established that plant cell wall degrading enzymes have only minor effects on the digestibility/extractability of soybean meal proteins, even when the viscosity of soybean meal slurries can be drastically reduced by these enzymes. This viscosity reducing effect is beneficial to the capability of most animals to utilize the feed effectively (*8*). Some proteins and carbohydrates remain insoluble even when large amounts of enzymes are added to the diet (*95*). The major reasons for this phenomenon are thought to be partly inherent, i.e. interactions between the different cell wall components, and partly induced by the molecular cross-linking (protein/protein, protein/carbohydrate) caused by the heat treatment. Effects of exogenous enzymes vary considerably, and accordingly there is a need for more research to pursue the understanding of the complex structural changes that occur when exogenous enzymes are used for SBM at *in vivo* as well as *in vitro* (*96*).

Enzymatic Digestibility of Soy Proteins

In vitro digestibility of soybean proteins has received little attention in the literature. In particular, the number of studies describing the digestibility of isolated soybean proteins

is limited. Proteolytic enzymes reported include pepsin, papain, trypsin, chymotrypsin plus various bacterial and fungal proteases (*97*). Without being complete, an overview of different studies on this subject is given in Table 5, including SBM proteins, protein isolates and purified proteins.

Table 5. Short literature overview on effects of proteolytic treatment on soybean meal protein, protein isolates and purified soy proteins.

Reference	**Substrate**	**Enzymes used**	**Conditions of hydrolysis and possible additional treatment**	**Main results/and or conclusions (hydrolysate compared to intact protein**
Marsman et al, 1997 (*14*)	Untoasted, toasted and extruded SBM	Esperase, Neutrase and Bio-Feed Pro	SBM suspensions (10%) in 0.05M Na-acetate buffer (pH 5), for Esperase also carbonate buffer (pH 9). Analysis: DSC, SDS-PAGE.	Toasted SBM: β-conglycinin well degraded – glycinin more resistant (polypeptide B more than A). Possible explanation: primarily non-covalent bonds broken, disulfide bonds more or less intact. Unheated: high resistance against degradation explained by native structure. Extruded: Both proteins well degraded – both non-covalent and disulfide bonds broken
Romagnolo et al, 1990 (*98*)	Processed SBM	Rumen microbes	Rumen bacterial fermentation, time course. Analysis: SDS-PAGE	β-conglycinin more susceptible to rumen degradation than glycinin that showed particular resistance within B-polypeptide.
Lallés et al, 1998 (*99*)	Heated SBM	Rumen microbes	Rumen bacterial fermentation (nylon bag placed in the rumen). Analysis: Western blotting (antibodies)	Glycinin: Sharp decrease in immunoreactive glycinin 2 h post feeding. Early outflow of glycinin composed of nearly intact B-polypeptides and partially degraded A-polypeptides. However, intact A-polypeptide and B-polypeptide detected at both 2 h and 6 h post feeding. β-Conglycinin: Rate considerably slower than glycinin.
Lee et al 2001 (*100*)	Defatted soybean meal	Alcalase and Flavourzyme	Acid pretreatment (0.05-0.2 N HCl), hydrolysis (0-24 h), pH 6.5. Analysis: DH and gel filtration	Protein hydrolysed primarily during first 5 h. DH and α-amino nitrogen increased after acid pre-treatment. Average peptide chain length: Alcalase (3 h), 7-8 amino acids; Alcalase/Flavourzyme (21 h): 3-5 amino acids.
Tsumura et al, 2004 (*101*)	Native soy protein isolate	Pepsin and papain	Dispersion in water (5% w/v). Pepsin incub: pH range 1.5-4.0, reaction 37°C for 30min. Papain incub: pH 7.0, 37-80°C for 5 min. Analysis: SDS-PAGE, DSC.	Glycinin fraction in native isolate selectively hydrolysed by pepsin in the range from pH 1.5-2.5. β-Conglycinin fraction selectively hydrolysed by papain at 70°C. Proteolysis significantly correlated with onset of denaturation of glycinin and β-conglycinin in SPI.

Table 5 continued

Bernardi-Don et al, 1991 (*97*)	Soy protein concentrate, denatured	*Aspergillus oryzae and Bacillus subtilis*	Protein conc. 6%, pH 6.8, 1 h, different enzyme/substrate ratios between 0.1-1:100. Analysis: DH and NSI	Solubility (NSI) greatly enhanced by both proteases (no discrimination between different soy proteins). Greatest effect up to DH 10. Bacterial protease more effective protein solubiliser than fungal protease – effect most remarkable at the highest DH values.
Shutov et al, 1996 (*102*)	Glycinin and β-conglycinin, purified	*Trypsin*	Substrate concentration (5 mg/ml) in 0.05 M Tris-HCl pH 8.0. Enzyme substrate ratio 1:600, 30°C. Analysis: SDS-PAGE	Four cleavage points identified in the A polypeptide of glycinin as well as the α'-chain of β-conglycinin.
Shutov et al, 1991 (*103*)	Glycinin	*Trypsin, chymotrypsin*	Protein conc. (5 mg/mL), heating to different degrees, incubation 60 min, 25°C, pH 8.2. Polypeptides separated by gel filtration.	Overall hexameric polypeptide structure retained. B polypeptides remained intact, A polypeptides reduced in size indicating that B is buried within the interior of the protein molecule (*104*, *105*).
Kim et al, 2003 (*106*)	Glycinin	*Trypsin*	Glycinin concentration 1% (w/w). Incubation with enzyme at 50°C, pH 7 for 4 h. Analysis: gel permeation, reverse phase chromatography, and DH.	Glycinin hydrolysate DH 12. Molecular size distribution from 200da to 1400 Da (~2-12 amino acids) showing varying hydrophobic character.
Hajos et al, 1996 (*107*)	Soy albumins fraction	*Pepsin*	Pepsin substrate ratio (100:1), pH 2, 2 h. Analysis: ELISA and SDS-PAGE	Immuno activity of KSTI significantly reduced. No significant reduction of BBI activity. One quarter of original KSTI content excluded from SDS-PAGE gel. BBI not reduced in molecular size.
Jensen et al, 1996 (*108*)	BBI, unheated	*Bovine trypsin*	BBI + 5% (molar) trypsin in different buffers, pH ranges (3.5-10.3). Primary analysis: HPCE	Primary sequence almost fully conserved. Cleavage of trypsin reactive site and more slowly in chymotrypsin reactive subdomains
Vaintraub and Yattara, 1995 (*109*)	KSTI, commercial	*Papain, subtilisin, pepsin*	KSTI 0.5% solution + 0.05M acetate buffer, pH 4.5 / 0.05 M Tris-HCl, pH 8.5. Enzyme substrate ratio 1:200 for pepsin and 1: 100 for papain and subtilisin. Analysis: SDS-PAGE and gel permeation	KSTI initially degraded into fragment retained in the molecule by covalent and disulfide bands. After extended hydrolysis degraded into smaller peptides. Fragment size (many retained inhibitor activity): Papain, (2.7 – 6.7 KDa); subtilisin, (3.9 – 8.8 kDa); pepsin (6.9 – 18.4).

Although it is difficult to compare the various results found for hydrolysis of soy proteins (different authors study different proteases on different substrates and at different conditions; Table 5), some general conclusions can be drawn.

Firstly, resistance to proteolysis of the peptide bonds of the major soy proteins depends largely on the extent of protein denaturation : native (high resistance, intact structure) > toasted (medium resistance, non-covalent bonds broken) > extruded (low resistance, non-covalent and disulfide bonds broken) (*14*). In toasted SBM β-conglycinin is effectively degraded by different endo-proteases (*14*). Heat treated β-conglycinin was also effectively degraded by proteases of rumen microbes (*98*), although Lallés and coworkers reported a considerable slower degradation rate for β-conglycinin than for glycinin by rumen microbes (*99*). Glycinin polypeptide B is more resistant to proteolysis than polypeptide A (*14*, *98*, *99*) possibly because polypeptide B is buried within the interior of the protein molecule (*104*, *105*).

Also for soy protein isolates proteolysis significantly correlated with the onset of denaturation of glycinin and β-conglycinin (*101*). As shown in studies with Alcalase and Flavourzyme, protein degradation progresses most rapidly within the first 5 h of incubation and a high degree of hydrolysis is only obtained for combinations of the two enzymes (*100*). Bacterial proteases are generally more powerful solubilisers than fungal proteases (*97*).

Secondly, the degradation of purified glycinin and β-conglycinin by subtilisin types of proteases (*14*) has many similarities to the degradation of these proteins in SBMs by rumen microbes (*98*, *99*). After heating at slightly alkaline pH the hexameric polypeptide structure of glycinin is conserved and B polypeptides remained largely intact (*103*). The α- and α' chains of β-conglycinin were also readily degraded by trypsin (*102*).

Finally, results of tryptic digestion of Bowman-Birk inhibitor showed that its primary sequence was almost fully conserved (*108*) whereas purified KSTI after prolonged incubation was degraded into smaller peptides by papain, subtilisin, and pepsin (*109*). However, in a pepsin-treated soy albumin fraction the immuno reactivity of KSTI was significantly reduced, whereas the molecule was only partially degraded. The BBI was not reduced in size and retained its immuno activity (*107*). The primary sequence of unheated BBI was also almost fully conserved after tryptic digestion (*108*).

In Vitro Testing of Enzymes

In vitro test systems are valuable complementary tools for the development of new enzyme applications for food and feed. *In vitro* models can be used to compare and rank new enzymes according to parameters of particular interest, including the capacity to degrade the target substrates at relevant conditions. The obvious strength of *in vitro* models is that experiments can be repeated at exactly the same conditions in series. In contrast, *in vivo* studies are subject to large physiological variations between individual animals and also within the same animal during its physiological development stages (*8*). Consequently a considerable number of animals are needed for *in vivo* testing in order to obtain statistically valid results. The capacity of *in vitro* models decreases greatly with increasing complexity of the model (i.e. how well it compares to an *in vivo* situation). Dynamic models such as the IFR Model Gut (Institute of Food Research, Norwich, UK) and the gastro intestinal model (GIT) from TNO (*110*) are used to study several parameters simultaneously, but have limited capacity. Naturally, the technical feasibility (and simplicity) inevitably plays a role when the need for high capacity is critical. Therefore, relatively simple batch models with a high number of replicates and a standardized set of conditions have been used for the experiments of this thesis.

Aim and Outline of Thesis

From the above, it is evident that much is known about what the different enzymes can do – but not so much about what they cannot do. Accordingly, there is a lack of knowledge about the molecular structure of the different soy polymers following enzymatic digestion and a lack on knowledge about the composition of enzyme resistant structures. A better knowledge on the latter is valuable for understanding if enzymes of interest in principle are capable of degrading resistant structures or the causes for their inefficiency. This could also help understanding if certain enzyme activities are lacking which might improve performance. Research in these areas should enable more efficient use of the current enzymes and the development of more efficient enzymes.

The first aim of this thesis is to broaden the knowledge about factors affecting the efficacy of enzymatic extraction of protein and carbohydrates from SBMs. The second aim is to investigate the effects of heat treatments on protein composition of enzyme-unextractable soybean meal fractions produced *in vitro* and *in vivo*.

In **Chapter 2,** the amounts and compositions of residues obtained after enzymatic treatment of unheated SBM and SBMs heated at different humidities are presented. High concentrations of commercial proteases and carbohydrases are used in a two-step hydrolysis procedure to obtain enzyme-unextractable material. The extractability of protein and carbohydrates from the meals is subsequently examined, and the unextractable residues are quantified and characterised with respect to amino acid and carbohydrate composition.

In **Chapter 3,** the enzymatic hydrolysis conditions are optimised for two commercial preparations, Alcalase and Flavourzyme, in order to increase protein solubilisation in a single step hydrolysis of SBM.

The experiments of **Chapter 4** further characterize the proteins in the enzyme-unextractable residues which resist extraction from the soybean matrix by enzymatic treatment. The residues are subjected to extraction by various solvents and the extracted proteins are analyzed by gel electrophoresis, chromatographic techniques and mass spectrometry. The solvent extractability of protein and carbohydrates from the residues is determined and the resulting residues are quantified and characterized for amino acid and carbohydrate composition.

Chapter 5 presents the molecular size and composition of proteinaceous material extracted from the insoluble components of a digesta sample obtained from pigs fed a feed consisting entirely of soybean meal. The molecular size of the alkali-extractable protein fraction is subsequently studied using gel permeation chromatography, gel electrophoresis, RPLC-MS, and MALDI-ToF MS. *In vitro* proteolysis of extracted, aggregated proteinaceous material is also studied.

In **Chapter 6,** the results obtained in the previous chapters are discussed.

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CHAPTER 2

ENZYMATIC EXTRACTABILITY OF SOYBEAN MEAL PROTEINS AND CARBOHYDRATES: HEAT AND HUMIDITY EFFECTS [♣]

[♣] Fischer,M.; Kofod,L.V.; Schols,H.A.; Piersma,S.R.; Gruppen,H.; Voragen,A.G.J. Enzymatic extractability of soybean meal proteins and carbohydrates: heat and humidity effects.
Journal of Agricultural and Food Chemistry **2001**, *49*, 4463-4469.

ABSTRACT

To study the incomplete enzymatic extractability of proteins and carbohydrates of thermally treated soybean meals, one unheated and three heat-treated soybean meals were produced. To obtain truly enzyme resistant material the meals were extracted by a repeated hydrolysis procedure using excessive concentrations of different combinations of commercial protease and carbohydrase preparations. The water extractability of protein from the different meals varied considerably (13 - 67%). For all soybean meals enzymatic treatment extracted most of the original protein (89 - 94%). Carbohydrase preparations did not improve protein extraction. High humidity heat treatment led to a more effective enzymatic extraction, which seemed to correlate to the extent of protein denaturation. Results with purified proteins indicated that the soybean meal matrix affects the enzymatic extraction of protein from the meals. Interactions between protein and other components (e.g. cellulose) may explain the incomplete enzymatic extractability of protein from the meals.

Keywords: soybean meal; heat treatment; hydrolysis; extraction; enzymatic residue; protease; carbohydrase; composition; protein; carbohydrate; amino acid

INTRODUCTION

The *in vitro* protein digestibility of soybean meals by enzymes has been shown to vary with thermal processing conditions. As a result, enzymatic extraction by commercial enzymes, i.e. the degradation and solubilization of SBM protein, is often incomplete (*1*). Fractions containing enzyme unextractable protein represent a loss of valuable protein for the manufacturers of enzymatic soy hydrolysates. An in-depth characterisation of the unextractable residue is required to obtain a knowledge base to improve the protein yield during hydrolysis of soybean meals.

Defatted soybean meal (SBM) contains approximately 50% protein (w/w) which is mainly composed of glycinin and β-conglycinin. The nutritional value of unprocessed soybean meal is limited by the presence of antinutritional factors (ANFs) such as trypsin inhibitors, lectins and oligosaccharides (*2*). In addition to protein, SBM contains approximately 16% polysaccharides. A large part of the polysaccharides is cellulose and more than half represents pectic substances. The latter can be divided into rhamnogalacturonans containing arabinan and arabinogalactan sidechains, xylogalacturonans, and rhamnogalacturonans type II (*3*, *4*). Together these structures form a complex matrix, which form agglomerates with the cell wall proteins (*5*). The complex matrix composition of the native soybean meal is suspected to affect protein availability and extractability by enzymes (*1*).

To improve the nutritional value of the unheated meal, soybeans are subjected to thermal treatments such as toasting and extrusion (*1*, *6*, *7*, *8*). Depending on temperature and humidity conditions during heat treatment, the components of the soybean matrix may interact resulting in a reduced enzymatic degradability and extractability of the proteins (*9*, *10*). Generally, the effects of heat treatment on solubility and the proteolytic degradation of pure soy proteins, concentrates and isolates are well described in literature (*7*, *11*, *12*, *13*, *14*, *15*). Purified native proteins may show some resistance towards proteolytic degradation, but the enzymatic degradability of most proteins improves by appropriate heat treatment at high humidity followed by proteolysis (*16*, *17*). Hydrolysates of soy isolates produced with single proteases (e.g. trypsin, pepsin, chymotrypsin, and papain) often show a limited degree of hydrolysis (*18*, *19*). A higher degree of hydrolysis can be obtained with combinations of endo- and exo protease containing preparations such as Alcalase and Flavourzyme (*20*).

Less work has been published covering the effects of heat treatment and subsequent enzymatic proteolysis of SBM protein compared to purified proteins and protein isolates. In most cases the composition of the extracted material is the subject of interest (*11*). Marsman *et al.* (*1*) studied the *in vitro* accessibility of the water unextractable solids (WUS) from untreated, toasted, and extruded soybean meals for different protease and carbohydrase activities; they showed that the proteins in the extruded sample were more rapidly and completely degraded compared to the toasted and untreated soybean meals. Recently, Lee (*21*) characterized hydrolysates of protein dispersions, produced from defatted SBM and treated with Alcalase and Flavourzyme. The amount of free amino acid, dipeptide, and tripeptide accounted for almost half of the proteins in the hydrolysate, and the oligopeptides (360 - 2,000 Da) constituted 40%. In both studies no attention was given to the enzyme unextractable material.

Therefore, the aim of this study is to determine the amount and composition of residues obtained after enzymatic treatment of unheated and SBMs heat-treated at different humidities. High concentrations of commercial protease and carbohydrase enzyme preparations are used in a repeated hydrolysis procedure to obtain truly enzyme unextractable material. The extractability of protein and carbohydrates from the meals is subsequently examined, and the unextractable residues are quantified and characterised with respect to amino acid and carbohydrate composition.

MATERIALS AND METHODS

Soybean Meal Preparation and Heat Treatment

Unheated soybean meal was produced according to Lakemond (*22*) from unheated Williams 82 soybeans (from the 1994 harvest, stored at -20 °C). The final product had a protein content (N x 6.25) of 49% (w/w) and a particle size of 0.5 mm. It was denoted USBM and stored at -20°C. Subsequently, two other meals were prepared from this USBM. The first was heat-treated at high humidity: USBM (200 g) was mixed with 1800 mL water in 2-L screwcap bottles, stirred for 1 h and subsequently autoclaved with closed lid for 15 min at 125 °C in a table autoclave. After the mixture was autoclaved the suspension was cooled in an ice bath. The product was denoted SBM-H and stored at -20 °C or used directly. The second heat-treated meal was produced at low humidity:

USBM (250 g) was freeze-dried overnight and subsequently transferred to a desiccator. The water content was adjusted to 15% relative humidity by equilibrating the meal above a saturated potassium nitrate solution (31.6 g in 100 mL water). Equilibration was allowed for three days at 25 °C. The meal was placed in an airtight container and heat-treated in a table oven at 125 °C for 15 min. The product was denoted SBM-L and stored at -20 °C. A third heat-treated SBM, termed NN, was obtained from a local mill. It had a protein content (N x 6.25) of 49% (w/w), was conditioned at 68 °C for 10 min and pelletized (3 mm x 10 mm) at 85.2 - 86.9 °C. The meal was stored at -20 °C.

Isolation and Heat Treatment of Soybean Proteins

Glycinin and β-Conglycinin. Glycinin and β-conglycinin were isolated from USBM essentially according to procedures described by Thanh and Shibasaki (*23*). The resulting protein solution of 11S glycinin (12 mg/mL) showed a purity >90% by SDS-PAGE analysis. The solution was stored at -20 °C.

The resulting protein solution of crude β-conglycinin (50% pure) was further purified by affinity chromatography. A column (26 mm x 200 mm) was packed with 100 mL of ConA Sepharose (Amersham Pharmacia Biosystems, Sweden) and washed with 35 mM potassium phosphate buffer pH 7.6 containing 0.4 M NaCl and 2 mM β-mercaptoethanol. Crude β-conglycinin (200 mL) was added at a flowrate of 2 mL/min. The absorbance of the eluate was monitored at 280 nm. After removing unbound material with the washing buffer the column was eluted isocratically with 0.5 M α-D-glucopyranoside (Sigma, MO, USA) in the same buffer. The eluate was collected and washed by diafiltration using a 400-mL ultrafiltration cell (Amicon, CA, USA) with a 3 kDa membrane at a nitrogen pressure of 2 Bar. The final protein concentration was 13 mg/mL. SDS-PAGE showed a purity of 85%. The solution was stored at -20 °C.

Kunitz Trypsin Inhibitor and Lectin. Kunitz trypsin inhibitor (KSTI) from soy was purified from a commercial product (Prod. no. 93618, Fluka Biochemica, Germany). An ÄKTA Explorer system equipped with a Superdex 75 Hiload column (16 mm x 700 mm) (both from Amersham Pharmacia Biosystems, Sweden) was used. KSTI (1 g) was dissolved in 30 mL of the 35 mM potassium phosphate elution buffer at pH 7.6. Several injections of 5 mL were put onto the column, which was eluted at a flowrate of 10 mL/min. The absorbance of the eluate was monitored at 280 nm. Fractions eluting from

the column were analyzed by SDS-PAGE and the fractions containing essentially pure protein were pooled. These pooled fractions were diafiltrated with Millipore purified water using a 100 mL ultrafiltration cell (Amicon, CA, USA) with a 3 kDa membrane at a nitrogen pressure of 2 Bar. The recovery of protein was 75% with a final concentration of 5.5 mg/mL. The solution was stored at -20 °C.

Soybean lectin was purified from unheated soybean meal (Prod. no. S-9633, Sigma, MO, USA) according to Gordon (*24*) with affinity chromatography using N-ε-aminocaprooyl-β-D-galactopyranosylamine Sepharose (Sigma). The fractions eluting from the column were analyzed by SDS-PAGE and fractions containing essentially pure protein were pooled. The lectin protein solution (4.8 mg/mL) was stored at -20 °C.

Heat Treatment. The purified proteins were subjected to heat treatment at high or low humidity. In each case, an amount equivalent to 5 mg protein was weighed into a 2 mL eppendorf tube. For heat treatment at high humidity the volume was adjusted to 2 mL by addition of 35 mM potassium phosphate buffer (pH 7.6). The tubes were heat-treated in a table autoclave for 20 min at 125 °C. Before heat treatment at low humidity the purified proteins were freeze-dried in the tube and subsequently heat-treated in the eppendorf tubes in an oven with closed lid for 20 min at 125 °C. Samples were cooled at room temperature and stored at -20 °C.

Enzymes

Four enzyme preparations were selected for the experiments. Alcalase Food Grade (A) is a preparation from *Bacillus licheniformis*. The main component, Subtilisin A is an endoproteinase. The pH optimum is between pH 6.5 and 8.5. Flavourzyme (F) is a protease complex from *Aspergillus oryzae*. It contains endoproteinases and exopeptidases and has a pH optimum of between pH 5.0 and 7.0. Energex (E) from *Aspergillus aculeatus* and Biofeed Plus (B) from *Humicola insolens* are carbohydrase preparations, which hydrolyze a broad range of carbohydrate polymers. All enzymes were from Novozymes A/S (Bagsvaerd, Denmark).

Enzymatic Hydrolysis

Purified Proteins. The efficiency of the Alcalase and Flavourzyme protease combination for hydrolysis of the unheated and the heat-treated purified proteins was examined (in

duplicate). Proteins subjected to high-humidity heat treatment were used directly after heat processing (as described above). For these and for the unheated material an amount equivalent to 5 mg protein was pipetted into an Eppendorf tube and made up to 2 mL by 35 mM potassium phosphate buffer (pH 7.6). The proteins subjected to heat treatment at low humidity (see above) were taken from the freezer and solubilized in 50 µL 8 M urea. Next, the volume was adjusted to 2 mL using 35 mM potassium phosphate buffer (pH 7.6) to a final urea concentration of 0.2 M. Alcalase (2.5%) and Flavourzyme (5%) were added (expressed as volume of enzyme product / weight of protein). Hydrolysis took place in an Eppendorf Thermomixer comfort (Eppendorf, Germany) at 40 °C for 16 h at 700 rpm. The hydrolysates were analyzed by SDS-PAGE.

Efficiency of Different Enzyme Combinations. A repeated hydrolysis of the NN meal was performed to find the enzyme combination, which could extract most protein from the meals in subsequent experiments. Meal (200 g) was mixed with 1800 mL water in 2-L screw cap bottles. Chloramphenicol (100 mg/L) was added to prevent microbial growth. The suspensions were adjusted to pH 7 with 1 M NaOH and placed in a water bath at 40 °C, and stirred for 1 h. At this point samples were taken to determine the effects of the different heat treatments on the extractability of proteins and carbohydrates in water prior to enzymatic treatment of the meals. Subsequently, Alcalase (2.5%) and Flavourzyme, Energex and Biofeed Plus (5%), respectively, were added (expressed as volume of enzyme product / weight of protein). Different combinations of the four enzyme preparations were tested. The mixture containing Alcalase and Flavourzyme was termed AF and the mixture containing Alcalase, Flavourzyme, Energex and Biofeed Plus was termed AFEB. The used combinations were AF-AF, AF-AFEB, AFEB-AF, and AFEB-AFEB. After enzyme addition hydrolysis took place at the pH of the suspension at 40 °C for 16 h with constant stirring. The pH was adjusted to 7, and the extracted material was removed by centrifugation (20 min; 15.000 x g; 4 °C). Supernatants were discarded. Residues were washed 3 times with 500 mL ice-cold deionised water and freeze-dried before the second hydrolysis was started. The residues were dispersed in water at same water/substrate ratio used for the first hydrolysis. The pH of the dispersion was adjusted to 7, and the residue was enzyme treated again at conditions similar to the first hydrolysis. After hydrolysis the pH was adjusted to 7 and supernatants and residues were separated by centrifugation (20 min; 15.000 x g; 4 °C). Supernatants were discarded. Residues were washed and freeze-dried as described above.

The enzyme combination resulting in the highest extraction of protein from NN was subsequently used to hydrolyse the NN, USBM, SBM-H and SBM-L meals. A repeated enzymatic hydrolysis was performed with the AFEB-AFEB enzyme combination according to the procedures described above.

The enzymatic extraction of dry matter (EDM), protein (EP) and carbohydrates (EC) from the different enzyme treatments was calculated by subtracting the values of the residues from the values of the original material. All hydrolyses were performed in duplicate.

Statistical Variation: The reproducibility of the enzymatic hydrolysis has previously been examined in triplicate. The enzymatic protein extraction (EP) has a standard deviation of less than 1% and the standard deviation of EDM and EC is between 0.2% and 0.5%. This should be considered during interpretation of the results.

Differential Scanning Calorimetry (DSC)

The extent of protein heat denaturation in the meals was determined by differential scanning calorimetry in a micro-DSC (Setaram, France). The four SBMs were suspended in a 35 mM potassium phosphate buffer (pH 7.6), containing 0.1 M NaCl (I = 0.2). The protein concentration of the SBM suspensions was 50 mg/mL. The stainless steel vessels contained 0.9 mL of suspension. The samples were scanned from 20 °C to 115 °C at a scanning rate of 1.2 K min^{-1} and subsequently cooled to 20 °C at the same rate. The peak denaturation temperature (Tp), the temperature of the maximum heat capacity, was read from the curves. The peak areas of the unheated meal (USBM) were used to calculate the extent of protein heat denaturation in the other meals.

Dry Matter (DM)

Dry matter was defined as the weight (Mettler AE 240, Switzerland) of meals and residues after freeze-drying.

Protein

The protein content (N x 6.25) of the final residues was determined by a semi-automated Kjeldahl method. Approximately 25 mg of the dry samples was destructed in concentrated sulfuric acid at 200-385 °C according to the Kjeldahl method (*25*). The

released NH_3 was determined with an ammonia-hypochlorite-salicylate reaction on a Skalar 5101 auto-analyzer (Skalar, The Netherlands).
Protein contents are the mean of three determinations.

Carbohydrate Composition

Neutral Carbohydrates. The polysaccharides were hydrolyzed by pretreatment with 72% (w/w) 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 neutral carbohydrates were converted to their alditol acetates and analyzed by gas chromatography (*26*). Inositol was used as internal standard. Alditol acetates were separated on a 3 m x 2 mm (i.d.) glass column (packed with Chrome WAW 80-100 mesh coated with 3% OV275) in a Carlo Erba Fractovap 2300 GC operated at 200 °C and equipped with a flame ionization detector (FID) set at 270 °C.
Uronic Acids. The uronic acid content was determined using an automated *m*-hydroxy biphenyl assay (*27*). For the procedure 96% (w/w) H_2SO_4 containing 0.0125 M sodium tetraborate was used to quantify glucuronic as well as galacturonic acid residues.
Carbohydrate contents are the mean of three determinations.

Amino Acid Analysis

The amino acid composition was determined using an automated derivatizer analyzer system (Model 420A) with a 130A separation unit and a 920 data module. All equipment was from Applied Biosystems (Foster City, CA, USA). Each sample (10 mg) was hydrolyzed in 100 µL 6 M HCl for 16 h and subsequently derivatized by PITC. Separation was performed using a C18 reversed-phase column (21 mm x 220 mm) at a flowrate of 300 µL / min. The absorbance of the eluate was monitored at 254 nm. Amino acid standard H (Pierce, IL, USA) was used for identification.
Amino acid contents are the mean of six determinations.

SDS-PAGE

SDS-PAGE was performed on a Protean-system (Bio-Rad, CA, USA) using 10-20% pre-cast gels (Bio-Rad). Gels were processed at 200 V, 20 mA for 50 min, stained by Coomassie Brilliant Blue over night, and then destained with 30% methanol and 10%

acetic acid in deionised water according to the instructions of the manufacturer. Gels were scanned using a Computing Densitometer (Molecular Dynamics, CA, USA).

RESULTS AND DISCUSSION

Enzymatic Degradability of Purified Proteins

Prior to studying the enzymatic extractability of the unheated and heat-treated SBMs, the degradability of the purified proteins was examined after heat treatment at comparable conditions. SDS-PAGE analysis (results not shown) revealed that the unheated, as well as the heat-treated, glycinin and β-conglycinin proteins were all degraded to fragments below the lower separation limit of the gel (approximately 10 kDa). Also, KSTI heat-treated at high humidity and low humidity and lectin heat-treated at high humidity were fully degraded (no bands detected). In contrast to this, the unheated KSTI and especially the unheated and low humidity heat-treated lectin were partially resisting enzymatic degradation (Figure 1A-C).

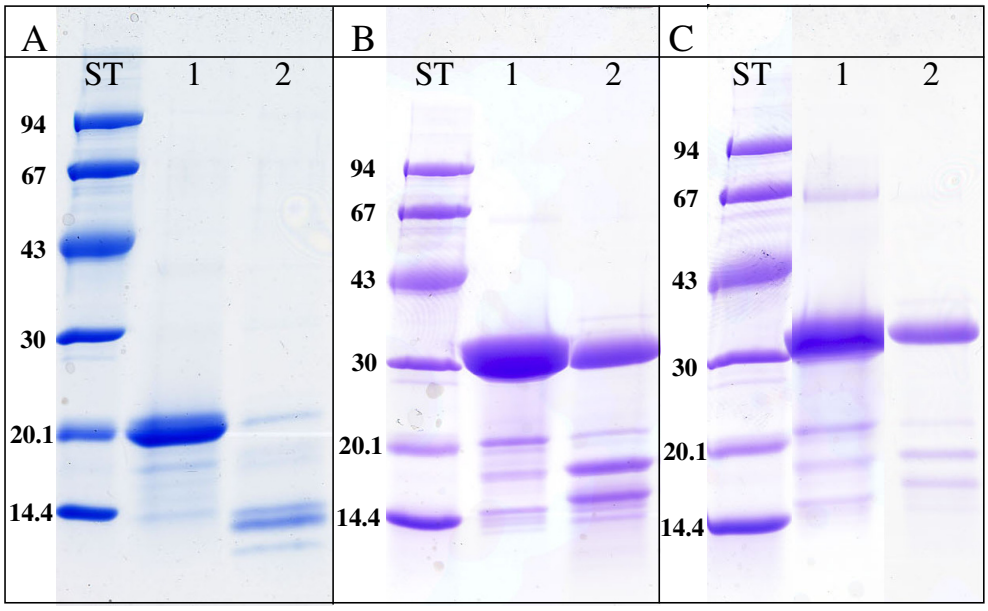

Figure 1. SDS-PAGE gels showing native KSTI (A), unheated lectin (B), and low humidity heat-treated lectin (C). The contents of the lanes are: ST, molecular weight standard (94, 67, 43, 30, 20.1, and 14.4 kDa). 1, protein solution before hydrolysis; 2. enzymatic hydrolysate (without separation).

The observed resistance of these two proteins is supported by Hessing *et al.* (*17*) and by Armour *et al.* (*28*) who found that KSTI and lectin activity was resisting proteolytic degradation, especially after heat treatment at dry conditions.

The results show that all major soy proteins heat-treated at high humidity were well degraded after incubation with the Alcalase and Flavourzyme protease mixture. The observed resistance by the unheated and low humidity heat-treated KSTI and lectin proteins indicate that a complete enzymatic extraction of these proteins during proteolytic treatment of unheated and low humidity heat-treated SBMs may be difficult to obtain.

Efficiency of Different Enzyme Combinations for Extraction of SBMs

Selection of Enzyme Combination. To establish the hydrolysis conditions for optimal protein extraction the NN meal was hydrolyzed by different combinations of protease and carbohydrase preparations. The results are presented in Table 1.

Table 1. Effects of different combinations of proteases and carbohydrases on the extractability of dry matter (EDM), protein (EP) and carbohydrates (EC)

enzymes[a]		EDM[b]	EP[c]	EC[d]
AF	-	67	83	44
AF	AF	77	95	49
AF	AFEB	82	96	56
AFEB	-	74	83	64
AFEB	AF	86	96	74
AFEB	AFEB	86	96	75

[a] *Enzymes: AF: Alcalase + Flavourzyme; AFEB: Alcalase + Flavourzyme + Energex + Biofeed Plus. All data are based on double determinations*
[b] *Expressed as gram dry matter extracted per 100 g SBM*
[c] *Calculated from protein content (N x 6.25). Expressed as % extracted of total protein present in SBM*
[d] *Calculated from sugar content. Expressed as % extracted of total carbohydrates present in SBM*

The extraction of protein (EP) after the first hydrolysis step reached 83% both with (AFEB) and without carbohydrases (AF). After the repeated hydrolysis EP reached 95 - 96%, independent of the combination of enzymes used, indicating that the majority of the initial protein could be extracted without the need for a carbohydrase enzyme preparation.

The extraction of carbohydrates (EC) was 44% for the AF treatment. The AF-AF combination gave an EC of 49% and 56% for AF-AFEB. With the use of proteases and carbohydrases (AFEB) the EC reached 64%. The AFEB-AF and the AFEB-AFEB combinations both resulted in an EC of 74 - 75%. The difference in the final extraction between the AF-AF and AF-AFEB combinations and the AFEB-AF and AFEB-AFEB combinations shows that proteases and carbohydrases are needed together (the AFEB combination) in the first round of the hydrolysis to obtain a high extraction of carbohydrates after the repeated hydrolysis.

The highest extraction of protein and carbohydrates, and therefore the most resistant residue, was obtained with the AFEB-AFEB enzyme combination. This combination was selected as the standard hydrolysis procedure for the subsequent experiments.

Enzymatic Extraction of Heat-Treated SBMs. To differentiate between aqueous and enzymatic extraction, the effects of the different heat treatments on the water extractability of proteins and carbohydrates prior to enzymatic treatment was determined (Table 2). The protein of USBM (67%) was the most water-extractable followed by SBM-H (30%), NN (20%) and SBM-L (13%). The results show that, compared to USBM, any kind of heat treatment is unfavorable for the extractability of protein from the SBMs and, moreover, that the heat induced reduction of extractability varies with the presence or absence of water during heat treatment (*9*, *10*, *29*). The unheated material was, not surprisingly, the most extractable because the proteins are in a condition free from thermally induced aggregation or cross-linking. It has previously been shown that heat treatment of USBM sharply decreases nitrogen extractability (*30*). Also, the extractability of carbohydrates from the SBMs in water varied with the heat treatment conditions. About 40% of the carbohydrates were extracted from the unheated material (USBM). The extractability was reduced to 20% by heat treatment at low humidity (SBM-L) but was increased to 51% when heat treatment was done at high humidity (SBM-H). Most likely, pectic structures were extracted by the autoclavation (*31*).

Independent of the heat treatment, the enzymes were very effective for extraction of proteins from all meals (Table 2).

Table 2. Extraction of dry matter (EDM), protein (EP) and carbohydrates (EC) from the unheated and heated soybean meals before enzymatic treatment (in water) and after enzymatic treatment with Alcalase, Flavourzyme, Energex and Biofeed Plus

	before enzymes			after enzymes		
	EDM[a]	EP[b]	EC[c]	EDM[a]	EP[b]	EC[c]
NN	38	20	34	88	92	81
USBM	60	67	40	85	89	76
SBM-H	49	30	51	90	94	85
SBM-L	33	13	20	82	89	78

[a] *Expressed as gram dry matter extracted per100 g SBM. All data are based on double determinations*
[b] *Calculated from protein content (N x 6.25). Expressed as % extracted of total protein present in SBM*
[c] *Calculated from sugar content. Expressed as % extracted of total carbohydrates present in SBM*

The EP of USBM increased from the 67% extracted in water to 89% after enzymatic treatment. For the heat-treated meals the EP increased from 30% to 94% for SBM-H and from 13% to 89% for SBM-L. The EP of NN increased from 20% to 92%. The extraordinary high EP found for SBM-H and NN indicate that the wet autoclavation affected the enzymatic extractability of these substrates positively.

The extractability of carbohydrates from the USBM increased from 40% in water to 76% after enzymatic treatment. Before enzymatic treatment the heat treatment at low humidity (SBM-L) reduced the EC by 50% (compared to USBM) but after enzymatic treatment the ECs of USBM and SBM-L were comparable (76% vs 78%). Also, the EC of NN and SBM-H were comparable (81% vs 85%) showing the positive effect of high humidity heat treatment on enzymatic extractability.

A comparison of USBM and SBM-L shows that none of the proteins, which became unextractable in water upon heat treatment at low humidity, remained enzyme unextracable. This means that, although dry heat treatment reduces aqueous EP strongly it does not make the proteins less susceptible to enzymatic extraction, as the protein of both of these meals was 89% extractable. SBM-H and NN shared the highest enzymatic protein extractability (92% and 94%). Both substrates were heat processed at relatively high humidity, which indicates that this condition renders the meals more susceptible to enzymatic degradation, as previously suggested (*1*, *30*). Compared to the three other meals, the larger particle size of NN (pellets) may have reduced the extraction of protein and carbohydrates in water prior to enzymatic treatment. However, the data indicate that

the difference in particle size did not prevent enzymatic extraction of proteins from this meal. This observation confirms the efficiency of the enzyme combination for protein extraction.

Heat Denaturation and Enzymatic Extraction

The extent of protein denaturation of the meals after heat treatment was monitored by DSC. The DSC thermograms of the four SBMs are shown in Figure 2.

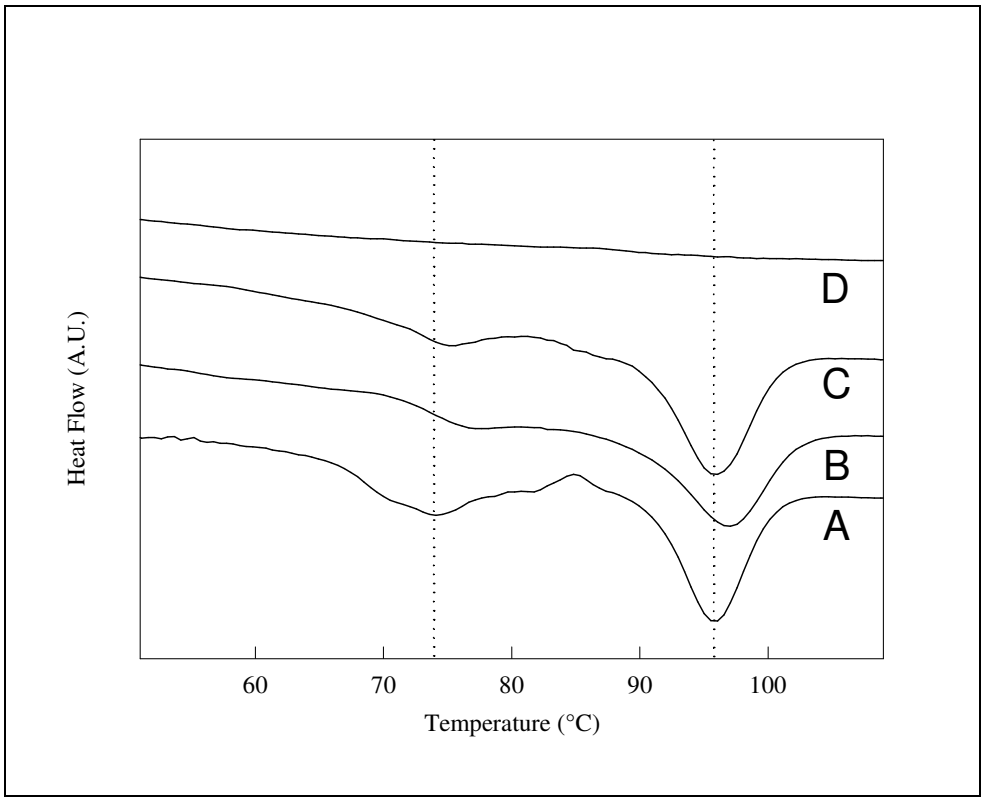

Figure 2. DSC-thermograms of soybean meal suspensions (50 mg protein / mL) in 35 mM potassium phosphate buffer containing 0.1 M NaCl (pH 7.6), I = 0.2 M. A, USBM; B, NN; C, SBM-L; D, SBM-H. Scanning rate was 1.2 K min^{-1}.

The curve of USBM shows two endothermic transitions with peak temperatures (Tp) of 74 °C and 95 °C corresponding to denaturation of the major proteins, 7S β-conglycinin and 11S glycinin, respectively (*32*, *33*). Whereas the SBM-H (high humidity) resulted in complete denaturation of both 7S and 11S globulins, incomplete denaturation was found after low humidity heat treatment (SBM-L). Compared to that of USBM, the β-conglycinin peak area of NN and SBM-L were reduced by 79% and 86%, respectively. Compared to USBM the glycinin peak area of NN was reduced by 18%; the glycinin

peak area of SBM-L was unchanged after the heat treatment. The absence of transition peaks in SBM-H shows the efficient, irreversible protein unfolding occurring during high humidity heat treatment.

Comparison of the enzymatic extractability of the four meals presented in Table 2 and the denaturation state of the proteins suggests a correlation between the reduced enzymatic extractability of the proteins and the observed incomplete protein denaturation. The protein of the fully heat denatured meal (SBM-H) was the most enzyme extractable (94%) and the unheated USBM and the poorly denatured SBM-L meals were the least extractable (89%).

The previous results on purified proteins showed that they were fully degraded after appropriate heat treatment at high humidity. In contrast, even after heat processing which resulted in complete protein denaturation (SBM-H), a complete enzymatic extraction of the meal protein (94% $\rightarrow$ 100%) was never reached. Therefore, the composition of the enzyme unextractable residues was analysed in more detail.

Composition of Enzyme Unextractable Material

Protein. The amino acid composition of the two original meals and the obtained enzyme unextractable residues are presented in Table 3.

The residues contained 15 - 20% protein. The amino acid compositions of the meals were comparable to those reported in literature (*7*). Enzymatic extraction of NN caused an increase in the proportion of the hydrophobic amino acids (*34*) in the residue. Gly, Ala, Val, and Leu increased from 30% of total in the NN to 41% in the enzymatic residue. This change was accompanied by a decrease in the proportion of hydrophilic amino acids Lys, Arg, His (*34*) from 14% in NN to 10% in the residue.

The proportion of hydrophobic amino acids in the USBM increased from 30% in the SBM to 35% in the residue. This change was accompanied by a minor decrease in the proportion of the hydrophilic amino acids in the residue. For SBM-H the content of hydrophobic amino acids in the residue was 38%. The proportion of hydrophilic amino acids decreased insignificantly. The proportion of hydrophobic and hydrophilic amino acids for SBM-L was more or less unchanged.

Table 3. Protein content and amino acid composition (molar %) of the NN and USBM soybean meals (SBMs) and the four residues obtained after enzymatic extraction

	SBMs[a]		residues[a]				SPI[b]	
	NN	USBM	NN	USBM	SBM-H	SBM-L	av.	SD
Protein[c]	49.4	49.2	15.3	19.9	17.3	18.3	n.d.	n.d.
Ala	7.5	7.4	10.1	8.3	10.1	6.7	5.96	0.27
Arg	5.7	5.5	4.0	5.4	4.0	6.6	5.73	0.43
Asx[d]	10.1	10.8	7.5	9.4	8.1	7.8	10.96	0.44
Cys	0.9	0.8	1.5	0.4	0.5	1.0	1.00	0.14
Glx[e]	12.0	13.3	7.7	11.4	7.8	7.3	16.88	0.14
Gly	8.9	8.3	11.2	9.4	10.4	9.9	7.00	0.21
His	2.3	2.2	1.5	1.7	1.8	2.7	2.21	0.26
Ile	4.5	4.4	4.7	4.6	4.8	4.9	4.74	0.34
Leu	8.1	8.4	11.8	10.4	11.4	6.2	8.12	0.34
Lys	6.2	6.5	4.6	6.6	4.7	7.0	5.44	0.44
Met	0.6	0.5	0.6	0.6	1.0	1.6	1.38	0.22
Phe	4.4	4.3	4.7	4.4	4.5	6.1	4.40	0.26
Pro	6.9	6.4	7.4	6.5	9.6	6.6	6.42	0.51
Ser	8.3	7.8	7.8	7.2	7.1	9.2	6.47	0.26
Thr	5.1	4.8	5.3	4.6	4.1	6.8	4.17	0.17
Trp[f]	-	-	-	-	-	-	0.83	0.09
Tyr	2.7	2.7	2.3	2.4	2.7	3.3	2.95	0.29
Val	5.9	5.8	7.4	6.5	7.4	6.5	5.37	0.40

All data are based on six determinations
[a] *Standard deviations were generally between 1 and 5 %, except for Asx and Glx (3-10 %) and Met (10-20 %)*
[b] *Average of thirteen isolates (SPI), adapted from Henn and Netto, 1998 (7).*
[c] *Calculated from Kjeldahl N x 6.25. Expressed in % of dry matter*
[d] *The sum of asparagine + aspartic acid*
[e] *The sum of glutamine + glutamic acid*
[f] *Fully destroyed during hydrolysis*

Generally, the increased hydrophobic character of the enzymatic residues suggests that the residues were enriched in hydrophobic amino acids or hydrophobic peptides. Previous studies on the degradability of the purified proteins showed that KSTI and lectin might resist complete enzymatic degradation if they were not heat-treated at high humidity. A comparison of the amino acid composition of KSTI and lectin (Swissprot, Switzerland) to the amino acid composition of the residues did not indicate that the residues were enriched in either of these two proteins.

Carbohydrates. The carbohydrate composition of the meals and enzyme unextractable solids was analysed as seen in Table 4.

Table 4. Carbohydrate content and molar carbohydrate composition of soybean meals (SBMs) and the four enzyme unextractable residues (RES)

				Carbohydrates[d]							
sample	DM[a]	C[b] (%)	C[c] (g)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
SBMs											
NN	100.0	29.4	29.4	3 (0.8)	0 (0.1)	7 (1.8)	8 (1.8)	4 (1.1)	22 (6.5)	43 (12.8)	13 (4.4)
USBM	100.0	25.6	25.6	2 (0.5)	1 (0.2)	8 (1.0)	6 (1.3)	3 (1.1)	31 (8.2)	31 (8.3)	18 (5.1)
RES											
NN	12.4	44.0	5.5	3 (0.1)	0 (0.0)	6 (0.3)	16 (0.7)	2 (0.1)	3 (0.2)	61 (3.4)	9 (0.6)
USBM	15.5	40.9	6.3	3 (0.2)	3 (0.2)	7 (0.4)	11 (0.6)	2 (0.2)	7 (0.5)	52 (3.4)	15 (1.1)
SBM-H	10.0	38.0	3.8	3 (0.1)	0 (0.0)	4 (0.1)	9 (0.3)	3 (0.1)	4 (0.1)	64 (2.5)	12 (0.5)
SBM-L	18.0	33.0	5.9	3 (0.2)	2 (0.1)	7 (0.3)	11 (0.5)	2 (0.1)	8 (0.5)	51 (3.0)	16 (1.1)

All data are based on triple determinations
[a] *Dry matter (expressed in grams of 100 g soybean meal)*
[b] *Carbohydrate content (expressed in % w/w of DM). Determined as the sum of neutral sugars + uronic acids*
[c] *Carbohydrate content (expressed in grams)*
[d] *Composition of carbohydrates, shown as molar % (yield in grams /100 g meal is shown in parentheses)*

The meals were generally rich in Glc, Gal, UA, Ara and contained low levels of Man, Rha and Fuc. Compared to NN, USBM had 25% more uronic acid, 30% more Gal and 25% less Glc. The enzymatic treatment of the meals extracted a large proportion of the carbohydrates. Compared to that of the meals the absolute carbohydrate content of the residues was reduced by 75 - 85%. After enzymatic extraction, Glc was the most abundant carbohydrate constituent in the enzymatic residues (51% to 64%), followed by UA and Xyl. The level of Gal and Ara was low in all residues showing that arabinans and arabinogalactans were well extracted. Little change was detected for the Rha, Fuc, and Man content of the meals and residues.

The residues of NN and SBM-H had the highest proportion of Glc. The proportion of cellulosic and non-cellulosic glucose was determined for the meal and enzymatic residue of NN. Approximately 36% of the Glc in the meal originated from cellulose (*4*) and more than 90% of the Glc in the residue was cellulose showing that an estimated, 20% of the initial cellulose had been extracted during the enzymatic treatment. The results

show that the cellulose was only extracted to a minor extent by the cellulases of the two carbohydrase preparations.

In conclusion, the enzymatic residues can be divided into two groups: '*high humidity*' and '*unheated + low humidity*', based on the conditions for heat treatment. High-humidity heat treatment leads to a more effective enzymatic extraction. However, in all cases the protein extraction from the meals was incomplete and, taking into account the results of the purified proteins, it is clear that the soybean meal matrix affects the enzymatic extraction of protein from the meals. We speculate that interactions between protein and other components e.g. the cellulose, which a major component of the residues) may explain the incomplete enzymatic extractability of protein from the meals. This is a topic of future investigation.

ACKNOWLEDGEMENTS

This research was financially supported by Danish Academy of Technical Sciences, (Lyngby, Denmark). Gerrit van Koningsveld is thanked for helpful suggestions to the manuscript. Jolan de Groot and Jan Cozijnzen are thanked for help with protein purification and carbohydrate analysis. Ina Nørgaard from Novozymes A/S is thanked for skillful assistance with the amino acid analysis.

ABBREVIATIONS USED

A, Alcalase; ANFs, antinutritional factors; B, Biofeed Plus; DM, dry matter; DSC, differential scanning calorimetry; E, Energex; F, Flavourzyme; KSTI, Kunitz trypsin inhibitor; SBM, soybean meal; SBM-H, soybean meal heat-treated at high humidity; SBM-L, soybean meal heat-treated at low humidity; EC, extraction of carbohydrate; EDM, extraction of dry matter; EP, extraction of protein; Tp, transition temperature; USBM, unheated soybean meal

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CHAPTER 3

OPTIMISING CONDITIONS FOR HYDROLYSIS OF SOYBEAN MEAL PROTEINS WITH EXO- AND ENDO-PROTEINASE PREPARATIONS ♣

♣ Fischer,M.; Voragen,A.G.J.; Kofod,L.V.; de Rooij,G.; Gruppen,H. Optimising conditions for hydrolysis of soybean meal proteins with exo- and endo-proteinase preparations. *Submitted* **2006**.

ABSTRACT

With the purpose of obtaining a high solubilisation of protein and/or high degree of hydrolysis from soybean meal by two commercial protease preparations the effects of time, pH and dose of the two enzyme products were studied. Protein solubilisation was favored by a relatively high pH (7.5) reflecting the pH optimum of the endo-proteinase and emphasizing the importance of this type of enzyme to reach a final high level of protein solubilisation. Experiments indicated that the efficacy of the hydrolysis is to some extent negatively affected by the degradation products of the proteins. The ratio of the endo/exo proteases in mixture was found to be an important parameter, which could affect both protein solubilisation and degree of hydrolysis in both negative and positive direction. A combined objective of high protein solubilisation and high degree of hydrolysis in a single hydrolysis product may be conflicting.

Finally, two models were developed in order to predict protein solubilisation and degree of hydrolysis as function of dose of the proteases. The protein solubilisation model was tested and results indicated a slight improvement of solubilisation using the prediction of the model although further optimization is needed. Models of this kind could have a potential in commercial production of enzyme hydrolysed vegetable proteins to control development of protein solubilisation or degree of hydrolysis depending on the objective of the hydrolysis.

Keywords: degree of hydrolysis; endo-proteinase; exo-proteinase, protein; solubilisation, soybean meal,

INTRODUCTION

Soybean is a rich source of protein and is, therefore, widely used as an ingredient in foods and as a protein source for animal feed. Appropriate processing of the soybean to produce soybean meal (SBM) is crucial for the final quality and nutritional value of soy proteins. Different heat treatments may explain differences in protein solubility of SBMs, but also differences in processing procedures for dehulling and flaking conditions are known to affect the solubility of the proteins and their enzymatic degradability. Toasting of soybean meal results in completely different protein properties compared to pelleting and extrusion (*1*, *2*, *3*, *4*, *5*). The Protein Dispersibility Index (PDI) is often used to determine the solubility of vegetable protein preparations (*6*, *7*, *8*). The PDI for raw materials is generally between 70 and 90% and decreases generally to about 50% after 20 min at 85°C (*2*).

The *in vitro* degradability and *in vivo* digestibility of processed soy proteins is increased by enzymes. Enzymes have multiple advantages compared to other agents including a high specificity, effectiveness at low concentrations and under mild conditions. In addition, being in general considered as safe, it is not necessary to remove the enzymes from the finished products (*9*, *10*).

The solubilisation of protein from defatted SBM by enzymes is generally between 84% and 90% (*11*). Of the different soy proteins β-conglycinin is in generally readily degraded enzymatically, whereas glycinin shows more resistance to proteolysis (*3*, *12*). The basic polypeptides of glycinin are less readily degraded than the acidic polypeptides (*3*). The relatively high resistance of the basic polypeptides against proteolytic attack is possibly due to the tendency of these polypeptides to from large insoluble complexes (*13*) that render them less prone to enzymatic hydrolysis (*14*).

Among the protease preparations that are commonly used for enzymatic hydrolysis of vegetable proteins are Alcalase and Flavourzyme (*15*, *16*). Although Alcalase, derived from *Bacillus sp.*, is most active at slight alkaline pH, the enzyme remains fairly active down to ~pH 5. Most food/feed systems, including soybean meals, have a neutral or slightly acidic pH. In most of these systems Alcalase still has an activity of 80% or higher (*17*). Flavourzyme is derived from *Aspergillus oryzae* (*15*) and has a pH optimum of about 5-7 (*9*), hence a high activity in most food/feed systems.

The effects of Alcalase and Flavourzyme on protein solubilisation from SBMs have been previously subject of study (*18*). The initial pH of hydrolysis was pH 7 but during

enzymatic processing the pH was allowed to float freely resulting in a gradual pH decrease to about pH 6.5. Upon incubation, the protein solubilisation reached 83% of the total protein present in the meal. By reincubating the residue obtained from the first hydrolysis with fresh enzymes the total protein solubilisation reached 95%. The results indicated an inhibition or inactivation of protease activity and it was hypothesized that components inherently present in the meal or proteolytic degradation products of the SBM protein were affecting the activity of the proteases (*18*).

Since enzymatic solubilisation of protein from SBM is influenced by several parameters, including quality of the substrate, choice of enzymes, doses and ratios of the enzyme products and various other conditions such as the pH applied in the process it appears that a one-fits- all solution is improbable. In addition, the economical perspective (cost-benefit) of the process is often limiting to the amount of enzymes which can be used in industrial protein hydrolysis.

Our specific objectives were to enhance the solubilisation of protein from SBM using two commercial protease preparations and to determine the effects of time, pH and enzyme doses on the efficacy of the solubilisation and degree of hydrolysis in a single step hydrolysis of SBM protein using Alcalase and Flavourzyme.

MATERIALS

Substrates. The soybean meal used was a pelletised SBM processed in a local pilot plant. The meal was conditioned at 68°C and pelletised at about 85 °C for 10 min. The meal contained 42.7% crude protein (N x 6.25).

A casein isolate (Merck, Germany) containing 89.2% crude protein according to the manufacturer was used for protease inhibition studies.

Enzymes. Two commercial protease preparations were selected for the experiments. Alcalase Food grade (Alcalase) is a protease preparation from *Bacillus licheniformis* composed of mainly endoproteinases. Its main component is Subtilisin A. The product has an activity optimum from pH 6.5–8.5. Flavourzyme (Flavourzyme) is a protease complex derived from *Aspergillus oryzae*. Flavourzyme contains several proteolytic ac-

tivities, mainly endoproteinases and exopeptidases and has an activity optimum from pH 5−7 (*18*). Both enzymes were from Novozymes A/S (Bagsvaerd, Denmark).

METHODS

Protein Hydrolysis

The efficacy of protein solubilisation from the SBM by Alcalase and Flavourzyme was optimized in a series of experiments. The first experiments were conducted on a relatively large scale. Later experiments were downscaled to ease sample handling.
Chloramphenicol (0.3 mM) was added to all SBM preparations to reduce the influence of proteolytic activities originating from microbial contaminants in the meals.

General Hydrolysis Procedure. Unless stated otherwise, SBM was suspended in water in a ratio of 1:9 (w/w). After stirring for 1h the pH was adjusted to 7.0, which is about the natural pH of the soybean meal suspended in water. A representative sample was taken while stirring. The suspension was transferred to a water bath (40°C) to equilibrate for 30 min before enzymes were added (pH was still ~7). Different doses of the two enzyme preparations were applied in separate experiments. Enzymes dosages were calculated as percentage of enzyme solution relative to approximate amount of protein in the samples (v/w). Different enzyme/substrate combinations were used (details under results). After enzyme addition the hydrolysis proceeded for 16h with constant stirring (300 rpm). When the incubation was completed the pH was recorded and all samples were adjusted back to pH 7 prior to separation by centrifugation (8000g; 4°C; 25 minutes). Supernatants were stored at -20°C and residues were washed with Millipore water in a ratio of 1:9 (w/w). Residues were recovered by centrifugation (8000g; 4°C; 25 minutes) and were freeze-dried. The protein content of the residues and the degree of hydrolysis of the solubilised proteins were subsequently determined (see analyses).

Time Optimization. To optimize the incubation time, 50 g of SBM in 450 g water was treated according to *General Hydrolysis Procedure* using 2.5% Alcalase and 5% Flavourzyme. After enzyme addition aliquots (10 mL) were taken from the suspension at 10, 20, 30, 40, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, and 1200 min. Immedi-

ately after sampling, the enzymes in the aliquots were inactivated by heat treatment (90°C; 15 min) This is sufficient to inactivate proteolytic activities in the protease preparations (*19*, *20*). Further separation of the samples by centrifugation and washing of the residues were as described under *General Hydrolysis Procedure*. Experiments were conducted in duplicate.

pH Optimization. The effect of fluctuating pH around neutral (pH 7) was studied for the narrow range between 6.5 and 7.5. SBM (10g) was suspended in 90 mL MilliQ water. The pH was adjusted using 1M $NaHCO_3$. After adjustment the pH remained constant within 0.1 pH unit over the entire incubation period. The pH was adjusted to 7 prior to separation of the soluble material fraction from the solid particles by centrifugation. Further treatment of the samples was as described under *General Hydrolysis Procedure* and protein solubilisation and degree of hydrolysis was determined. All pH experiments were conducted in triplicate.

*Sequential Hydrolysis***:** To study the stability of protease activities the effect of a standard hydrolysis of the SBM by Alcalase and Flavourzyme was compared to a sequential hydrolysis procedure. For the "*standard"* treatment SBM (50 g) was suspended in 450 g water and enzymes (2.5% Alcalase and 5% Flavourzyme) were added. The conditions for the "*sequential*" treatment were as "*standard*" during the first 10h but with another (equal) dose of the two enzymes added at 10h. The start pH for hydrolysis was 7 and hydrolysis and separation was performed essentially as described under *General Hydrolysis Procedure.* Aliquots were collected at 10 and 20 h. Experiment was conducted in triplicate.

Effect of Soluble Peptides on Efficacy of Protein Solubilisation: The effect of SBM degradation products released into the supernatant by the two protease preparations on protease efficacy was studied. Casein isolate, generally known as a readily hydrolysable substrate (*21*), and SBM were used as substrates. A supernatant produced according to *General Hydrolysis Procedure* (2.5% Alcalase and 5% Flavourzyme) was isolated by centrifugation and inactivated by heat treatment (90°C; 15 min) as previously described. Cooled supernatant or water (45 mL) was mixed with either casein (5 g) or SBM (5 g) before Alcalase (2.5%) and Flavourzyme (5%) were added to the samples. The start pH

for hydrolysis was 7 and hydrolysis and separation was performed essentially as described under *General Hydrolysis Procedure*. Aliquots were collected at 10 and 20 h. Experiment was conducted in triplicate.

Optimising Enzyme/Substrate Ratio. Different ratios of concentrations of Alcalase and Flavourzyme were tested in order to build a statistical model that can be used for predicting the enzyme dose combination that will result in the highest possible solubilisation of protein. The experiment was performed as a statistical response surface design using SAS-JMP (SAS Institute, Heidelberg, Germany). 10 enzyme treatments were defined according to the following principle:
The specific type of response surface design used is called a two-factor central composite design. Central composite designs are intended to fit full quadratic models. They place their trials at corners, the center point, and on a sphere enclosing the cube above the centers of faces (*22*). Figure 1 shows the central composite design for two experimental factors.

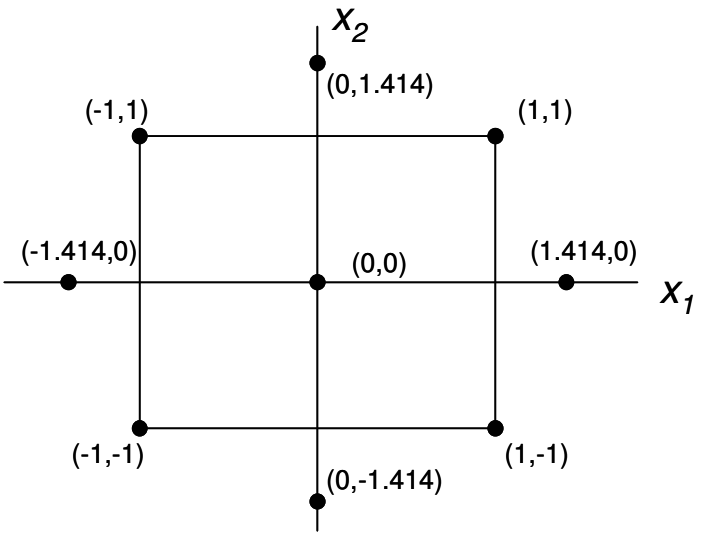

Figure 1. Central composite design in two factors.

The enzyme concentration (2.5% Alcalase and 5% Flavourzyme), assumed to give the highest solubilisation of protein, is coded as (1,1), and the lowest concentration for both enzymes is zero which is coded -1.414.

The central composite design determines which specific enzyme dose combinations should be made in order to build a second-order model of the data after hydrolysis. En-

zymes are supposedly more effective at relatively high dose and consequently a higher importance was given to the lower enzyme/substrate ratios. Therefore, a relatively high number of occurrences for ratios below average are seen in Table 1. In addition to the ten samples required for this design four samples representing ”extreme” values were produced. These additional samples are placed in two of the four extreme corners of the design, i.e. in (-1.414,-1.414) and (1.414,1.414).

Table 1. Design with translation between coded variable and enzyme doses (%) together with number of occurrences for each experimental condition

Coded Variables	Alcalase	Flavourzyme	Number of occurences
(-1.414,0)	0.0	2.93	1
(1.414,0)	2.93	2.93	1
(-1,1)	0.43	0.86	1
(0,-1.414)	1.46	0.0	1
(1,1)	2.50	5.0	1
(0,0)	1.46	2.93	2
(0,1.414)	1.46	5.86	1
(1,-1)	2.50	0.86	1
(-1,1)	0.43	5.0	1
(-1.414, -1.414)	0.0	0.0	2
(1.414,1.414)	2.93	5.86	2

Using the enzymes doses the SBM was enzymatically processed essentially according to *General Hydrolysis Procedure* using 10 g of SBM in 90 g water. After incubation further treatment of the samples proceeded as described under *General Hydrolysis Procedure* and protein solubilisation and DH in the samples were determined.

The results from this experiment were used in an attempt to develop two models, one to predict protein solubilisation and one to predict degree of hydrolysis, respectively, as a function of the dose of the two protease preparations. The “optimal” dose combination of Alcalase and Flavourzyme for protein solubilisation predicted by the protein solubilisation model was finally tested in an experiment essentially as described under *Time Optimization* and under *General Hydrolysis Procedure*.

ANALYSES

Dry Matter. Dry matter was defined as the weight of meals and residues immediately after freeze-drying. The solubilisation of dry matter by the different treatment was calculated as: $(DM_{total} - DM_{residue}) / DM_{total}$ x 100%.

Protein Determination. The protein content of SBM (N x 6.25) and casein (N x 6.38) and of the different enzymatic residues was determined by a semi-automated Kjeldahl method (*23*). Dry samples (100 mg) were destructed in concentrated sulfuric acid at 200-385 °C. The released NH_3 was determined with an ammonia-hypochloritesalicylate reaction on a Skalar 5101 autoanalyzer (Skalar, Breda, The Netherlands). The solubilisation of protein by the different treatments was calculated as: $(P_{total} - P_{residue}) / P_{total}$ x 100%.

The protein contents determined have a standard deviation of about 0.2%, which should be considered during interpretation of the results. Protein contents are the means of three determinations.

Degree of Hydrolysis. The degree of hydrolysis (DH) of the protein hydrolysates in different samples was determined using an semi-automated micro titer plate based colorimetric OPA method (*24*). The OPA reagent was prepared as follows: 7.620 g di-sodium tetraborate decahydrate and 200 mg sodium dodecyl sulphate (SDS) were dissolved in 150 mL deionised water. 160 mg *o*-phthaldialdehyde 97% (OPA) was dissolved in 4 mL ethanol. The OPA solution was transferred quantitatively to the above-mentioned solution. 176 mg dithiothreitol 99% (DTT) was added to the solution that was made up to 200 mL with deionised water. A serine standard (0.9516 meqv/L) was prepared by solubilizing 50 mg serine (Merck, Germany) in 500 mL deionized water.

The sample solution was prepared by diluting each sample to an absorbance (280 nm) of about 0.5. Generally, supernatants were diluted (100 ×) using an automated Tecan dilution station (Männedorf, Schwitzerland). All other spectrophotometer readings were performed at 340 nm using deionized water as the control. Aliquots (25 µL), were dispensed into a micro titer plate. The micro titer plate was inserted into an iEMS MF reader (Labsystems, Finland) and 200µL of OPA reagent was automatically dispensed. Plates were shaken (2 min; 700 rpm) before measuring absorbance at 340 nm. Finally,

the DH (total number of peptide bonds h_{tot}=7.8 (*17*)) was calculated using L-serine for the standard curve (*25*). Eightfold determination of all samples was carried out.

RESULTS AND DISCUSSION

Effects of Time

The time course of solubilisation of protein, dry matter and degree of hydrolysis is presented in Figure 2.

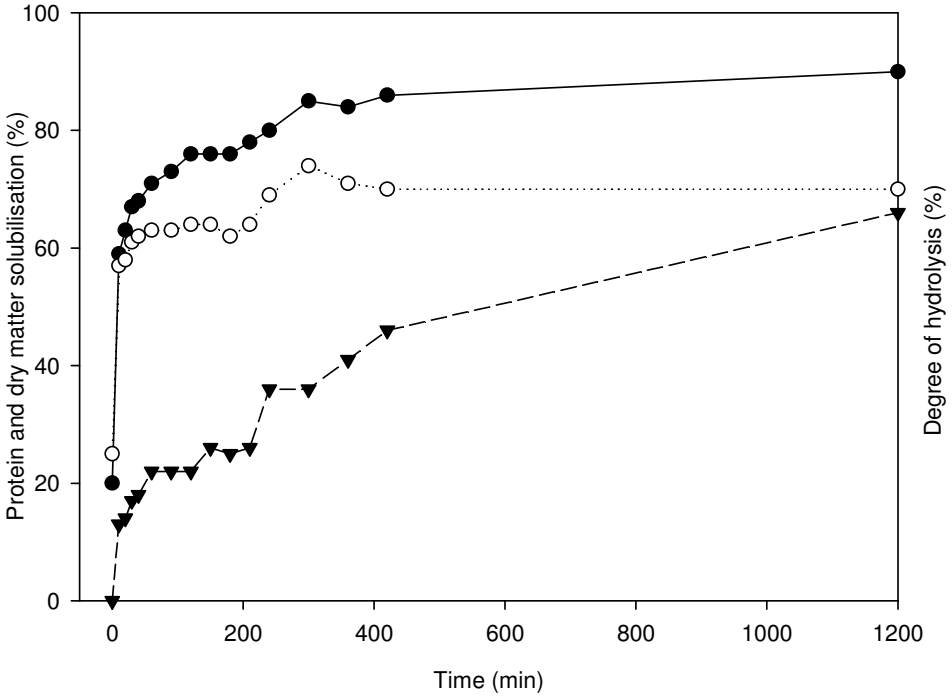

Figure 2. Solubilisation of protein (—●—), dry matter (···○···) and development of DH (−▼−) plotted against time (n=2). Enzyme dose, 2.5% Alcalase and 5% Flavourzyme.

The figure shows that enzymatic solubilisation of the protein progresses rapidly during the first hours of the hydrolysis. At the start of the hydrolysis ~20% of the protein in the SBM was water soluble, whereas 10 min after addition of the proteases 59% of protein was soluble and after 60 min solubilisation exceeded 70%. At 240 min the solubilisation had reached 80% increasing to 86% at 420 min. Maximum solubilisation of protein (90%) was reached at 1200 min, indicating that proteases were still active at the end of the experiment. However, between 420 and 1200 min only a limited amount (4%) of protein was solubilised. A decrease of pH (results not shown) from pH 6.4 to 6.1 was

observed between 800 and 1200 min. This pH decrease could partly be explained by the increased DH.

In contrast to the rapid solubilisation of protein from the beginning the degree of hydrolysis increased at a more moderate rate. In the time span from 10 min to 1200 min DH increased steadily from DH 13 to 66 showing that development of very high DH in protein hydrolysates is a relatively time demanding process.

From the data it is obvious that the rate of proteolytic solubilisation of protein proceeds very fast in the early phase of the incubation but becomes steadily slower over time. This could indicate either a gradual inhibition or inactivation of proteolytic activities by degradation products released over time or/and that the remaining fraction of insoluble SBM protein bebecomes increasingly more difficult to access for the proteases (*3*, *18*, *26*). Due to the increasing risk of microbial contamination over time it was not considered relevant to increase the incubation time further. Therefore, subsequent experiments were focused on the effects on protein solubilisation and degree of hydrolysis by higher enzyme dose and optimized pH conditions to better match the pH optima of the different activities of the protease preparations.

Optimization of pH

A study was conducted to compare the influence of pH on the protein solubilising effect of Alcalase and Flavourzyme in the narrow pH range between 6.5 and 7.5. The results are presented in Table 2.

Table 2. Effects of pH on protein solubilisation and DH

pH	protein sol. (av. ± SD)	DH (%) (av. ± SD)
6.5	91.0 ± 1.0	68.0 ± 4.6
7.0	91.7 ± 0.7	70.0 ± 4.6
7.5	93.4 ± 0.5	55.7 ± 4.9

Protein sol = protein solubilisation; DH (%) = degree of hydrolysis. The pH was kept constant within 0.1 pH unit over the entire incubation and was adjusted to 7.0 prior to centrifugation.

Following the incubation the differences in protein solubilisation between the different pH values were minor. The highest protein solubilisation (93.4%) was reached after 20 h at pH 7.5, whereas protein solubilisation was lower (91.1 and 91.7%) for pH 6.5 and 7.0, respectively. Probably the differences observed can be ascribed to the pH activity optimum (pH 7.5 to 8) of Alcalase, the endo-proteinase, making this protease increasingly more effective for protein solubilisation with increasing pH (*27*).

In an earlier publication (*18*), an extent of protein solubilisation of about 95% was obtained at comparable levels of Alcalase (2.5%) and Flavourzyme (5.0%). To reach such high protein solubilisation level, however, a repeated incubation of the SBM with proteases at pH 7 was necessary. The results of the present manuscript show the positive effect on protein solubilisation by performing hydrolysis at constant pH since protein solubilisation was higher (about 6 – 8%) for all pH values (6.5 – 7.5) compared to hydrolysis at less controlled pH conditions (start pH 7 → end pH 6.5) (*18*).

As regards DH there was no significant difference between the levels (about 68-70%) reached for pH 6.5 and pH 7.0, respectively. A significantly lower DH (56%) was, however, observed at pH 7.5. The results probably reflect the pH activity optimum (pH 5-7) of Flavourzyme leading to a significantly lower activity of Flavourzyme at pH 7.5 compared to pH 7.0 and 6.5. The decrease in protein solubilisation at lower incubation pH (but at high DH) could also indicate that a high DH may be unfavourable to obtain high protein solubilisation because the solubilisation is counteracted by a precipitation of aggregated peptides (*16*, *28*, *29*, *30*).

Sequential Hydrolysis

The stability of Alcalase and Flavourzyme activity was studied by comparing the effect of a standard hydrolysis to a sequential hydrolysis for which fresh enzymes were added at 10h. The results are presented in Table 3.

Table 3. Protein solubilisation and DH for two conditions of hydrolysis of SBM by Alcalase and Flavourzyme

	10 h		20 h	
treatment	protein sol. (av. ± SD)	DH (%)	protein sol. (av. ± SD)	DH (%)
"standard"	83.0 ± 0.2	51	89.4 ± 0.1	68
"sequential"	83.0 ± 0.1	52	89.0 ± 0.5	75

Protein sol = protein solubilisation; DH (%) = degree of hydrolysis.
Sample pH: start, pH 7.0; final, pH ~6.5.

No significant differences (89.4 vs. 89.0%) in protein solubilisation was observed between the two treatments at the endpoint (20 h) of the incubation. Accordingly there was no additional protein solubilising effect of adding fresh enzymes at 10h (sequential). The conditions for the first 10 h of incubation are identical for the treatments, which are also reflected in the protein solubilisation level (83%) at this time.

While having no effect on protein solubilisation the sequential treatment resulted in a DH increase of about 7% (from 68 – 75%). This is higher than the DH (66%) obtained in Figure 2 and, since protein solubilisation was not affected by the additional dose of the enzymes, it is speculated that the increase is primarily due to the addition of fresh exo-acting proteolytic activities from Flavourzyme.

Even with a double dose of proteases, of which half the amount was added after 10h to boost the reaction, the protein solubilisation in the present experiment was not increased to a higher level. This might indicate that protease activities are to some extent affected by degradation products of the intact soy proteins which are brought into solution by means of the action of Alcalase and Flavourzyme (*18*). This hypothesis was studied further

Effect of Soluble Peptides on Efficiency of Protein Solubilisation

The effects of the addition of soluble peptidesderived from one of the previous trials (2.5% Alcalase and 5% Flavourzyme) on the enzymatic solubilisation of protein from SBM and a casein isolate was studied. The results are presented in Table 4.

Table 4. Development of protein solubilisation and DH during hydrolysis of SBM and casein isolate by Alcalase and Flavourzyme in water and in supernatant (containing soluble SBM peptides)

conditions	10h	20 h	
	protein sol. (av. ± SD)	protein sol. (av. ± SD)	DH (%)
Casein + supernatant	95.2 ± 1.1	91.8 ± 0.8	54
Casein + water	97.7 ± 0.4	96.5 ± 0.4	47
SBM + supernatant	84.0 ± 0.2	92.9 ± 0.2	68
SBM + water	86.4 ± 0.1	93.5 ± 0.1	66

Protein sol = protein solubilisation; DH (%) = degree of hydrolysis.
Protein numbers are corrected for the contribution of protein from the supernatant.

After 20 h incubation the highest solubilisation of casein (96.5%) was seen for the casein + water treatment. With a protein solubilisation of 91.8% a negative effect (4.7%) was seen when casein was hydrolysed in the presence of soluble SBM peptides (supernatant). Also, at 10 h this effect is seen (decrease from 97.7 to 95.2%). A higher protein solubilisation level from the casein isolate at 10 h compared to 20 h show that this substrate is readily hydrolysed and that the reaction can be optimized by reducing the reaction time. This decrease could be explained by increasing aggregation of peptides between 10 and 20 h (*31*, *32*).

Also for the SBM differences in protein solubilisation were seen between the SBM + water and SBM + soluble SBM peptides treatments. At 10 h, protein solubilisation was 86.4 and 84% for the two treatments, respectively. After 20 h, however, protein solubilisation had increased to 93.5% for the water treatment and 92.9% for the soluble SBM peptides treatment. The final levels of protein solubilisation in this experiment were unusually high for the SBM hydrolysis. We have no obvious explanation for this. The results show that enzymatic solubilisation of SBM protein in water progresses at lower rate (86.4%) compared to casein (97.7%) after 10 h. The same is true for the treatment in the presence of soluble SBM peptides (84 vs. 95.2%). After 20 h in water, however,

the final difference in solubilisation between the two substrates was only 3% (casein 96.5% vs. SBM 93.5%).

From this experiment it can be concluded that the solubilisation of protein (20 h) from casein isolate as well as SBM is to some extent negatively affected by the soluble SBM peptides present in the supernatant. However, the earlier results on the effects of pH indicated that this effect may be less pronounced or can be compensated for when pH is set and held at 7.5.

Prediction of Protein Solubilisation

The protein solubilisation and the DH values achieved by the various combinations of different concentrations of Alcalase and Flavourzyme (Table 1) are presented in Table 5.

Table 5. Protein solubilisation and DH for different doses of Alcalase and Flavourzyme

	enzyme dose (%)			
sample	Alcalase	Flavourzyme	protein sol.	DH (%)
1	0.0	2.93	84.5	41
2	2.93	2.93	90.1	57
3	0.43	0.86	86.1	31
4	1.46	0.0	82.3	15
5	2.50	5.0	85.3	62
6	1.46	2.93	83.6	48
7	1.46	5.86	85.3	66
8	2.50	0.86	82.7	41
9	1.46	2.93	89.2	52
10	0.43	5.0	88.6	59
11	0.0	0.0	20.2	0
12	0.0	0.0	19.3	0
13	2.93	5.86	84.7	67
14	2.93	5.86	86.3	68

Protein sol = protein solubilisation; DH (%) = degree of hydrolysis.

The different combinations of Alcalase and Flavourzyme resulted in a solubilisation of protein ranging from 82 to 90%. The solubilisation of the soy protein in water prior to

enzyme addition was about 20% (samples 11 and 12). The highest solubilisation (90.1%) was seen for 2.93% of both Alcalase and Flavourzyme. For Flavourzyme alone (sample 1, 2.93%) protein solubilisation was 84.5%. Protein solubilisation by a relatively small amount of only Alcalase (sample 4, 1.46%) was 82.3%. With 1.46% Alcalase and higher concentration of Flavourzyme (sample 9, 2.93%) protein solubilisation increased from 82 to 89%. However, when the dose of Flavourzyme was further increased to 5.86% protein solubilisation decreased to 85.3% indicating that Flavourzyme doses above 2.93% have a negative effect on protein solubilisation (in combination with Alcalase).

With respect to DH, the highest DH values (66-68%) were reached in the samples (7, 13, and 14) with the highest Flavourzyme dose (5.86%). When only one of the proteases was used Flavourzyme (sample 1, 2.93%) reached a DH of 41, whereas Alcalase (sample 4, 1.46%), not surprisingly, had a limited effect (15%) on DH. In sample 3 with an approximately 3 times lower Alcalase dose (0.43%) compared to sample 1 and a low Flavourzyme dose (0.86%) a DH of 31 was reached making the DH increasing effect of the exopeptidase in the combination obvious. The results clearly reflect the capability of Flavourzyme to increase DH in protein hydrolysates (*21*). They also emphasize that high doses of Flavourzyme are not necessarily beneficial to the protein solubilisation.

From the data obtained (Table 5) a protein solubilisation model was developed and the capability of the model to predict protein solubilisation as a function of Alcalase and Flavourzyme doses was subsequently tested. According to the surface response analysis of SAS-JMP protein solubilisation can be predicted using the following equation (also presented as a chart in Figure 3):

$$\boldsymbol{P\,sol = 33.6881 + 30.3628*Alc + 18.0238*Flav - 4.5961*Alc^2 - 1.5565*Flav^2 - 3.2979*Alc*Flav}$$

Where *P sol* = protein solubilisation (%), *Alc* = Alcalase dose; and *Flav* = Flavourzyme dose (%). The terms *Alc, Flav, Flav*^{2}, and *Alc*Flav* in the model are all significant. *Alc*^{2} is not significant but is used to get a better fit of the model. The fact that the interaction effect between Alcalase dose and Flavourzyme dose is significant indicates that effect of one enzyme depends on the level of the other. Furthermore, the fact that the second-order effect of Flavourzyme is significant whereas the second-order effect of Alcalase is

not indicates that the effect of adding more Flavourzyme levels out in contrast to the effect of adding more Alcalase.

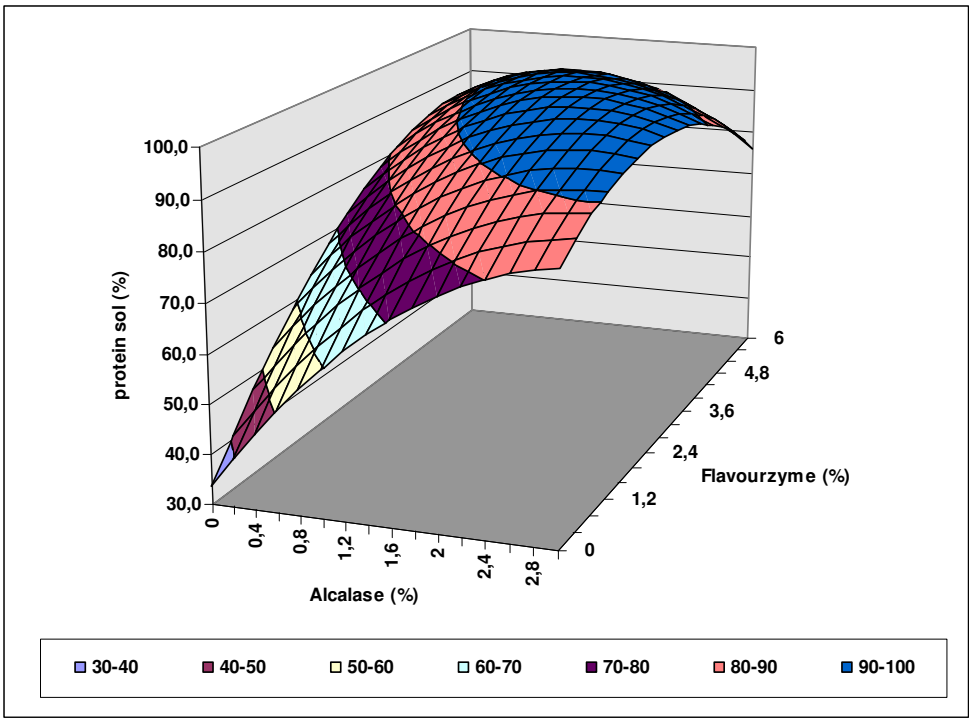

Figure 3. Protein solubilisation as a function of different concentrations of Alcalase and Flavourzyme. Summary of fit: R Square = 0.76; R Square adj. = 0.61; Root Mean Square Error 14.9; Mean of Response = 76.3; Observations = 14.

According to the model the highest protein solubilisation (96%) is predicted for doses of 2.0% Alcalase and 3.6% Flavourzyme. With 0% Alcalase and 2% Flavourzyme protein solubilisation should reach 63%, whereas 2% Alcalase and 0% Flavourzyme protein solubilisation should reach 76%. Alcalase has the highest slope in the model and is, therefore, the most important enzyme for protein solubilisation. The model predicts a decrease in protein solubilisation for Alcalase doses of 2-2.4% in combination with Flavourzyme doses exceeding ~3%. The model as fitted can explain 76% of the variability in protein solubilisation.

Based on the results from Table 5 also an equation was developed to predict the degree of hydrolysis as a function of Alcalase and Flavourzyme doses. The degree of hydrolyis can, according to the statistical analysis, be predicted using the following equation (also presented as a chart in Figure 4):

$$DH=0.82848 + 12.8980*Alc + 17.2649*Flav - 1.1603*Flav^2 - 0.8721*Alc^2 - 1.5724*Alc*Flav$$

Where *DH* = Degree of Hydrolysis *Alc* = Alcalase dose; and *Flav* = Flavourzyme dose (%).

As was the case for protein solubilisation the terms *Alc, Flav, Flav*^{2}, and *Alc*Flav* in the model are all significant. *Alc*^{2} is not significant but is used to get a better fit of the model. The fact that the interaction effect between Alcalase dose and Flavourzyme dose is significant indicates that effect of one enzyme depends on the level of the other.

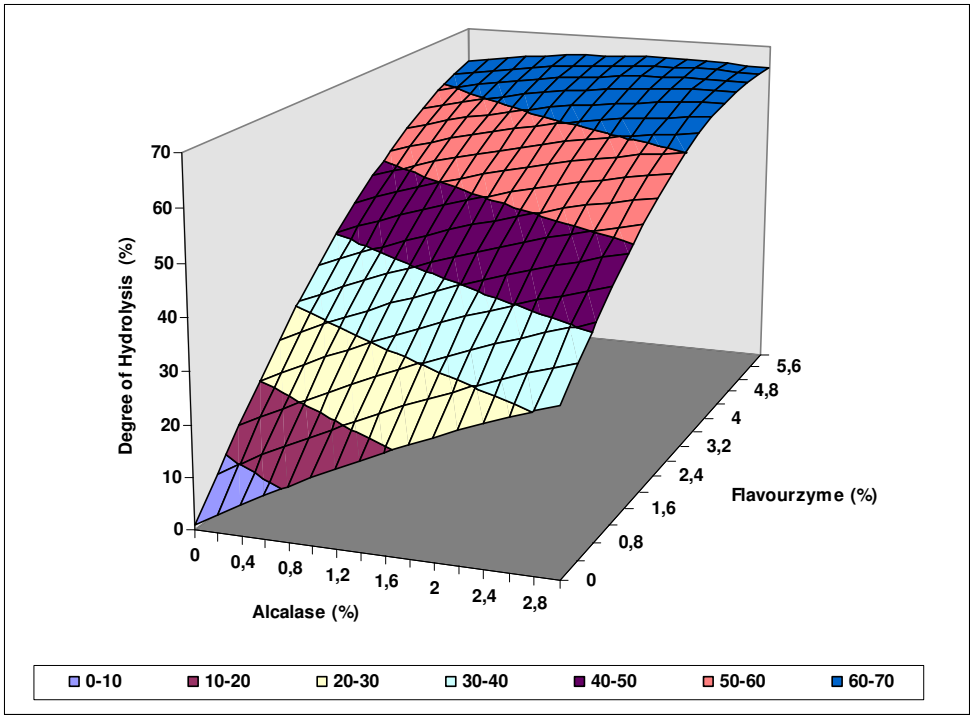

Figure 4. DH as a function of different concentrations of Alcalase and Flavourzyme. Summary of fit: R Square = 0.97; R Square adj. = 0.95; Root Mean Square Error 5.09; Mean of Response = 43.04; Observations = 14.

The figure clearly shows the larger effect of Flavourzyme on DH compared to Alcalase. In combination with Alcalase the DH increases steadily with increasing doses of Flavourzyme but the effect levers out for the higher dose region of Flavourzyme. This could possibly be explained by an increased level of aggregation and precipitation of the peptides released, which may in turn also affect protein solubilisation negatively. Lack of potential hydrolysable peptide bonds may also be part of the cause. The model as fitted explains 97% of the variability in the DH.

The chart of Figure 3 predicts that maximum protein solubilisation (96%) is reached with doses of 2.0% Alcalase and 3.6% Flavourzyme. The time courses of solubilisation of protein and degree of hydrolysis for these doses together with those of 2.5% Alcalase and 5% Flavourzyme are presented in Figure 5.

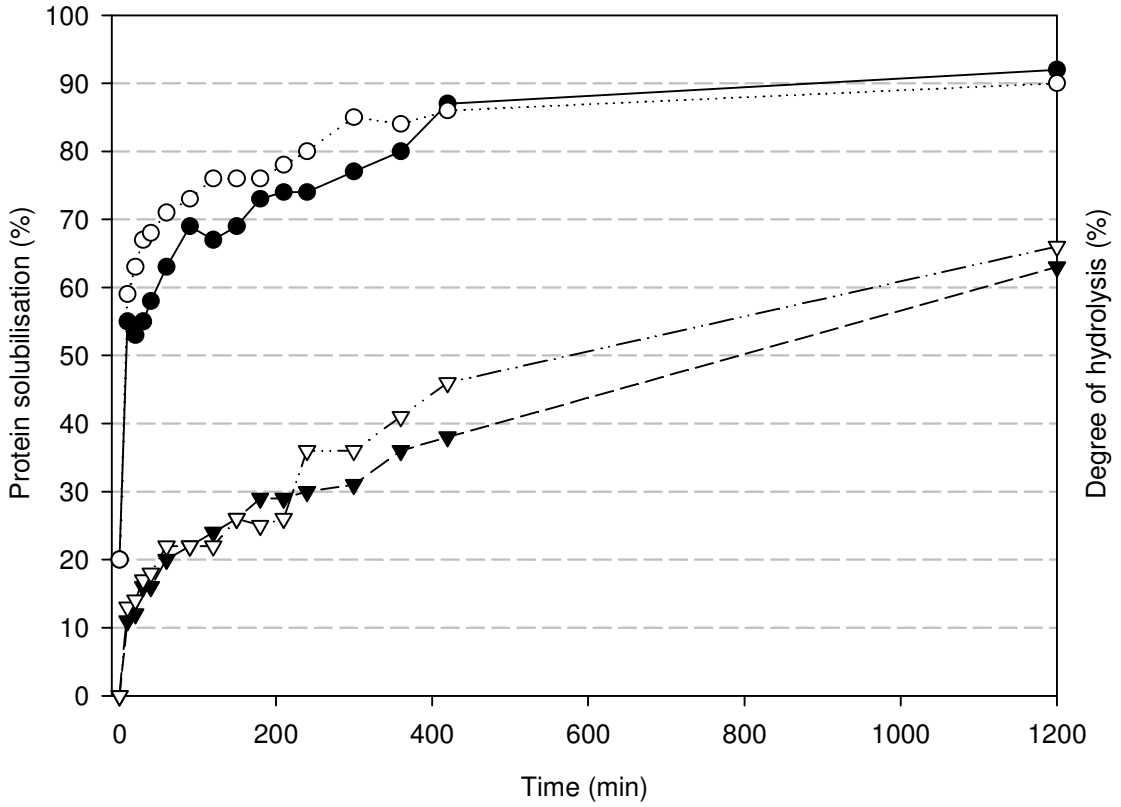

Figure 5. Protein solubilisation and degree of hydrolysis plotted against time (n=2) and compared to data from Figure 2. —●— = protein sol. for 2.0% Alcalase + 3.6% Flavourzyme; ···○··· = protein sol. for 2.5% Alcalase + 5% Flavourzyme); −▼− = DH for 2.0% Alcalase + 3.6% Flavourzyme; ━▽━ = DH for 2.5% Alcalase + 5% Flavourzyme.

From the two protein solubilisation curves it appears that protein solubilisation proceeded somewhat faster in the beginning for 2.5% Alcalase and 5% Flavourzyme compared to 2.0% Alcalase and 3.6% Flavourzyme. This is in line with the assumptions of the model. At the endpoint of the experiment (1200 min) the solubilisation of protein by 2% Alcalase and 3.6% Flavourzyme was 92%. This is only insignificantly higher than the 90% reached using 2.5% Alcalase and 5% Flavourzyme, but evidently not as high as the 96% predicted by Figure 3 although it still shows that the model has potential.

The results show that protein solubilisation can potentially reach >90% in a single step if hydrolysis conditions are set correctly and when enzyme dosing is optimized. Evi-

dently optimisation of SBM protein hydrolysis is a complicated topic and the final level of either protein solubilisation or degree of hydrolysis in the end product can be largely influenced by careful adjustment of the different process parameters. Economical considerations were not part of this study but the cost of enzymes will in many cases put a limitation to the degrees of freedom when designing the process. In this connection models offer approximation of solubilisation of soybean proteins by different doses of Alcalase and Flavourzyme in real conditions and could be useful tools in commercial production of enzyme hydrolysed vegetable protein. However, due to the complexity of the process, further evaluation of the here presented models is needed.

CONCLUSIONS

The presented data show that effective protein solubilisation is not only a matter of selecting efficient endo- and exo-proteinases. The enzymes can be overdosed and the ratio between the enzymes is equally important depending of the need for high protein solubilisation or high DH in the final hydrolysed protein product. The control of pH was found to be an important parameter for protein solubilisation as well as DH. To better understand the limitations of the enzymes, further attention could be given to the fraction of the soy proteins resisting enzymatic solubilisation.

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CHAPTER 4

AGGREGATION OF PEPTIDES DURING HYDROLYSIS AS A CAUSE OF REDUCED ENZYMATIC EXTRACTABILITY OF SOYBEAN MEAL PROTEINS [♣]

[♣] Fischer,M.; Gruppen,H.; Piersma,S.R.; Kofod,L.V.; Schols,H.A.; Voragen,A.G.J. Aggregation of peptides during hydrolysis as a cause of reduced enzymatic extractability of soybean meal proteins. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 4512-4519.

ABSTRACT

With the purpose of analyzing the size and composition of enzyme-unextractable proteins in differently heat-treated soybean meals, a selection of extractants was screened for their ability to extract these proteins from enzyme-unextractable residues. The largest effects were obtained with urea, urea plus β-mercaptoethanol, and dilute alkali; the latter extracted up to 87% of the enzyme-unextractable protein. Gel permeation chromatography indicated that a large proportion of the extracted material was of high molecular weight. However, the combined results from gel electrophoresis, LC-MS, and MALDI-ToF MS showed that the extracted protein material was composed of aggregated peptides. The largest aggregates were observed in the enzymatic residues originating from meals heat-treated at high humidity. Extracted aggregates were fully degraded upon subsequent proteolytic treatment.

Keywords: soybean meal; heat treatment; enzymatic hydrolysis; extraction; protein; amino acids; peptides; mass spectrometry.

INTRODUCTION

The proteins of soybean meals can be extracted enzymatically to a high extent, but even with excessive enzyme concentrations the enzymatic extraction of soybean meal protein remains incomplete (*1*, *2*). The unextractable material represents a loss of valuable protein, for example for manufacturers of enzymatic soy protein hydrolysates.

We have previously analyzed the composition of residues of unheated meal and soybean meals heat-treated at controlled humidity conditions after extensive enzymatic extraction with commercial protease and carbohydrase preparations (*1*). Before the enzymatic treatment, the extractability in water of the proteins of the different meals varied between 13% and 67%. However, after enzymatic treatment, an almost equally high extractability of protein from the meals (89-94%) was observed. Protein comprised 15-20% (w/w) of the residues and had a hydrophobic amino acid profile. Depending on the heat treatment conditions the cellulose content of the residues accounted for 17-27% (w/w) (*1*). Although the basic polypeptide of glycinin has been reported to be more resistant toward proteolytic breakdown than the acidic polypeptide (*3*, *4*, *5*) the observed resistance to enzymatic extraction could not be ascribed to resistance of a single polypeptide to proteolytic breakdown. The cause of the observed resistance of the protein to further enzymatic extraction has not yet been elucidated (*1*).

The aim of the present work is to further characterize these, previously obtained, enzyme-unextractable residues and possibly identify proteins that resist extraction from the soybean matrix by enzymatic treatment. The residues are subjected to extraction by various solvents, and the molecular weight of the extracted proteinaceous material is determined by gel electrophoresis, different chromatographic techniques, and mass spectrometry. The extractability of protein and carbohydrates from the residues by different solvents is determined, and the resulting residues are quantified and characterized with respect to amino acid and carbohydrate composition.

MATERIALS AND METHODS

Materials

Enzyme-unextractable solids (EUS) were prepared from four differently heat-treated soybean meals by a repeated hydrolysis of the meals using excessive concentrations of Alcalase (A), Flavourzyme (F), Energex (E), and Biofeed Plus (B) as previously described (*1*). After separation the obtained EUS fractions were washed and freeze-dried.

The enzymes used were commercial protease and carbohydrase preparations from Novozymes A/S (Bagsvaerd, Denmark). The meals were USBM, a defatted unheated soybean meal, SBM-H, a meal heat-treated at high humidity, and SBM-L, a meal heat-treated at low humidity (the two latter were prepared by heating of USBM). NN, a defatted pelletized meal, was obtained from a local supplier (*1*).

Extractions

Comparison of Extractants. Five extractants, including a chaotrophic reagent, a reducing agent, a detergent, acid, and alkali, were tested for their ability to extract protein from the EUS. The extractants were 6 M urea in 35 mM potassium phosphate buffer, pH 7.6; 6 M urea in 35 mM potassium phosphate buffer, pH 7.6 plus 5 mM β-mercaptoethanol; detergent Triton X100 (1%); pH 12 (dilute NaOH) and pH 2 (dilute HCl). For extraction by urea, urea plus β-mercaptoethanol, and detergent, the four residues (150 mg) were suspended in 1500 μL extractant in an Eppendorf tube and shaken in an Eppendorf Thermomixer comfort (Hamburg, Germany) for 1 h at 700 rpm and 25 °C. Supernatants were separated from the residues by centrifugation (20 min; 12000g; 25 °C).

For extraction at pH 2 the residues (150 mg) were suspended in 1200μL Millipore water. Subsequently, droplets of 0.1 M HCl were added with simultaneous shaking. After reaching pH 2 the volumes were made up to 1500 μL with water. Samples were shaken for 10 min in an Eppendorf Thermomixer comfort (700 rpm) and were separated by centrifugation (20 min; 12000g; 25 °C). The supernatants were quickly adjusted to pH 7.6 using 0.1 M NaOH and stored at −20 ˚C for further analyses. Samples were denoted pH 2 (10 min).

For extraction at pH 12 the effect of incubation time was examined. To reach pH 12 ± 0.1 each of the four EUS samples (500 mg) was mixed with 20 mL 0.1 M NaOH. The

alkaline solutions contained 50 mM $NaBH_4$ to prevent peeling of the polysaccharides (*6*). Extraction took place for 10 min and 1 h, respectively, at 25 °C. The suspensions were centrifuged (10 min; 12000g; 25 °C), and the residues were washed and freeze-dried. The supernatants from the alkaline extractions were adjusted to pH 7.6 using 0.1 M HCl and stored at -20 °C for further analysis. Samples were denoted pH 12 (10 min) and pH 12 (1 h).

All residues obtained after extraction were freeze-dried, denoted extractant unextractable solids (EXUS), and stored in a dessicator at 4 °C for further analysis. The extractability of protein (EP) and carbohydrates was calculated by subtracting the respective amount present in the EXUS from the amount present in the EUS. All extractions were performed in duplicate.

Statistical Variation. The extraction procedures have a standard deviation of ~0.5%. Determinations of proteins and carbohydrates have a standard deviation of 0.2% and 0.5%, respectively. This should be considered during interpretation of the results.

Enzymatic Degradability of Extracted Proteins

The enzymatic degradability of the extracted proteins was examined by incubating an extract with commercial protease preparations. A pH 12 extract (10 min) of NN was adjusted to pH 7.6 and subjected to enzymatic hydrolysis. Alcalase (2.5 μL) and Flavourzyme (5 μL) were added to an extract containing 100 mg of protein. Reaction was allowed for 5 h. After centrifugation (10 min; 12000g; 25 °C), the extract and the hydrolysate were analyzed by gel permeation chromatography.

Dry Matter

Dry matter was defined as the weight of meals and residues immediately after freeze-drying.

Protein

The protein content (N x 6.25) of the final residues was determined by a semi-automated Kjeldahl method (*7*). The dry samples (25 mg) were destructed in concentrated sulfuric acid at 200-385 °C. The released NH_3 was determined with an ammonia-hypochlorite-salicylate reaction on a Skalar 5101 auto-analyzer (Skalar, Breda, The Netherlands). Protein contents are means of three determinations.

Carbohydrate Composition

Neutral Carbohydrates. Polysaccharides were hydrolyzed by pre-treatment with 72% (w/w) 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 neutral sugars were converted to their alditol acetates and analyzed by gas chromatography (*8*). Inositol was used as internal standard. Alditol acetates were separated on a DB-225 [5 m × 0.53 mm internal diameter; film thickness 1.0 μm] (J&W Scientific Folsom, CA, USA) on a CE Instruments GC 8000 TOP (ThermoQuest Italia, Milan, Italy). It was operated at 200 ºC and equipped with a flame ionization detector (ThermoQuest Italia) set at 270 ºC.

Uronic Acids. The uronic acid content was determined using an automated *m*-hydroxy biphenyl assay (*9*). For the procedure, 96% (w/w) H_2SO_4 was used containing 0.0125 M sodium tetraborate in order to quantify glucuronic as well as galacturonic acid residues. Carbohydrate contents are means of three determinations.

Amino Acid Analysis

The amino acid composition was determined using an automated Model 420A derivatizer analyzer system with a 130A separation unit and a 920 data module. All equipment was from Applied Biosystems (Foster City, CA, USA). Each sample (10 mg) was hydrolyzed in 100 μL of 6 M HCl for 16 h at 100 °C and subsequently derivatized by phenyl isothiocyanate. Separation was performed using a C18 reversed phase Brownlee Speri-5 PTC column [2.1 mm x 220 mm] (Perkin Elmer, Wellesley, MA) at a flow rate of 300 μL/min. The absorbance of the eluate was monitored at 254 nm. Amino acid standard H from Pierce (Rockford, IL, USA) was used for identification. Amino acid contents are means of six determinations.

Statistical Analysis. Effects of experimental conditions and intrinsic amino acid properties on the molar proportions of amino acids in the residues were tested using a "repeated measures analyses of variance" (*10*). Within-subject factors were "before (EUS) and after (EXUS) alkali extraction", denoted "extraction" and "enzymatic SBM residues" (NN, USBM, SBM-H, and SBM-L), denoted "residues". Three groups of amino acids (hydrophobic, hydrophilic, and "neutral") (*11*) constituted a between subjects factor "level of amino acid hydrophobicity", denoted "hydrophobicity". Prior to this analysis, proportions were arcsine transformed to correct for non-linearity of the data. Analy-

ses were performed using SPSS software (SPSS Version 8.0, SPSS Inc, Chicago IL, USA). The significance level was 5%.

SDS-PAGE

SDS-PAGE was performed on a Protean-system (BioRad, Hercules, CA, USA) using 10-20% Tris-HCl and 10-20% Tris-Tricine pre-cast gels (BioRad). According to the manufacturer the gels have an optimized separation range of 10-100 kDa and 1-40 kDa, respectively. The two gel types were processed at 200 V, 20 mA for 50 min. The Tris-HCl gels were stained by Coomassie Brilliant Blue o/n and destained with 30% (v/v) methanol and 10% (v/v) acetic acid in deionised water according to the instructions of the manufacturer. Tris-Tricine gels were developed using a PlusOne protein silver staining kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the instructions of the manufacturer. Gels were scanned using a Computing Densitometer from Molecular Dynamics (Sunnyvale, CA, USA).

N-terminal Sequencing

Sequencing was used for identification of proteins, which could be extracted from the EUS. The extracts were boiled in reducing sample buffer and loaded onto a 10-20% gradient gel (BioRad, Hercules, CA, USA). The gel was processed according to the instructions of the manufacturer. Blotting was performed using a Mini Trans-Blot Cell (BioRad) and Hybond-P, a PVDF transfer membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein transfer buffer was a 40 mM Tris buffer containing 40 mM boric acid and 1 mM EDTA, pH 8.3. The gel and the membrane were prepared for transfer according to the instructions of the manufacturer and subsequently processed for 1 h at 100 V / 0.3 A with cooling. The membrane was stained for 5 min using a standard Coomassie Brilliant Blue staining solution, with 5% acetic acid, followed by destaining for 1 min with 50% methanol containing 10% acetic acid. The membrane was air-dried, and bands at 20 and 14 kDa were selected for sequencing, which was performed at the E.C. Slater Institute (Amsterdam, The Netherlands) using an automated N-terminal Edman degradation method. Analysis was performed using a Procise 494A from Applied Biosystems (San José, CA, USA)

Molecular Weight Determination

Extractions. The molecular weight distribution of the urea, urea plus β-mercaptoethanol, and pH 12 (1 h) extracts was determined using a Superdex 75 column [3.2 mm x 300 mm] connected to an ÄKTA purifier system (all equipment from Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were diluted (10 times) and eluted using three different buffers: buffer a), potassium phosphate buffer (35 mM) containing 0.1 M NaCl, pH 7.6; buffer b), buffer a + 6 M urea; and buffer c), buffer a containing 6 M urea and 5 mM β-mercaptoethanol.

For analysis, the pH 12 extracts (1 h) were diluted in buffers a-c. The extracts obtained with urea and urea plus β mercaptoethanol were diluted in urea (buffer b). The diluted samples were allowed to equilibrate for 3 h before injection (25 μL) onto the column. Elution was performed with the respective buffers at a flow rate of 80 μL/min. The absorbance of the eluate was monitored at 214 and 280 nm.

Proteolysis of Extracts. The molecular weight distribution before and after hydrolysis of a pH 12 (10 min) extract and a urea extract of SBM-H was determined using a Superdex 75 column [3.2 mm x 300 mm] connected to a SMART-system (Amersham Pharmacia Biotech). Extracts and hydrolysates were diluted 20 times with the elution buffer before 50 μL was applied onto the column. Separation took place at a flow rate of 80 μL/min using a 35 mM potassium phosphate buffer containing 0.1 M NaCl, pH 7.6. The absorbance of the eluate was monitored at 214 and 280 nm. All presented chromatograms were verified by duplicate injections.

Mass Spectrometry

Liquid Chromatography–Mass Spectrometry (LC-MS). The size of the proteins extracted by urea, urea plus β-mercaptoethanol and pH 12 (1 h) was determined by LC-MS using a C18 column [2.1 mm x 250 mm] from Vydac (Hesperia, CA, USA) connected to HPLC system from Spectra Physics (San José, CA, USA). Solutions of 0.03% (v/v) trifluoroacetic acid (TFA) in water and 0.03% (v/v) TFA in acetonitrile were used for elution. The extracts were diluted two times with the first eluent. A flow rate of 0.2 mL/min was used in a linear gradient with the second eluent rising from 0.8 to 80% over a period of 60 min. The absorbance of the eluate was monitored at 214 and 280 nm. Mass spectrometric analysis was performed using a MAT 95 LCQ ion trap mass spectrometer from Thermo Finnigan (San José, CA, USA). Analysis was run in the

positive electrospray mode using a spray voltage of 2.5 kV and a capillary temperature of 200 °C. The apparatus was controlled and data were processed by Xcalibur software.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-ToF MS). Protein and peptides were cleaned up from the urea and pH 12 extracts using ZipTip C18 reversed phase tips from Millipore (Bedford, MA, USA) according to the instructions of the manufacturer. MALDI-ToF MS spectra were recorded on a PerSeptive Voyager DE-RP (PerSeptive Biosystems, Framingham, MA, USA) mass spectrometer equipped with delayed extraction technology. The spectra were acquired in linear positive-ion mode. Per spectrum 256 laser shots were summed. The following instrumental settings were used: accelerating voltage 25000 V; grid voltage, 91.5%; guide wire, 0.3%; and extraction time, 200 ns. Sinapinic acid and 2,5-dihydroxybenzoic acid were used as matrices. The sinapinic acid matrix solution was prepared by dissolving 10 mg matrix in 1 mL 50% (v/v) aqueous acetonitrile containing 0.3% (v/v) TFA. The 2,5-dihydroxybenzoic acid was dissolved in 1 mL distilled water containing 0.3% (v/v) TFA. Samples were prepared using the dried droplet method by mixing 9 μL matrix solution with 1 μL sample solution. Aliquots of 1μL were transferred to a gold-coated welled sample plate and were allowed to crystallize under atmospheric pressure at room temperature. All samples were spotted in duplicate. Spectra were calibrated externally using the $[M+H^+]^+$ and $[M+2H^+]^{2+}$ peaks in the spectrum of a mixture of bovine insulin (5734.6 Da), thioredoxin (11674.5 Da) and apo-myoglobin (16952.6 Da) (calibration mixture 3, PerSeptive Biosystems). For identification of smaller peptides a mixture of angiotensin (1297.5 Da), ACTH (2094.5 Da, 2466.7 Da, and 3660.2 Da, respectively), and bovine insulin (5734.6) was used for external calibration (calibration mixture 2, PerSeptive Biosystems). The spectra were recorded under identical conditions.

RESULTS AND DISCUSSION

Effects of Different Extractants

Extractability of Protein from Residues. The extractability of protein (EP) was determined after incubation with the different extractants as shown in Table 1.

Table 1. Extractability of protein from the enzyme-unextractable solids of differently heated soybean meals by the various extractants

	extractability of protein (%) [1]					
	urea	urea + β-ME	Triton	pH 12 (10 min)	pH 2 (10min)	pH 12 (1h)
NN	18	14	4	50	4	66
USBM	20	32	28	50	20	74
SBM-H	17	21	2	49	3	87
SBM-L	25	39	15	49	15	72

[1] *Expressed as proportion (%) of total protein originally present in the residue. The standard deviation of the extraction procedures was ~0.5%.*

On the basis of the extractability of protein from the four enzymatic residues by either urea, urea plus β-mercaptoethanol, detergent Triton X100, or pH 2 (10 min), two groups could be distinguished. The residues of unheated plus low humidity heat-treated meals (USBM and SBM-L) constituted one group, and the residues of meals heat-treated at high humidity (SBM-H and NN) constituted the other group. Urea extracted 20-25% of the protein of the USBM and SBM-L residues, increasing to 32-39% in the presence of β-mercaptoethanol. Conversely, urea had less effect on NN and SBM-H residues (17-18%), and for these residues the extraction did not improve significantly with addition of β-mercaptoethanol. The results show that, by elimination of hydrogen bonds (chaotropic reagent), protein material could be extracted from both groups of residues. Furthermore, this suggests that disulfide bonds were retaining protein in the enzymatic residues of the unheated and low-humidity heat-treated meals. The detergent solution extracted 15-27% of the protein from the SBM-L and USBM residues. For the SBM-H and NN residues it was only 2-4%. The acid treatment extracted 15-20% of the protein from SBM-L and USBM residues, whereas little protein was extracted from the SBM-H and NN residues (3-4%).

The highest extraction of protein from the residues was obtained with the alkali. By pH 12 (10 min) between 49 and 50% of the enzyme-unextractable protein was extracted, increasing to 66-87% with pH 12 (1 h). As much as 89-93% of the protein could be extracted by increasing the concentration to 1 M and 6 M NaOH, respectively (results not shown). However, these harsh alkaline conditions resulted in splitting of the peptide backbone as observed by SDS-PAGE using purified soy glycinin and Kunitz protease

inhibitor as reference proteins. With pH 12 (1 h) no splitting of the backbone was observed by SDS-PAGE (no further results shown). It should, however, be realized that even at relatively low concentrations of alkali, deamidation of proteins occurs (*12*). Extraction with urea and urea plus β-mercaptoethanol does not affect the primary structure of proteins (*13*). Therefore, these two extracts were included with the pH 12 (1 h) extracts for further characterization of the molecular weight of the extracted material.

Composition of Residues

Protein Composition. The molar amino acid composition of the enzyme-unextractable solids (EUS) and the extractant unextractable solids (EXUS) resulting from pH 12 (1 h) extraction was determined as shown in Table 2.

Table 2. Protein content and amino acid composition (molar %) of the enzyme-unextractable solids (EUS) and the extractant-unextractable solids (EXUS) obtained after extraction of EUS with pH 12 (1 h)

	EUS[a]				EXUS[a]			
	NN	USBM	SBM-H	SBM-L	NN	USBM	SBM-H	SBM-L
protein[b]	15.3	19.9	17.3	18.3	8.1	7.3	4.5	7.1
Ala	10.1	8.3	10.1	6.7	10.6	11.4	7.3	11.2
Arg	4.0	5.4	4.0	6.6	2.4	3.2	2.4	3.0
Asx[c]	7.5	9.4	8.1	7.8	5.2	5.6	5.2	5.3
Cys	1.5	0.4	0.5	1.0	0.2	0.2	0.2	0.2
Glx[d]	7.7	11.4	7.8	7.3	8.2	5.8	16.8	6.6
Gly	11.2	9.4	10.4	9.9	14.0	11.8	10.4	11.8
His	1.5	1.7	1.8	2.7	1.4	1.1	2.7	1.3
Ile	4.7	4.6	4.8	4.9	4.1	5.2	2.6	4.7
Leu	11.8	10.4	11.4	6.2	13.0	15.1	9.0	15.2
Lys	4.6	6.6	4.7	7.0	3.9	3.6	7.9	4.4
Met	0.6	0.6	1.0	1.6	0.5	0.7	0.2	1.0
Phe	4.7	4.4	4.5	6.1	4.4	5.4	3.9	5.1
Pro	7.4	6.5	9.6	6.6	8.3	7.2	9.7	7.0
Ser	7.8	7.2	7.1	9.2	8.4	7.4	8.5	7.6
Thr	5.3	4.6	4.1	6.8	5.6	6.0	3.5	5.5
Trp[e]	-	-	-	-	-	-	-	-
Tyr	2.3	2.4	2.7	3.3	2.6	2.2	4.4	2.6
Val	7.4	6.5	7.4	6.5	7.3	8.0	6.0	7.7

All data are based on six determinations
[a] *Standard deviations were generally between 1 and 5%, except for Asx and Glx (3-10%) and Met (10-20 %)*
[b] *Calculated from Kjeldahl N x 6.25. Expressed as % of dry matter*
[c] *The sum of asparagine + aspartic acid*
[d] *The sum of glutamine + glutamic acid*
[e] *Fully destroyed during hydrolysis*

To understand whether or not akaline treatment was selectively extracting proteinaceous material containing specific types of amino acids, the proportions of hydrophilic (Arg, His, and Lys), hydrophobic (Gly, Ala, Val, Leu, and Ile) and neutral amino acids (*11*) in the residues before extraction were compared with the respective proportions after extraction. A significant effect was found for the Extraction × Hydrophobicity interaction: $F(2,14) = 4.09$, $p = 0.040$. This implies systematically changing proportions of the three hydrophobicity categories upon alkaline extraction, regardless of the identity of the ini-

tial residue (NN, USBM, SBM-H, or SBM-L). As shown in Figure 1, this interaction is caused by a general increase upon alkaline extraction of the proportions of hydrophobic amino acids at the cost of a decrease of proportions of hydrophilic amino acids.

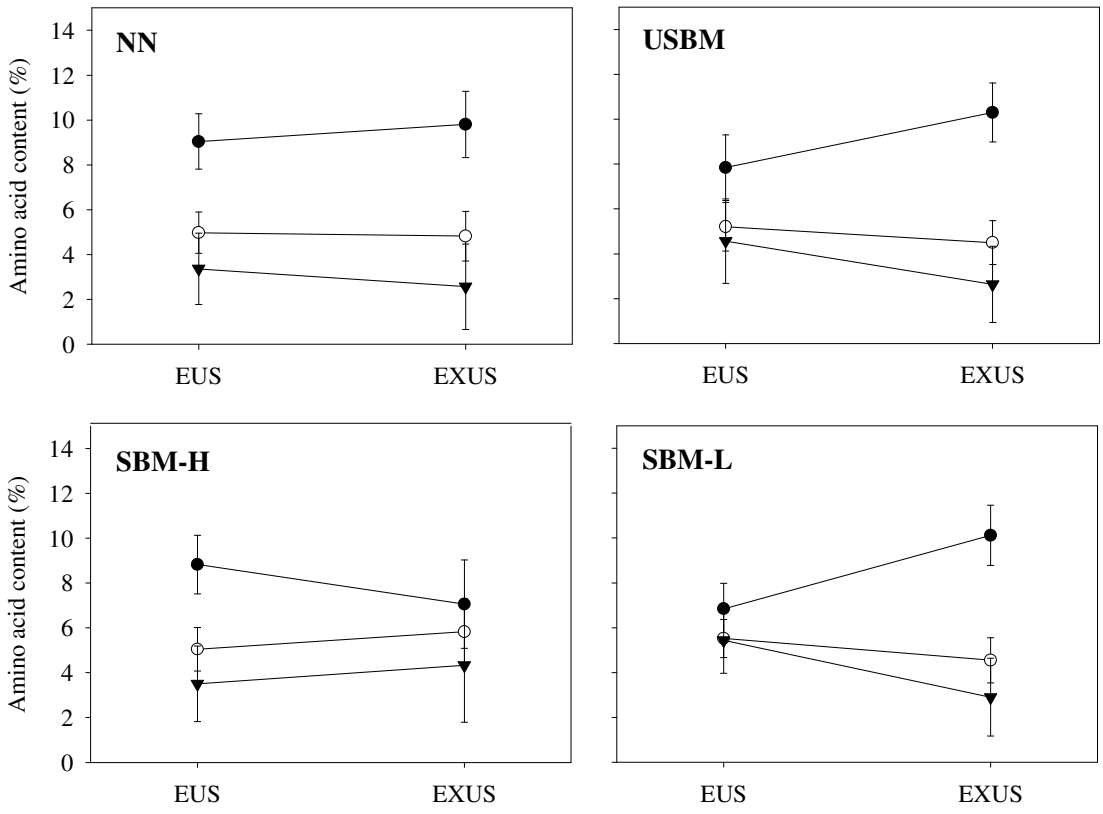

Figure 1. Average molar percentage (± standard error) for each category of amino acids for the NN, USBM, SBM-H, and SBM-L residues: (●) hydrophobic amino acids; (O) neutral amino acids; (▼), hydrophilic amino acids.

However, this general tendency does not apply for the SBM-H residue, which, upon extraction, shows a decrease of the relative proportion of hydrophobic amino acids together with an increase of the relative proportion of hydrophilic amino acids. This contrasting effect is reflected by the significant three-way extraction × hydrophobicity × residue interaction: $F(6, 42) = 4.71$, $p = 0.001$.

The results did not show a significantly different composition of amino acids in the four EUS residues: $F(3, 42) = 0.071$, $p = 0.975$. From this it should be concluded that compared to the NN, USBM, and SBM-L residues, the high-humidity condition of heat treatment used for SBM-H results in a different amino acid composition of the extractant-unextractable solids.

Carbohydrate Composition. The molar monosaccharide composition of carbohydrates in the residues after hydrolysis (EUS) and the residues (EXUS) after extraction with pH 12 (1 h) is shown in Table 3.

Table 3. Yield of dry matter and carbohydrates after enzymic hydrolysis and extraction with pH 12 (1 h) and molar carbohydrate compositions of enzyme-unextractable solids (EUS) and extractant-unextractable solids (EXUS)

				carbohydrates (molar %)							
	yield[a]	C[b] (%)	C[c] (g)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
EUS											
NN	12.4	44.0	5.5	3	0	6	16	2	3	61	9
USBM	15.5	40.9	6.3	3	3	7	11	2	7	52	15
SBM-H	10.0	38.0	3.8	3	0	4	9	3	4	64	12
SBM-L	18.0	33.0	5.9	3	2	7	11	2	8	51	16
EXUS											
NN	8.0	68.0	5.4	1	0	6	14	1	1	71	7
USBM	10.9	58.0	6.3	2	0	6	10	2	5	62	15
SBM-H	5.1	71.0	3.6	1	0	4	8	2	2	75	9
SBM-L	13.1	44.0	5.8	3	0	6	9	2	6	60	15

All data are based on double determinations
[a] *Yield of dry matter. Expressed in grams of 100g SBM*
[b] *Carbohydrate content (%). Calculated as the sum of neutral sugars + uronic acids. Expressed in per-cent of DM*
[c] *Carbohydrate content in absolute amounts (expressed in g)*

The total carbohydrate content of the different EUS samples ranged from 33% (SBM-L) to 44% (NN). In the EXUS samples the carbohydrate content ranged from 58% (USBM) to 71% (SBM-H), primarily due to the effective extraction of protein by the alkali. However, in absolute amounts, the treatment with pH 12 (1 h) did not extract much carbohydrate. Chemical extraction of soy carbohydrates would demand higher concentration of alkali (*14*). The molar composition of the EUS showed that glucose was, by far, the most abundant of the constituent sugars (51-64%) followed by uronic acid (9-16%) and xylose (9-16%). Upon extraction (EXUS) the molar proportion of glucose increased to 60-75%, while the xylose content remained steady at 8-14%. It has previously been shown that >90% of the glucose of the residues is cellulose (*1*).

Molecular Weight of Extracted Material

Gel Permeation Chromatography. The molecular weight distribution (280 nm) of the protein material extracted by pH 12 (1 h) was determined after dilution and elution in phosphate buffer, urea, and urea plus β-mercaptoethanol containing buffers as shown in Figure 2A,B, curves a-c.

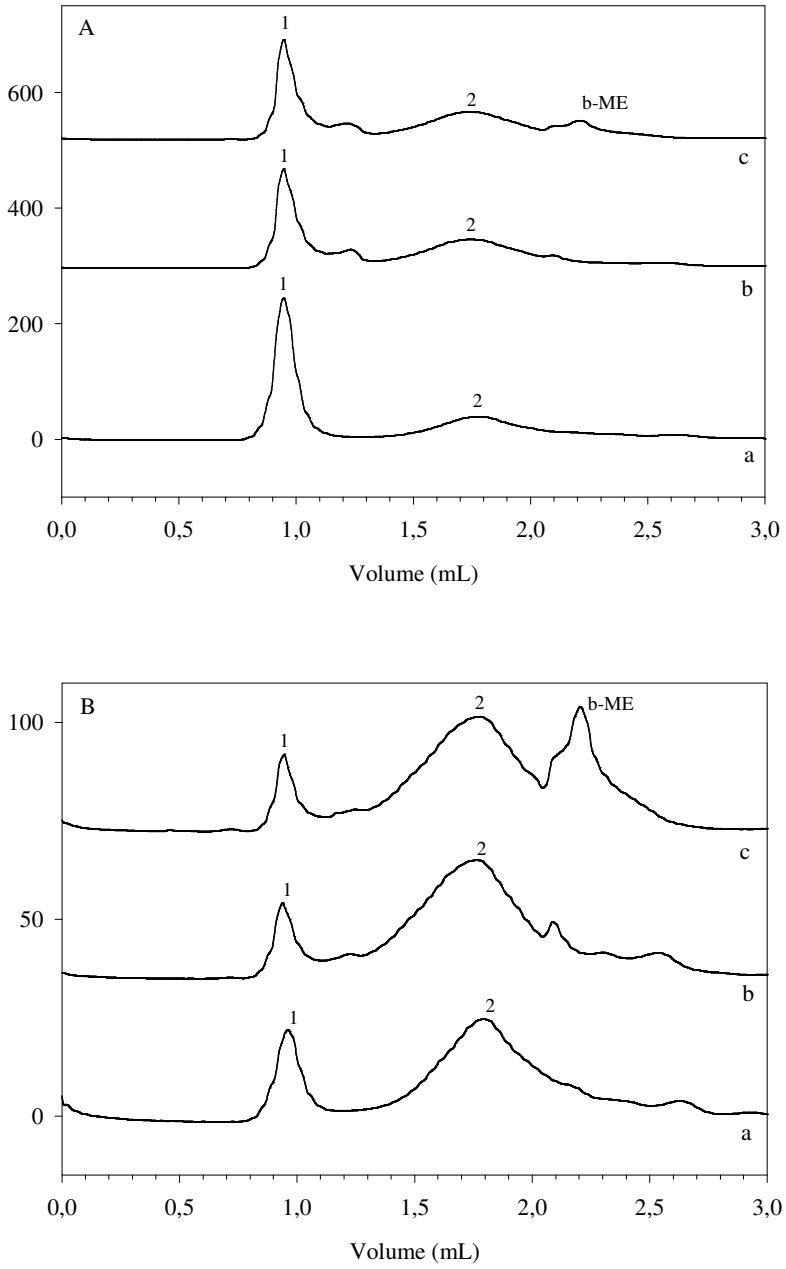

Figure 2. Gel permeation chromatograms (Superdex 75) of pH 12 (1 h) extracts of SBM-H (A) and USBM (B). Extracts were diluted and eluted in three different buffers: (a) 35mM potassium phosphate buffer containing 0.1M NaCl, pH 7.6; (b) buffer a + 6M urea, pH 7.6; (c) buffer a + 6M urea + 5mM β-mercaptoethanol, pH 7.6. Eluting peaks are denoted 1 and 2, respectively [V_0 = 0.9 mL and V_t = 1.9 mL].

After dilution and elution in phosphate buffer, two major peaks were eluting from the pH 12 (1 h) extract of high-humidity heat-treated samples (SBM-H and NN). The chromatogram obtained for SBM-H is shown in Figure 2A. Peak 1 had an apparent molecular weight >70 kDa (eluting at V_0). The peak contained >75% of the extracted material, based on 280 nm absorption. Peak 2 contained material with a molecular weight of <14 kDa.

Compared to the chromatogram obtained with phosphate buffer, dilution and elution in urea buffer or urea plus β-mercaptoethanol (Figure 2A, curves b and c) did, to some extent, dissolve part of the high molecular weight material (peak 1 was smaller for curves b and c than for curve a). Furthermore, more material was recovered in Peak 2 and a minor peak appeared at 1.2 mL (corresponding to a molecular weight of ~30 kDa). The results indicate that, to some extent, hydrogen bonds (and possibly other non-covalent bonds) were probably affecting the size of the extracted material (aggregation). The comparable curves for urea and urea plus β-mercaptoethanol show that S-S bridges were not affecting the size of the extracted material. The large peak observed in the urea plus β-mercaptoethanol extract at retention volume 2.3 mL is from β-mercaptoethanol itself.

Different proportions of the two major peaks were observed for the pH 12 (1 h) extracts of the residues of unheated (USBM) plus low humidity heat-treated (SBM-L) meals after dilution and elution in phosphate buffer as shown by USBM in Figure 2B, curve a. For USBM, peak 1 contained <25% of the extracted protein material. Consequently, the proportion (~75%) of material smaller than 14 kDa (peak 2) was much larger than observed for SBM-H (Figure 2A). In accordance with the results for SBM-H, dilution of the USBM extract in urea and urea plus β-mercaptoethanol (Fig. 2B, curves b and c) did to some extent affect the proportions of peaks 1 and 2 as seen by the appearance of two minor peaks at 1.2 mL and ~2.1 mL. This indicates that hydrogen bonds (and possibly other non-covalent bonds) were probably affecting the size of the extracted material. As for SBM-H, the results did not indicate that S-S bridges were present in the material.

The chromatograms (280 nm) obtained for the urea and the urea plus β-mercaptoethanol extracts of the SBM-H and USBM residues after dilution and elution in urea buffer are shown in Figure 3A,B, curves I and II.

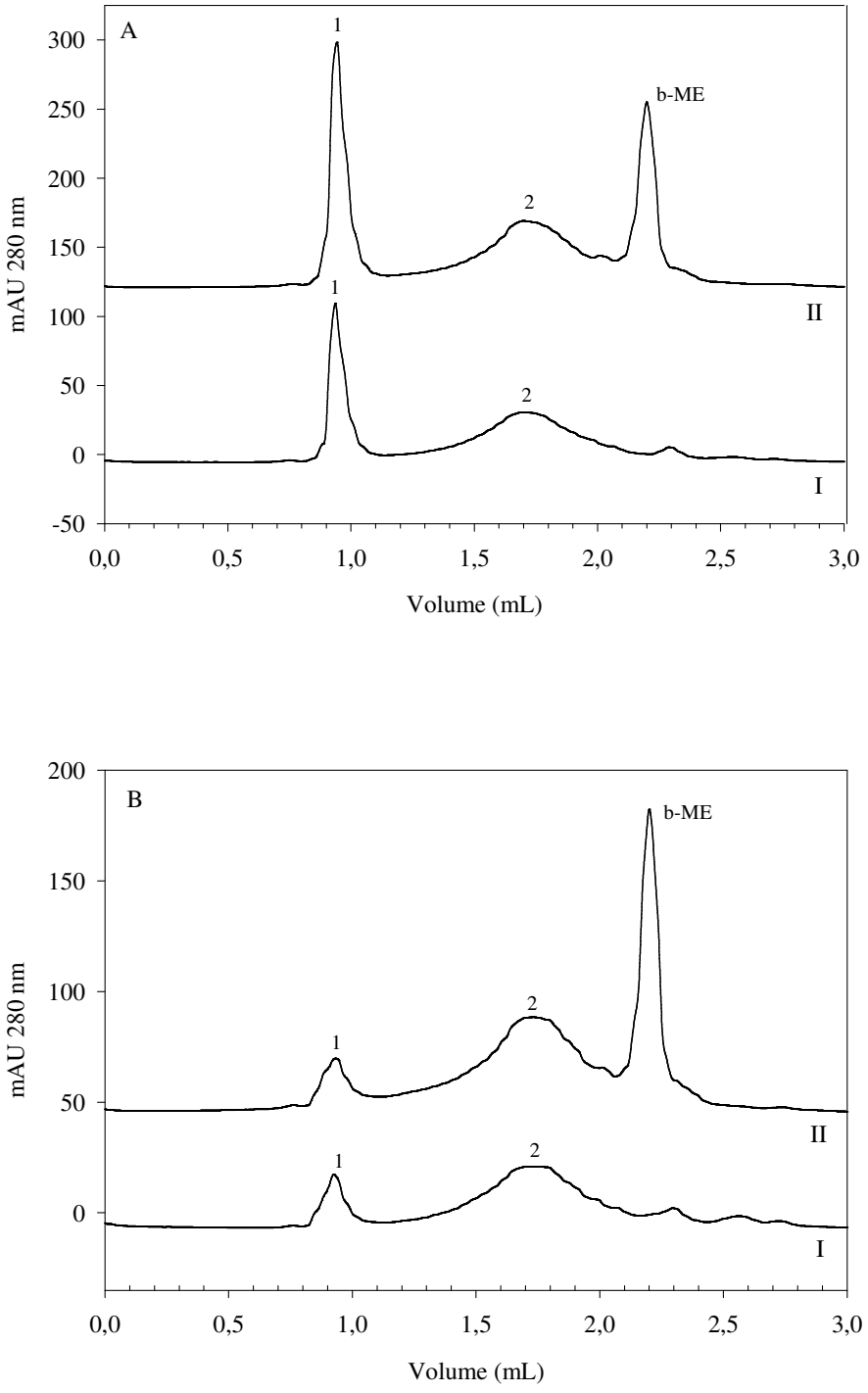

Figure 3. Gel permeation chromatograms (Superdex 75) of SBM-H (A) and USBM (B) extracts. The urea and urea and urea plus β-mercaptoethanol extracts are denoted by I and II, respectively. Extracts were diluted and eluted with 35mM potassium phosphate buffer containing 0.1M NaCl and 6M urea, pH 7.6. Eluting peaks are denoted 1 and 2, respectively [V_0 = 0.9 mL and V_t = 1.9 mL].

For both residues the peak profile was comparable to the pH 12 (1 h) extracts presented in Figure 2. A large proportion of protein material was contained in peak 1 (>70 kDa) of SBM-H. For the USBM the primary proportion of protein material eluted in peak 2 containing material smaller than 14 kDa. For both types of residues extraction by urea plus β-mercaptoethanol did not change the distribution of the peaks to any major extent.
The results indicate that the presence of high molecular weight proteinaceous material in the residues correlates with the use of high-humidity heat treatment (SBM-H and NN) of the soybean meals.

Gel Electrophoresis. The different extracts were subjected to SDS-PAGE as shown in Figure 4.

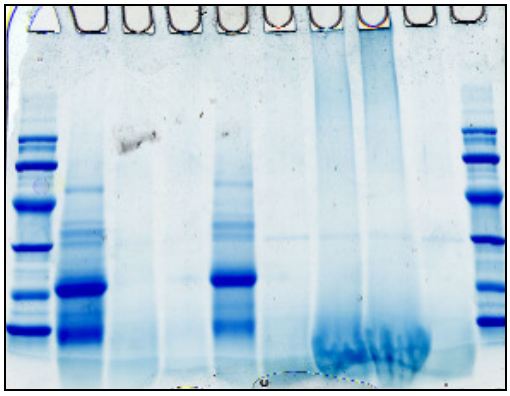

Figure 4. SDS-PAGE gels showing pH 2 and pH 12 extracts of the NN, USBM, SBM-H, and SBM-L residues: (lanes 1-4) pH 2; (lanes 5-8) pH 12. ST (starting from above): molecular markers 97, 67, 43, 30, 21, and 14 kDa.

The material extracted by pH 12 was smaller than the lower separation limit (10 kDa) of the gel as seen in lanes 5-8. For the NN and SBM-H samples a weak coloring was noticed below the 14 kDa protein marker at the bottom of lanes 6 and 7. The peptide gel contained no electrophoretically recognizable products (results not shown). Accordingly, at least 50% (Table 1) of the protein contained in the four different enzymatic residues had a molecular weight below 1 kDa (peptides < 8-10 amino acids). This finding clearly contradicts the molecular weights determined by the gel permeation chromatograms presented in Figures 2 and 3. No electrophoretically recognizable protein bands were observed by SDS-PAGE in the material extracted with urea, urea plus β-mercaptoethanol, and detergent Triton X 100 (results not shown). The results indicate that the two major peaks in the chromatograms contain aggregated peptides, which can-

not be dissolved by urea or urea plus β-mercaptoethanol at room temperature according to Figures 2 and 3. However, when the extracts are prepared for gel electrophoresis by boiling in SDS-PAGE sample buffer, the aggregates dissolve completely into low molecular weight peptides and / or amino acids.

Two strong protein bands (20 kDa and ~14 kDa) and several much weaker bands appeared in the pH 2 extracts of USBM (lane 1) and SBM-L (lane 4) as shown in Figure 4. The 20 and 14 kDa bands were subjected to N-terminal sequencing. The amino acid sequence for the 20 kDa band was G-I-D-E-T (Gly-Ile-Asp-Glu-Thr), identified as the N-terminus of the basic polypeptides B, B1A, and BX (*15*, *16*, *17*) from soy glycinin. The protein of the 14kDa band was not sufficiently pure for sequencing.

According to Table 1, the polypeptides extracted with pH 2 account maximally for 15-20% of the enzyme-unextractable protein in the residues of USBM and SBM-L. Interestingly, these extracted polypeptides were completely degraded when subjected to proteolysis with the protease preparations used for extraction of protein from the original SBMs (results not shown). This shows the efficiency of the used protease mixture and, in addition, that the resistance towards proteolytic extraction of the basic subunit of glycinin is not inherent to the protein but is caused by interactions of this protein with soy-matrix components.

Mass Spectrometry

LC-MS. The RP-HPLC chromatogram (214 nm) of the proteins and peptides of the urea, urea plus β-mercaptoethanol, and pH 12 (1 h) extracts of USBM are shown in Figure 5.

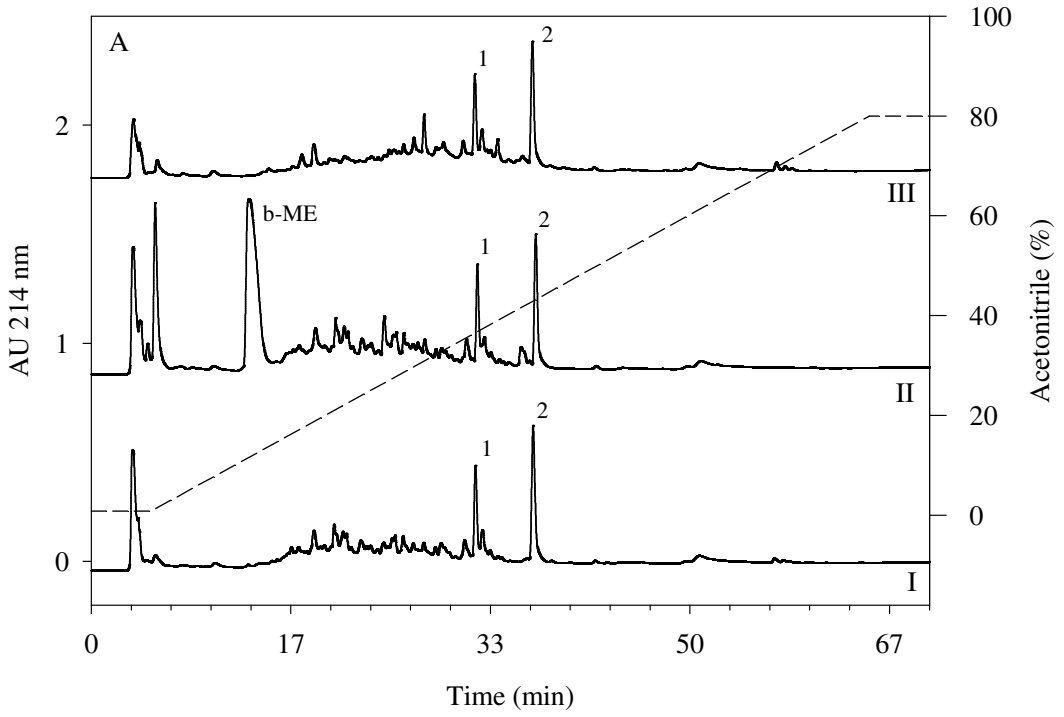

Figure 5. LC-MS reversed phase chromatograms for USBM after extraction by urea (I), urea + b-ME (II), and pH 12 (1 h) (III): peaks are denoted b-ME (for the β-mercaptoethanol peak) and 1 and 2 (for the peaks eluting at 36 and 42% acetonitrile concentrations, respectively).

Comparable results were obtained for the NN, SBM-H, and SBM-L residues. Essentially all proteinaceous material was eluting between 15 and 50% acetonitrile in line with the general range of peptide hydrophobicity (*18*). Qualitatively, the three chromatograms are comparable, though not identical. A complex mixture of peptides/proteins was eluting between 15 and 35% acetonitrile, depending on the extractant used. At 36 and 42% acetonitrile concentrations two more distinct peaks (marked 1 and 2, respectively) can be distinguished for all three extractants.

Mass spectra were acquired from 400-1800 Da over the whole chromatogram (Figure 6). In Figure 6, curve I, an example of a mass spectum of the USBM residue after extraction with urea is shown for peak 1 of Figure 5.

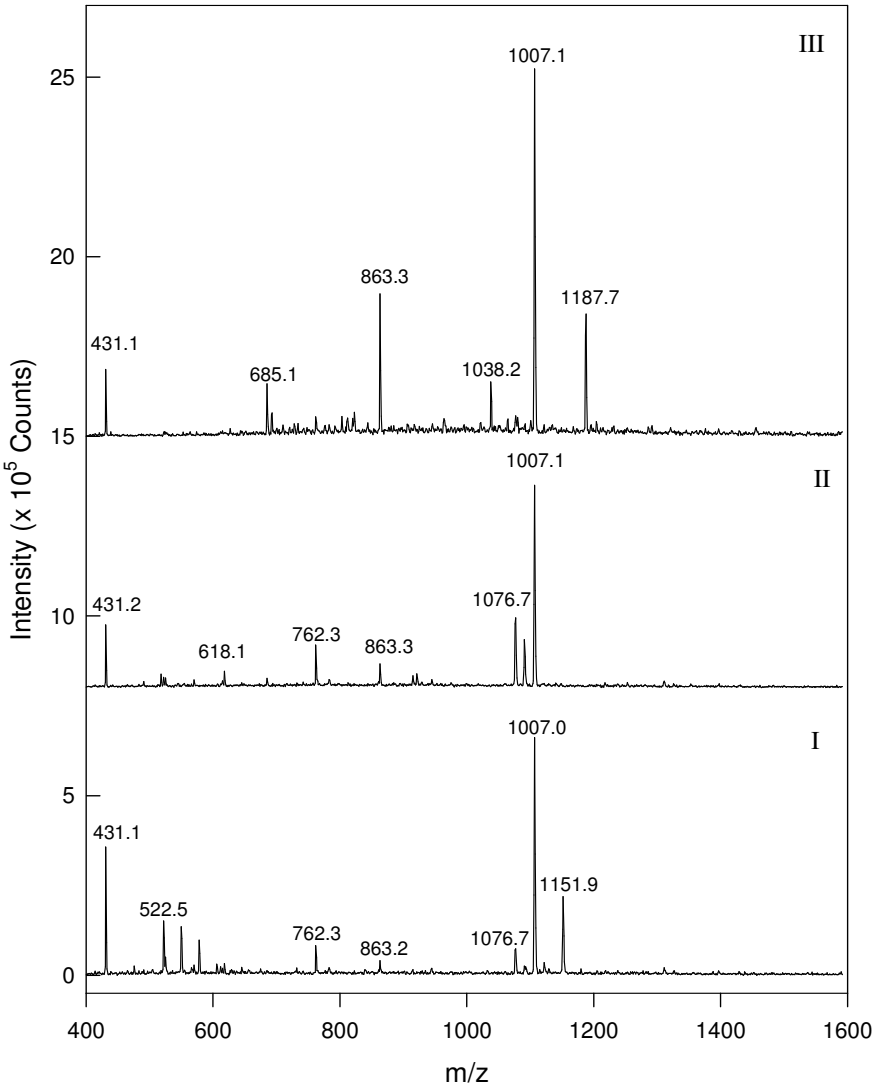

Figure 6. Recorded mass spectra for the peaks marked 1 of Figure 5.

The most predominant mass observed is 1007.0 Da, with additional less intense signals of 1151.9, 522.5 and 431.1 Da (no peaks were detected >1200 Da). All signals mentioned had a charge state of +1. Comparable mass spectra (curves II and III), with the predominant 1007 Da peak, were obtained for Peak 1 of the two other extractants. The mass spectra of peptides/proteins eluting between 15 and 50% acetonitrile revealed many peaks with masses ranging between 400 and 1500 Da (results not shown) indicating the presence of peptides. Tuning of the LCQ instrumental parameters was performed using the $[M+2H]^{2+}$ peak of angiotensin at 648 Da. Therefore, peaks of oligosaccharides were less likely to be observed.

MALDI-ToF MS. To verify the composition of the extracts a different type of ionization method was used for the urea and pH 12 (1h) extracts of USBM and SBM-H. The recorded MALDI-ToF mass spectra confirmed the absence of high molecular weight (>

2000 Da) material in the two extracts (results not shown). In none of the three extracts masses >2000 Da could be distinguished, indicating that the material released from the residues by these extractants was composed of peptides.

The instrumental conditions applied are comparable to Lakemond *et al* (*19*) in which the acidic (30-40 kDa) and basic (20 kDa) polypeptides of soy glycinin could be distinguished. This indicates that, with the chosen matrices and instrumental conditions, high molecular weight components would have been detected, if present.

Enzymatic Degradability of Aggregates

The enzymatic degradability by proteases of the aggregated peptides extracted from SBM-H with at pH 12 (10 min) was examined. The resulting chromatograms (280 nm) of the pH 12 extract and the resulting hydrolysate are shown in Figure 7.

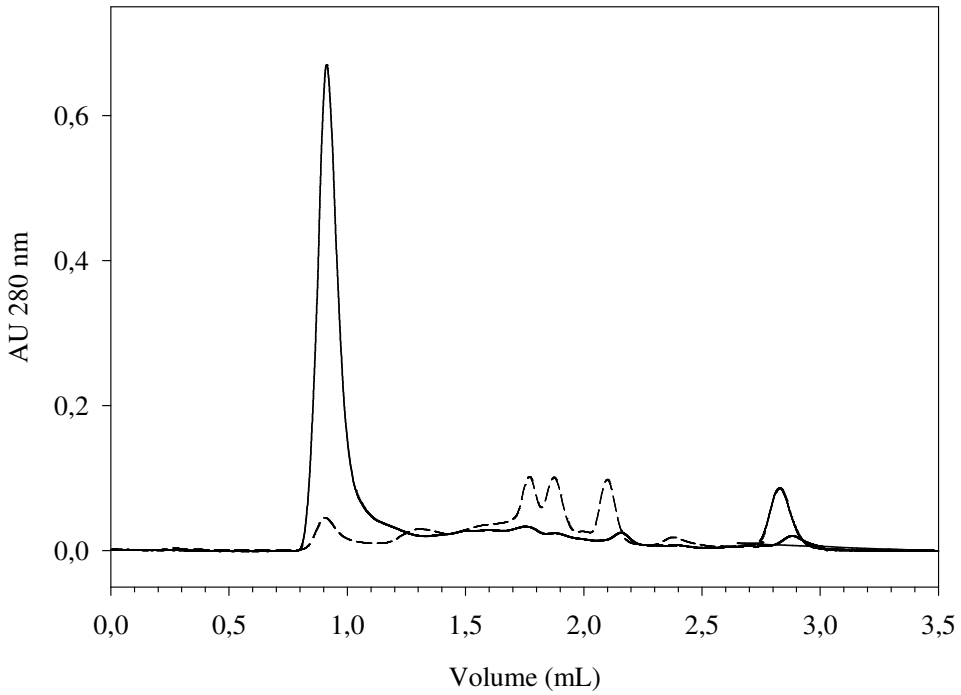

Figure 7. Gel permeation chromatogram (Superdex 75) of the pH 12 extract of the SBM-H residue and the enzymatic hydrolysate resulting after incubation with protease preparations: (solid line) pH 12 extract; (dashed line) hydrolysate [V_0 = 0.9 mL and V_t = 1.9 mL]. Samples were eluted with 35mM potassium phosphate buffer containing 0.1M NaCl, pH 7.6.

Aggregates of high molecular weight were observed in the alkali-extracted material but after proteolysis these aggregates were completely dissolved and four new peaks of much lower molecular weight appeared. Comparable results were obtained for the urea extract (results not shown) showing that the enzymatic degradation of the alkali-

extracted proteinaceous material should not be explained by alkali-induced deamidation of proteins during extraction.

It thus seems that the aggregates end up in the enzyme-unextractable residues during hydrolysis of the original meals simply because they are difficult to access for the enzymes and not because they resist enzymatic degradation. It could be speculated that interactions between peptide aggregates, formed during hydrolysis and other components of the enzyme-unextractable matrix somehow reduce the extractability of the protein material.

In conclusion, we believe that part of the extracted and hydrolyzed proteins become insoluble during hydrolysis because the peptides tend to aggregate after being released by the proteases (*20*, *21*). Aggregation of peptides is favored by the neutral pH conditions that exist during hydrolysis of the original SBMs. At higher (>9) pH conditions the peptides are more prone to solubilisation due to electrostatic repulsion (*22*), in line with the strong effect of alkaline extractants for protein extraction. Once extracted, the proteinaceous material can be hydrolysed by the enzymes. A high proportion of cellulose in the residues indicated a correlation between the insoluble cellulose and the formation of enzyme-unextractable peptides during hydrolysis of SBM proteins.

The mechanisms, which render the peptides insoluble during enzymatic hydrolysis thereby preventing a complete extraction of protein from soybean meals, are the topic of future experiments.

ACKNOWLEDGEMENTS

Harold Bult is greatly acknowledged for performing the statistical analysis of the amino acid data. Bas Kuipers is acknowledged for performing part of the experiments. Jan Cozijnzen from Wageningen University is thanked for help with carbohydrate analysis. This research was financially supported by Danish Academy of Technical Sciences, (Lyngby, Denmark).

ABBREVIATIONS USED

A, Alcalase; B, Biofeed Plus; DM, dry matter; E, Energex; EP, extractability of protein; EUS, enzyme-unextractable solids; EXUS, extractant unextractable solids; F, Fla-

vourzyme; SBM, soybean meal; SBM-H, soybean meal heat-treated at high humidity; SBM-L, soybean meal heat-treated at low humidity; USBM, unheated soybean meal.

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CHAPTER 5

PRESENCE OF INDIGESTIBLE PEPTIDE AGGREGATES OF SOYBEAN MEAL IN PIG ILEAL DIGESTA RESIDUE [♣]

[♣] Fischer,M.; Voragen,A.G.J.; Piersma,S.R.; Kofod,L.V.; Joergensen,C.I.; Guggenbuhl,P.; Nunes,C.S.; Gruppen,H. Presence of indigestible peptide aggregates of soybean meal in pig ileal digesta residue *Submitted to Journal of the Science of Food and Agriculture,* 2006.

ABSTRACT

With the purpose of analyzing the molecular size and composition, proteinaceous material was extracted from the insoluble components of a digesta sample obtained from pigs fed a feed consisting of only soybean meal. Gel permeation chromatography indicated that the alkali-extractable fraction of the proteinaceous material from the residue was of relatively high apparent molecular weight. However, the combined results from gel electrophoresis, RPLC-MS, and MALDI-ToF MS showed that the extracted protein material was in fact, to a high extent, composed of aggregated peptides. To our knowledge this has not previously been described. Aggregates extracted by dilute alkali were fully degraded upon subsequent proteolytic treatment. N-terminal sequencing of selected protein bands from SDS-PAGE gels indicated the presence of partly degraded β-conglycinin α-subunits in the residue.

Keywords: amino acids; enzymatic hydrolysis; ileal digesta; mass spectrometry; soybean meal; peptide aggregates; protein;

INTRODUCTION

Soybean meal (SBM) is a commonly used ingredient in animal nutrition. In feed applications the digestibility of the proteins is of primary importance. For this, the enzymatic accessibility of proteins in the feed matrix is a key issue. The use of exogenous enzymes in food and feed manufacturing has increased tremendously over the last decades. More recently, the enzymatic extractability of proteins from unheated and heat-treated soybean meals has been studied at *in vitro* conditions (*1*, *2*). Even with high dosages of carefully selected combinations of commercial endo- and exo-proteases, containing significant amounts of carbohydrase activities as well, the enzymatic extraction of protein remains incomplete (*1*). For heat-treated soybean meals (SBMs) it was shown that, independent of the heating conditions, a large proportion of the enzyme unextractable protein from SBM was composed of aggregated and precipitated peptides (*2*). Consequently, the proportion of undegraded or partly degraded polypeptides in the non-extracted fraction was limited. The majority of the aggregated soy peptides could be extracted by an alkaline treatment (0.1 mol litre^{-1} NaOH) and the aggregates solubilised could subsequently be degraded by the same proteases (*2*). It was hypothesized that interaction of the aggregates with other components of the soybean matrix could partly explain the precipitation observed and the incomplete proteolytic extraction of proteinaceous material from the soybean meals (*2*).

In vivo studies have shown that soybean meal proteins cannot be fully utilized by pigs (*3*, *4*, *5*, *6*). Most of the work published in this research area presents protein mass balances or focuses on the composition of the soluble part or enzyme extractable protein fractions of soybean meal in the gastro intestinal content. In view of that there is a scarcity of information on the composition of the insoluble proteinaceous material in digesta collected at the distal ileum from pigs fed a SBM feed. Obviously, this is proteinaceous material, which is not extracted by the hydrolytic activities in the digestive tract of the animal. In addition to unextracted dietary proteins, digesta of soybean meal might contain (partly degraded) digestive enzymes and other proteins e.g. mucin secreted by the animals. Mucin is a glycoprotein, secreted as part of the gastrointestinal mucus, that contributes to endogenous protein and carbohydrate levels in digesta (*7*, *8*, *9*). Mucin constitutes approximately 30, 7 to 22, 15 and 11 % of the endogenous threonine, proline, serine and protein, respectively, in ileal digest of pigs fed a protein free diet (*10*).

Mucin subunits, resulting from *in vivo* proteolysis of exposed regions of the protein, have been identified (*9*, *11*, *12*). Exhaustive proteolysis *in vitro* resulted in degradation of up to 30% of mucus protein (*8*, *13*). Still little is known about the recovery of mucin, or the extent of proteolytic digestion of mucin, at the distal ileum of pigs (*10*, *13*).

In order to investigate the reason why proteins present in soybean meals can not be fully utilized by pigs the present study focuses on the analysis of water-unextractable proteinaceous material from a digesta sample representing an average of 7 pigs fed a feed consisting only of SBM. The pooled digesta sample was analyzed with various analytical methods.

MATERIALS AND METHODS

Preparation of Pigs Digesta Residue

Digesta was collected from seven Large White growing pigs weighing on average 50.4 ± 3.1 kg at the beginning of the experiment. The animals were obtained from GAEC (Leclerc, Ostheim, France). Each animal was fitted with permanent cannulae in the gastrointestinal tract in the ileum 10 cm before the caecum as described by Nunes and coworkers (*14*). This operation allowed non-painful withdrawal of kinetic ileum contents. After surgery the animals were individually housed in cages allowing easy access to the intestinal cannulae for sampling. Between experiments animals were kept on a normal maintenance diet.

Only on the day of the trial each animal was fed 600 g of a SBM (Soy 44), a toasted meal obtained from a local supplier. The SBM was given at 7.45 a.m., after a 24 hours fasting period. Seven ileal contents samples were collected during the time intervals 30 minutes to 2 hours, 2 to 3 hours, 3 to 4 hours, 4 to 5 hours, 5 to 6 hours, 6 to 7 hours, and 7 to 8 hours post feeding. All samples collected from the seven pigs were kept on ice, pooled, mixed thoroughly and frozen at –80 °C.

To remove soluble digesta components the digesta was thawed and transferred to centrifuge tubes (500 ml) and centrifuged (30 min; 4000 g; 25 °C). The resulting residue was washed (three times) with 350 ml tap water, with intermediate centrifugation steps (30 min; 4000 g; 25 °C), until a clear supernatant was obtained. The residue was freeze-

dried and homogenized using a mortar and pestle. It was denoted 'Pigs Digesta Residue' (PDR) and stored at –20 °C for further analysis.

Extraction of Pigs Digesta Residue

PDR (500 mg) was suspended in 20 ml of 0.1 mol litre-1 NaOH. The suspension was stirred at 25 °C for 1 h and centrifuged (20 min; 10000 g; 25 °C). The supernatant obtained was adjusted to pH 7.6 with 1 mol litre-1 HCl and immediately subjected to further analysis. The remaining residue was washed twice with 50 ml of deionised water with intermediate centrifugation steps (20 min; 10000 g; 25 °C). The final residue was freeze-dried denoted 'Alkali-Unextractable Residue' (AUR) and stored at –20 °C for further analyses.

For LC-MS and MALDI-ToF MS analyses an additional extract was produced from PDR. PDR (150 mg) was suspended in 1.5 ml of 6 mol litre^{-1} urea in 35 mM potassium phosphate buffer, pH 7.6, in an Eppendorf tube and shaken in a Thermomixer comfort (Eppendorf, Germany) for 1 h at 700 rpm and 25 °C. Supernatants were separated from the residues by centrifugation (20 min; 12000 g; 25 °C) and were subjected to immediate analysis.

All extractions were performed in duplicate.

Enzymatic Degradability of Extracted Proteins

Two protease preparations were selected for the experiments. Alcalase Food Grade is a preparation from *Bacillus licheniformis*. It has mainly endoproteinase activities and an activity optimum between pH 6.5 and pH 8.5. Flavourzyme is a protease complex of endoproteinases and exopeptidases from *Aspergillus oryzae* with an activity optimum between pH 5.0 and pH 7.0. All enzymes were provided by Novozymes A/S (Bagsvaerd, Denmark) (*1*).

To study the enzymatic degradability of the proteinaceous alkali-extracted PDR material, an alkali extract was adjusted to pH 7.6 with 1 mol litre^{-1} HCl. Alcalase 2.4L FG (2.5 μlitre) and Flavourzyme 1000L (5 μlitre) (Novozymes A/S, Bagsvaerd, Denmark), were added to an alkali extract containing 100 mg of protein in a final volume of 1500 μlitre. Reaction was allowed for 5 h at 40 °C. The molecular size distribution of proteinaceous material in the parental extract and the hydrolysate obtained were subsequently determined by gel permeation chromatography.

Experiments were performed in duplicate.

Chemical Composition of Samples

The protein content (N x 6.25) of the different samples was determined by the Dumas combustion method using a NA 2100 Nitrogen and Protein Analyzer (ThermoQuest Italia, Milan, Italy). Approximately 20 mg of each sample was used for analysis.

Protein contents are means of three determinations.

Carbohydrate and amino acid composition of the different samples were determined according to previously described procedures (*2*). Carbohydrate contents are means of three determinations and amino acid contents are means of six determinations.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry of Soy 44 and PDR was performed using a micro-DSC (Setaram, France). Samples were suspended in 35 mmol litre^{-1} mM potassium phosphate buffer (pH 7.6) containing 0.1 mol litre^{-1} NaCl (I = 0.2). The protein concentration of the suspensions was 50 g litre^{-1}. Samples (0.9 mL) were scanned from 20 °C to 115 °C at a scanning rate of 1.2 K min^{-1} and subsequently cooled to 20 °C at a similar rate. The peak denaturation temperature (Tp), the temperature of the maximum heat flow, was read from the curve. The area of the major peaks of the Soy 44 sample was subsequently used to determine whether non-unfolded proteins, resisting proteolytic degradation, were present in the PDR.

Molecular Size Determinations

Gel Permeation Chromatography. The molecular size distribution of the proteinaceous material in the supernatants of the alkali-extracted PDR was determined using a Superdex 75 column (3.2 × 300 mm) connected to an ÄKTA-system. One set of samples was diluted (10 times) using 35 mmol litre^{-1} potassium phosphate buffer (pH 7.6) containing 0.1 mol litre^{-1} NaCl. A volume of 50 μlitre was applied onto the column. Another set of samples was diluted using 6 mol litre^{-1} urea in 35 mmol litre^{-1} potassium phosphate buffer (pH 7.6) containing 0.1 mol litre^{-1} NaCl.

The molecular size distribution before and after enzymatic hydrolysis of an alkaline PDR extract (25 °C; 1h) was determined using a Superdex 75 column (3.2 mm × 300 mm) connected to a SMART-system. Extract and hydrolysate were diluted (20 times)

using a 35 mmol litre^{-1} potassium phosphate buffer containing 0.1 mol litre^{-1} NaCl, pH 7.6.

For all samples separation took place at a flow rate of 80 μlitre min^{-1} using the respective dilution buffers. The absorbance of eluates was monitored at 214 and 280 nm. The void volume (V_0) and the total volume (V_t) of the column was determined using molecular weight standards. Presented chromatograms were verified by duplicate injections and processed using Unicorn software.

Chromatographic equipment, GPC columns and molecular standards and software were from GE Healthcare (Uppsala, Sweden).

SDS-PAGE. Analysis was performed using a Protean-system. Tris-HCl pre-cast gels (10-20%) were used for separation of proteins (10–100 kDa) and Tris-Tricine pre-cast gels (10-20%) were used for separation of peptides (1–40 kDa). Separation unit and gels were from BioRad (Hercules, CA, USA).

The PDR and the supernatant from the alkaline extraction were both subjected to analysis. The supernatant was mixed with sample buffer (1:1) and boiled for 5 min at 100 °C. An aliquot 25 μlitre was loaded onto the gel. To extract proteinaceous material from the PDR a sample (10 mg) was mixed in an Eppendorf tube with 500 μlitre of a reducing sample buffer containing SDS (25 g kg^{-1}) and β-mercaptoethanol (0.25 g kg^{-1}). The efficiency of protein extraction with this method was ~90%. The suspension was subsequently incubated (100 °C; 30 min) in a Thermomixer comfort (Eppendorf, Germany). Finally, the suspension was separated by centrifugation (10 min; 15000 g; 25 °C). An aliquot (25 μlitre) of the supernatant obtained was loaded onto the gel after appropriate dilution.

The two gel types were processed at 200 V, 20 mA for 50 min. Tris-HCl gels were stained by Coomassie Brilliant Blue and destained with 300 ml litre^{-1} methanol, 100 ml litre^{-1} acetic acid in deionised water according to the instructions of the manufacturer. The Tris-Tricine gels were developed by using a PlusOne protein silver staining kit (GE Healthcare, Uppsala, Sweden) according to the instructions of the manufacturer. Gels were scanned using a Computing Densitometer from Molecular Dynamics (Sunnyvale, CA, USA).

N-terminal Sequencing

Sequencing was used to identify selected polypeptides/proteins which could be extracted from the PDR by SDS-PAGE sample buffer according to above described procedures.

After cooling, the extract was re-heated for 5 min at 100 °C in reducing sample buffer and loaded onto a 10-20% Tris-HCl gradient gel (BioRad). The gel was processed according to the instructions of the manufacturer. Blotting was performed using a Mini Trans-Blot® Cell (BioRad) and Hybond-P, a PVDF transfer membrane (GE Healthcare, Uppsala, Sweden). The protein transfer buffer was a 40 mmol litre^{-1} Tris buffer (pH 8.3) containing 40 mmol litre^{-1} boric acid, and 1 mmol litre^{-1} EDTA. The gel and the membrane were prepared for transfer according to the instructions of the manufacturer and subsequently processed for 1 h at 100 V/0.3 A with cooling. The membrane was stained for 5 min using a standard Coomassie Brilliant Blue staining solution, with 5 ml litre^{-1} acetic acid, followed by destaining for 1 min with 500 ml litre^{-1} methanol containing 100 ml litre^{-1} acetic acid. The membrane was air-dried and bands were selected for sequencing. Due to N-terminal blockage of most of the extracted polypeptides selected polypeptide bands were cut-out from the PVDF membrane and incubated (24 h) in 50 μlitre of a saturated solution of CNBr in 700 ml litre^{-1} formic acid. The hereby obtained mixtures of peptides derived from the original polypeptide were still attached to the membrane. Samples were applied to the sequencing apparatus and subjected to an automated N-terminal Edman degradation method using a Procise 494A (Applied Biosystems, San José, CA, USA). Peptide sequences were identified using the FASTF program (the 'mixed peptides versus protein database' (http://fasta.bioch.virginia.edu) that compares an ordered peptide mixture, as obtained by Edman degradation of a CNBr cleavage of a protein, against a protein (fastf) or DNA (tfastf) database - and by general search in public sequence databases.

Liquid Chromatography Mass Spectrometry. The molecular mass of the alkali and urea extracted proteinaceous material from PDR was determined by liquid chromatography mass spectrometry (LC-MS). A 218MS C18 reversed phase column (2.1 mm × 250 mm) from Vydac (Hesperia, CA, USA) was connected to an HPLC system from Spectra Physics (San José, CA, USA). Mass spectrometric analysis was performed using a MAT 95 LCQ ion trap mass spectrometer from Thermo Finnigan (San José, CA, USA).

MALDI-ToF Mass Spectrometry. Protein and peptides were cleaned up from the alkali and urea extracts using ZipTip C18 reversed phase tips from Millipore (Bedford, MA, USA) according to the instructions of the manufacturer. Matrix Assisted Laser Desorption Ionization Mass Spectra (MALDI-ToF MS) were recorded on a PerSeptive Voyager DE-RP (PerSeptive Biosystems, Framingham, MA, USA) mass spectrometer equipped with delayed extraction technology. Sinapinic acid and α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) were used as matrices. All other instrumental settings, methods and procedures were performed as previously described (*2*).

Statistical Variation

The extraction procedures have a standard deviation of 0.5%. Determinations of proteins and carbohydrates have standard deviations of 0.2% and 0.5%, respectively (*2*).

RESULTS & DISCUSSION

Composition of Residue

Effect of Alkaline Extraction. The PDR contained 117 g kg^{-1} protein of which 76 g kg^{-1} (~65% (w/w) was alkali-extractable. A comparable solubilising effect of proteinaceous material by 0.1 mol $litre^{-1}$ NaOH has previously been found when studying the composition of protein in residues from an *in vitro* hydrolysis of SBM using high concentrations of proteases and carbohydrases (*2*). The carbohydrate content of PDR was 620 g kg^{-1}, of which 110 g kg^{-1} (~18% (w/w) was alkali-extractable. Accordingly, the carbohydrate content of AUR was 710 g kg^{-1}. In total 22% of the dry matter in PDR could be extracted with alkali.

Protein. The protein contents and amino acid compositions of the original Soy 44 soybean meal, PDR and AUR are presented in Table 1.

Table 1. Protein content (g kg^{-1}) and amino acid composition (molar %) of Soybean meal 44, PDR, AUR and crude mucin (CM)

	SBM	SD	PDR	SD	AUR	SD	CM[e]	SD
Protein[a]	480	-	117	-	52	-	n.d.	-
Ala	7.5	± 0.08	8.8	± 0.24	8.5	± 0.15	8.6	± 0.35
Arg	5.8	± 0.11	4.2	± 0.42	4.8	± 0.09	3.4	± 0.14
Asx[b]	12.8	± 0.09	7.6	± 0.40	10.2	± 0.50	10.1	± 0.09
Cys	1.0	± 0.07	0.6	± 0.12	0.6	± 0.06	n.d.	
Glx[c]	16.2	± 0.08	14.3	± 0.33	15.7	± 0.19	9.8	± 0.06
Gly	8.4	± 0.07	13.0	± 1.30	10.4	± 0.54	11.7	± 0.35
His	2.4	± 0.06	1.9	± 0.45	2.1	± 0.16	2.0	± 0.06
Ile	t	-	t	-	t	-	4.5	± 0.11
Leu	6.6	± 2.95	6.5	± 2.96	7.0	± 3.14	6.8	± 0.05
Lys	5.8	± 0.16	5.4	± 0.32	6.1	± 0.17	5.6	± 0.27
Met	t	-	t	-	t	-	1.1	± 0.10
Phe	4.5	± 0.06	3.9	± 0.34	4.5	± 0.14	3.2	± 0.04
Pro	6.6	± 0.11	9.1	± 0.18	7.7	± 0.18	9.5	± 0.99
Ser	7.6	± 0.07	8.1	± 0.49	7.5	± 0.14	10.2	± 0.31
Thr	5.1	± 0.07	4.7	± 0.52	4.9	± 0.08	12.9	± 0.56
Trp[d]	-	-	-	-	-	-	-	-
Tyr	2.7	± 0.06	2.6	± 0.14	2.5	± 0.07	2.8	± 0.14
Val	5.6	± 0.05	6.6	± 0.19	6.1	± 0.16	7.3	± 0.13

All data are based on six determinations. t=trace amount
[a] *Calculated from Kjeldahl N x 6.25. Expressed as g kg^{-1} of dry matter*
[b] *The sum of asparagine + aspartic acid*
[c] *The sum of glutamine + glutamic acid*
[d] *Fully destroyed during hydrolysis*
[e] *CM: crude mucin. Adapted from Lien et al,1997 (15)*

Soy 44 had a protein content of 480 g kg^{-1}. The protein content (w/w) of the PDR and AUR was 117 g kg^{-1} and 52 g kg^{-1}, respectively. The amino acid composition of the soybean meal proteins was comparable to data reported in literature (*16*). The comparable amino acid composition of Soy 44 and PDR (Table 1) indicated a non-specific extraction/degradation of soybean meal proteins by the digestive enzymes. In literature crude mucin (*9*, *10*) is reported to have a high total content (30-40 mol/100 mol) of threonine, serine and proline (Table 1). In the SBM and PDR residue the total content of threonine, serine and proline was 19.3 mol/100 mol) and 21.9 mol/100 mol), respectively. Based on these figures, there is no indication that the PDR is rich in proteina-

ceous material originating from mucin, This shows that the largest part of mucin in the ileal digsta stays in the soluble fraction of the digesta.

A comparable amino acid composition of the AUR and the PDR showed that the alkaline extraction procedure did not favour extraction of specific amino acids/ polypeptides from PDR.

Carbohydrates. The molar monosaccharide composition of the carbohydrate fraction of Soy 44 (Table 2) showed that, prior to ingestion by the pigs, glucose was the most abundant monosaccharide constituent (36%), followed by galactose (24%), uronic acids (17%), arabinose (10%), xylose (8%), and mannose (4%).

Table 2. Carbohydrate composition of Soy 44, PDR and AUR

	Carbohydrate composition[a]										
Sample	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[b]	GlcNac	GalNac	Total[c]
Soy 44	1	0	10	8	4	24	36	17	0	0	31
PDR	1	0	14	10	3	23	26	22	0	0	62
AUR	1	0	15	11	4	23	29	18	0	0	71
PM[d]	0	5	0	0	0	2	0	0	50	43	n.d.

All data are based on triple determinations
[a] *Composition of carbohydrates, presented as molar % (mol/100mol)*
[b] *UA (uronic acids) is the sum of glucuronic acid and galacturonic acid*
[c] *Carbohydrate content (expressed as g /100g dry matter $^{-1}$). Determined as the sum of neutral sugars + uronic acids*
[d] *PM: pure mucin. Compiled from Ronchetti et al, 1997 (17).*

A comparable composition was found for the PDR with a noticeable decrease (from 36 to 26%) in the molar proportion of glucose. Although starch only represents 3-4% of the total carbohydrates in soybean meal (*18*), this observed decrease in glucose is likely due to starch solubilisation and degradation by pancreatic amylase. Furthermore, water soluble oligosaccharides like stachyose and raffinose contain glucose (*18*). The molar monosaccharide composition significantly differed from that of mucin (Table 2), once more indicating that the PDR does not consist of large amounts of mucin.

As a result of alkaline extraction of protein material from PDR the proportion of carbohydrates in AUR was increased by about 15% (from 620 g kg^{-1} to 710 g kg^{-1}). Nevertheless a comparable molar composition of the PDR and AUR residues was found.

Protein Conformation

The DSC thermograms of Soy 44 and PDR are shown in Figure 1.

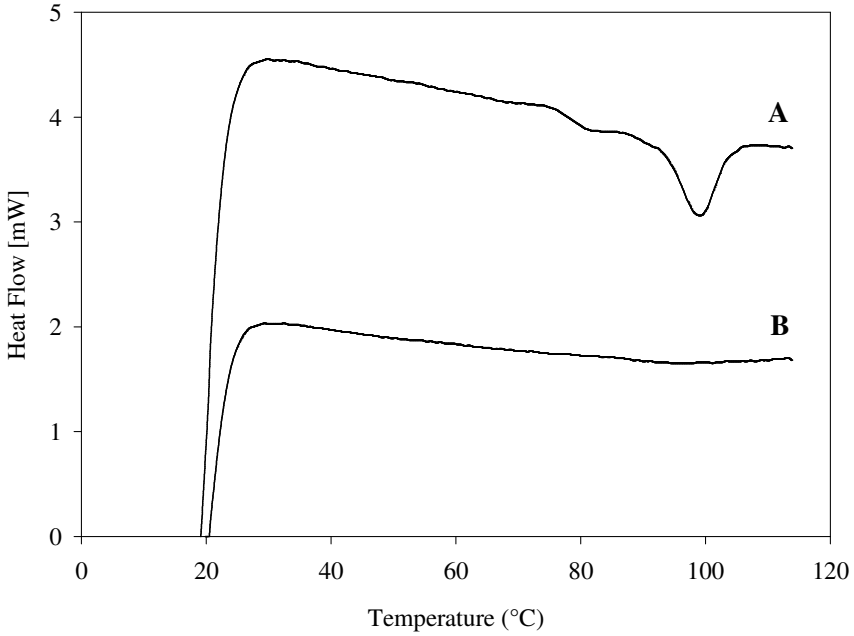

Figure 1. DSC-thermograms of Soy 44 (A) and PDR (B) protein suspensions.

Soy 44 (curve A) shows two endothermic transitions with peak temperatures (Tp) of approximately 80 °C and 100 °C. These peaks correspond to the denaturation of the major proteins, 7S β-conglycinin and 11S glycinin, respectively (*19*, *20*) and show the presence of intact proteins in the meal (*1*). The absence of transition peaks in PDR (curve B), however, show that intact soy proteins were no longer present in the PDR after passing the low pH conditions in the stomach and the action of the digestive enzymes through the stomach and small intestine. This finding is in line with previously published results (*1*) showing that, with an appropriate selection of proteases, incomplete unfolding of soy proteins is not limiting for the efficiency of proteolytic extraction of protein from SBMs by commercial protease preparations (*1*). This is in agreement with the observation that the efficiency of degradation of the major soy proteins de-

pends largely on the selection of proteases (*21*, *22*, *23*, *24*, *25*, *26*, *27*). The composition of the proteinaceous material of PDR was subsequently studied in further detail.

Molecular Size of Extracted Material

The gel permeation chromatograms obtained (280 nm) for the alkaline extract of the PDR using a phosphate/NaCl and a phosphate/NaCl/urea buffer system are shown in Figure 2.

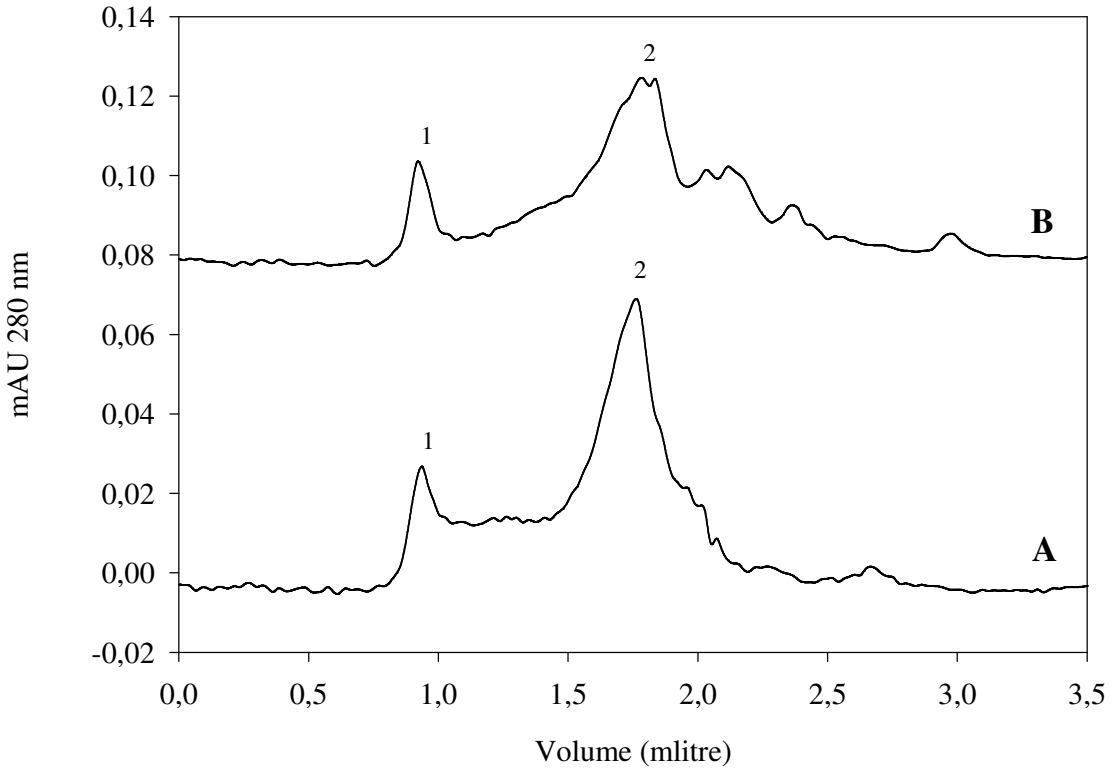

Figure 2. Gel permeation chromatograms of alkaline extract of (PDR) following dilution in two different buffers: A) 35mM potassium phosphate buffer containing 0.1M NaCl, pH 7.6 and B) buffer A + 6M urea, pH 7.6. Eluting peaks are denoted 1 and 2, respectively [V_0 = 0.9 ml and V_t = 1.9 ml]. AU = absorbance units.

For both buffers used two major peaks (marked 1 and 2) were found. After dilution and elution in phosphate buffer (Figure 2, curve A) peak 1, eluting at V_0 (> 70 kDa), contained approximately 15% of the extracted proteinaceous material. Peak 2, eluting between 1.5 and 1.9 ml retention volume, contained proteinaceous material (about 85% of total peak area) with apparent molecular masses ranging from 5 kDa to about 14 kDa. Dilution and elution of the alkaline extract in urea (Figure 2, curve B) clearly affected the appearance of the two major peaks, in particular of peak 2. The results show that

hydrogen bonds, which could be broken by urea, were present in the extracted material of peaks 1 and 2. A specific interaction of the extracted material with the column material caused the urea solubilised material to elute after the included volume (V_t ~1.9 ml) as seen by the appearance of several minor peaks eluting after peak 2 of curve B. This indicates a more hydrophobic character of the urea solubilised material.

SDS-PAGE analysis of the alkali-extracted proteinaceous material showed no electrophoretically recognizable products on a Tris-HCl (10–20%) protein gel (results not shown). Also, the Tris-tricine gel (10–20%) that is optimized for peptide identification revealed no peptide bands (results not shown). This shows that all protein material of the alkali extractable fraction (representing 65% of the proteinaceous material of the PDR) had a molecular size smaller than 1 kDa (equivalent to peptides of 8–10 amino acids). This does not comply with the apparent higher molecular size of the alkali-extracted material indicated by the gel permeation chromatograms of Figure 2. The apparent higher molecular size indicated by the GPC results is likely due to the presence of peptide aggregates, which are formed during proteolytic degradation of the soybean proteins (*2*), and which are only partly affected by the urea containing GPC buffer (*28*, *29*, *30*).

Several distinct polypeptide bands appeared in the electrophoresis gel of the PDR sample (Figure 3, lane 1).

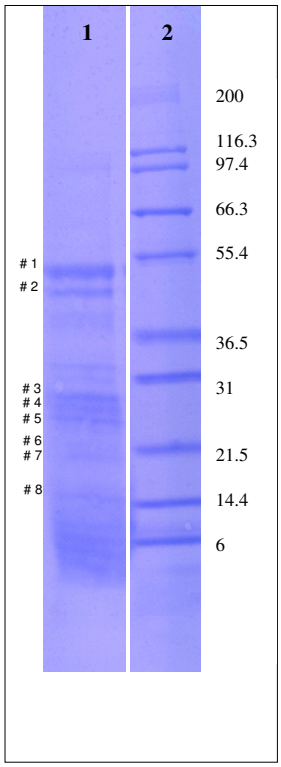

Figure 3. SDS-PAGE of PDR extract prepared using electrophoresis sample buffer. Lane 1, extract (diluted 10 times); Lane 2, molecular markers. N-terminally sequenced bands are marked #.

Since no electrophoretically recognizable polypeptides were observed in the alkaline extract, these bands represent material not extracted from PDR by 0.1 mol litre^{-1} NaOH. Polypeptide bands were selected for N-terminal sequencing. Database search lead to the following identifications (in cases where proteins could not be identified unambiguously based on the peptide sequences the sequences are shown): **#1**, 55 kDa, not identified. Mixed peptide sequences were e.g.: ADRNVADTDNT, YGPIAVTIQIG, THKLKFGGRKY; **#2**, 48 kDa, β-conglycinin α-subunit fragment (soy); **#3,** 30 kDa mix of: elastase 2 (pig), β-conglycinin α-subunit fragment (soy), trypsin precursor (pig); **#4**, 27 kDa 7S seed globulin precursor (soy); **#5**, 24 kDa trypsin precursor (pig); **#6**, 22 kDa, not identified and no sequence; **#7**, 18 kDa, β-conglycinin α-subunit fragment (soy); **#8**, 16 kDa, β-conglycinin α-subunit fragment (soy). In addition the bands in the MW range 6-16 kDa were sequenced together and contained a mix of soybean β-conglycinin α-subunit fragments.

The results show that part of the extracted polypeptides were endogenous digestive enzymes (elastase, trypsin) from the pigs, that apparently could not be removed during the intensive washing of the ileal digesta residue. A generally effective enzymatic degradation of the SBM proteins in the pig was indicated, since only two of the extractable pro-

tein bands originated from soy. In particular the high abundance of the α-subunit of β-conglycinin indicated that this protein is more resistant to proteolytic degradation by the digestive enzymes of the pigs than other subunits of the soy proteins. The fact that mucin protein was not present among the polypeptides indicated that the mucin protein is degraded to high extent or/and stays in the soluble fraction of the digesta, which is removed during the washing of the digesta.

Surprisingly, many of the protein bands from the digesta were N-terminally blocked and accordingly had to be digested with CNBr to obtain peptides with free N-terminals for sequencing and database identification. This indicates that the soy proteins, and maybe diet protein in general, are modified during stomach and intestinal passage in the pigs. Such modifications were not observed of polypeptides extracted from enzymatic residues produced with exogenous microbial proteases *in vitro* (*2*).

Molecular Masses of Alkali- and Urea-Extracted PDR

The reversed phase HPLC chromatograms (214 nm) obtained for the alkali- and urea-extracted proteinaceous material of PDR are presented in Figure 4, curves A and B.

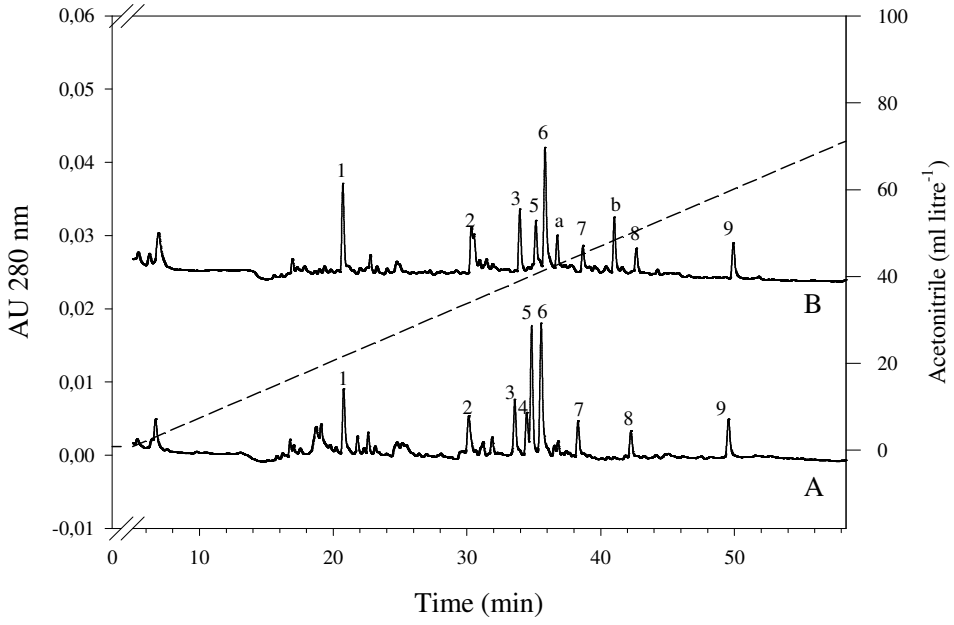

Figure 4. LC-MS chromatogram of PDR after alkaline (A) urea (B) extraction.

Comparable results were obtained for the two conditions of extraction. Essentially all proteinaceous material eluted between 150 ml litre^{-1} and 600 ml litre^{-1} acetonitrile,

which is in line with the general range of peptide hydrophobicity (*31*). Qualitatively, the two chromatograms are comparable, although not identical. Except for one distinct peak (peak 1) a complex mixture of low abundant peptide/protein material was eluting between 150 ml litre^{-1} and 280 ml litre^{-1} acetonitrile. For the alkaline extract (curve A), a range of distinct peaks (marked 2-9, respectively) is distinguished eluting between 300 ml litre^{-1} and 600 ml litre^{-1} acetonitrile. A comparable chromatogram was seen for the urea extracted proteinaceous material (curve B) except from the appearance of two additional peaks (marked a and b, respectively).

Mass spectra from 400 - 1800 Da were acquired for all peaks in the chromatogram confirming the low molecular size of proteinaceous material indicated by SDS-PAGE analysis. Typical mass spectra for selected peaks of PDR after extraction by 0.1 mol litre^{-1} NaOH are shown in Figure 5 for peak 2 eluting at 33 ml litre^{-1} acetonitrile (Figure 5A), peak 6 eluting at 40 ml litre^{-1} acetonitrile (Figure 5B), and peak 9 eluting at approximately 60 ml litre^{-1} acetonitrile (Figure 5C).

The most predominant masses observed are 453.27, 566.40, 605.20, 718.33, 854.07, 984.00, and 1268.80 Da. A series of additional, less abundant, signals were observed with masses ranging between the above mentioned masses. The 275.60 mass present in all mass spectra is a contamination from the plastic container in which the PDR was stored.

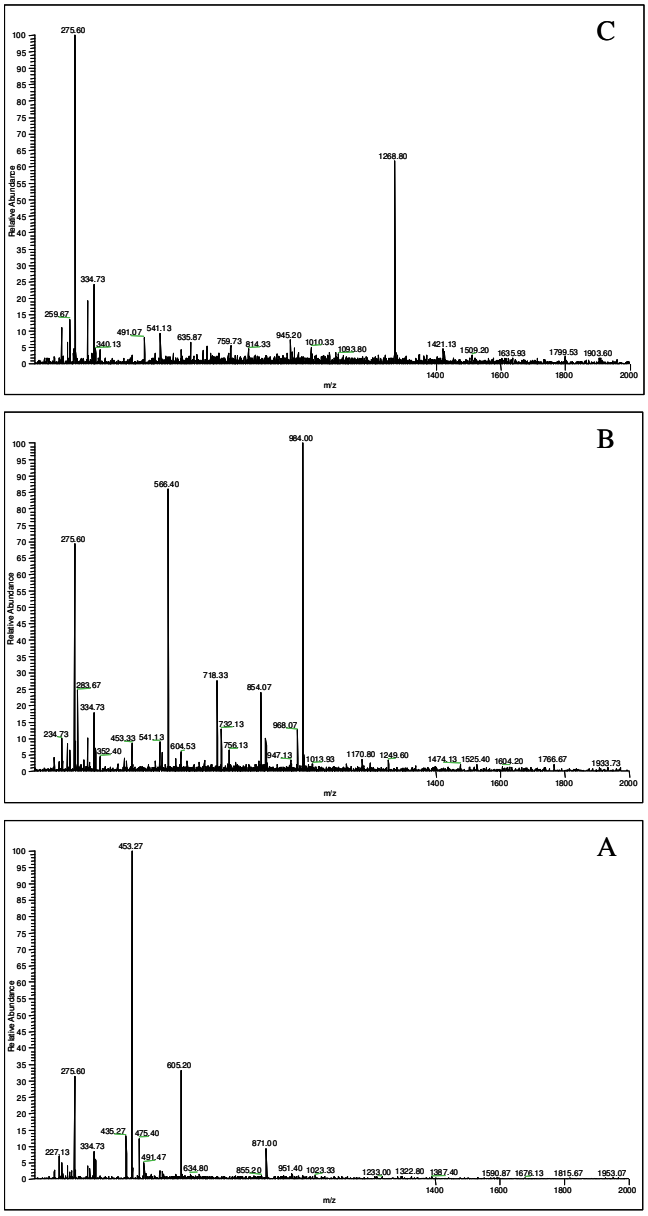

Figure 5. Recorded mass spectra of an alkaline extract of ileal pigs digesta residue (PDR). The three mass spectra shown (panels A-C) correspond to peaks 2, 6 and 9 of Figure 4.

Comparable mass spectra were acquired for the urea extract of PDR. Peak a of Figure 4, curve B, contained two predominant masses of 943.1 Da and 912.9 Da. In peak b the predominant masses were 230.2 Da and 459.3 Da (MS results not shown). The mass spectra of peptides / proteins eluting between 15 ml litre^{-1} and 60 ml litre^{-1} acetonitrile revealed many smaller peaks with masses ranging between 230 Da and approximately 1500 Da (results not shown) indicating the presence of low abundant peptides. The in-

strumental parameters of the LCQ were tuned using the $[M+2H]^{2+}$ peak of angiotensin at 648 Da. Therefore, peaks of oligosaccharides are not likely to be observed.

The low molecular size of the alkali- and urea-extracted proteinaceous material indicated by mass spectrometric measurements was subsequently confirmed by MALDI-ToF Mass Spectrometry, a different ionization method. The recorded MALDI-ToF mass spectra confirmed that all proteinaceous material extracted at both conditions had a molecular size <1800 Da (results not shown). It has previously been shown that the different subunits of soy glycinin are distinguishable at comparable instrumental settings (*32*).

Enzymatic Degradability of Aggregates

The enzymatic degradability by protease preparations of the alkali-extracted aggregated peptides in PDR was examined. The chromatograms (280 nm) of the extract and the hydrolysate produced are shown in Figure 6.

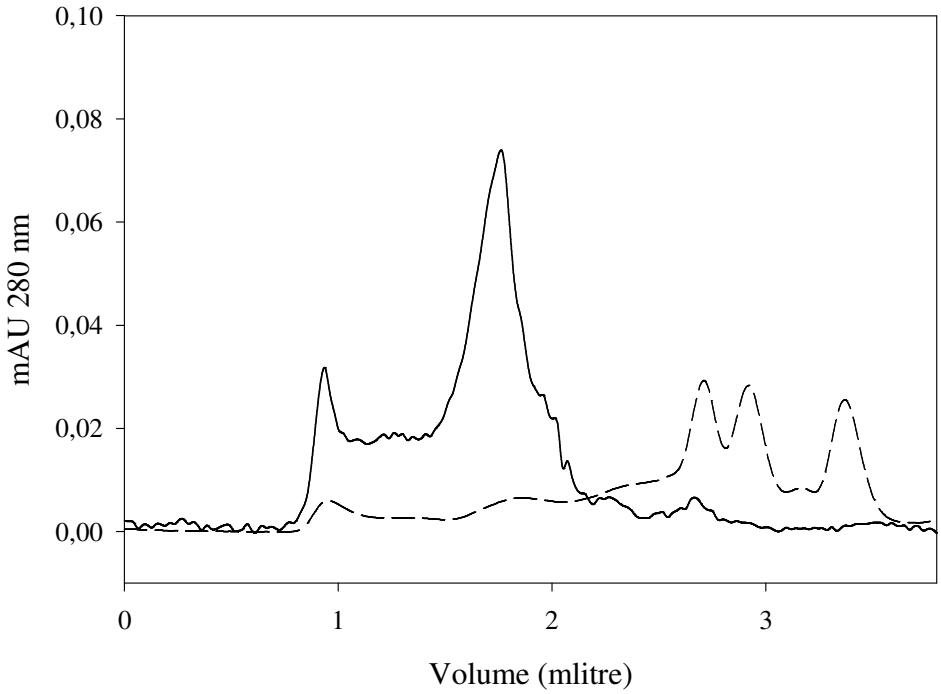

Figure 6. Gel permation chromatograms of alkaline extract of PDR and the enzymatic hydrolysate obtained after incubation of the extract with two commercial protease preparations. Solid line, alkaline extract; dashed line, enzymatic hydrolysate.

Aggregates of high molecular weight were observed in the alkali-extracted material, but after proteolysis these aggregates were almost completely degraded. As a result three

smaller peaks appeared with a retention volume corresponding to a lower molecular size. A hydrophobic character of the enzymatically degraded material was indicated, since all the new peaks were eluting after V_t (1.9 ml).

Peptide aggregates, of comparable molecular size to the ones from PDR, were also highly abundant in residues from *in vitro* hydrolysis of SBM proteins using two commercial protease preparations. Like for the protein material extracted from ileal digesta these other aggregates could also be degraded by commercial protease preparations (*2*). The comparable composition of the aggregated protein material from these two soy residues indicate that the aggregates of the alkaline extract of PDR are primarily derived from the soy proteins and do not contain much endogenous protein.

In conclusion, the results presented indicate a high efficiency of the digestive enzymes of the pig for extraction/degradation of SBM proteins. Naturally, a large proportion of the soluble protein material is readily absorbed by the animal or stays in the soluble fraction of the ileal sample, which was not the subject for investigation in the current study. From the present experiment, however, it is evident that insoluble peptide aggregates are formed during hydrolysis of the soy proteins in the stomach or the ileal part of the pigs gastro-intestinal system that become inaccessible to the digestive enzymes of the animals. Poor accessibility (steric hindrance) of the digestive enzymes to these peptide aggregates or interaction of the aggregated material with other components could partly explain the incomplete enzymatic protein extraction observed. We are currently investigating this subject.

ACKNOWLEDGEMENTS

This research was financially supported by Danish Academy of Technical Sciences, (Lyngby, Denmark).

ABBREVIATIONS USED

AUR, alkali-unextractable residue; DSC, Differential Scanning Calorimetry**;** GPC, gel permeation chromatography; HPLC, high performance liquid chromatography; RPLC-MS, reversed phase liquid chromatography - mass spectrometry; MALDI-ToF MS, matrix assisted laser desorption / ionization time-of-flight mass spectrometry; PDR, pigs digesta residue; SBM, soybean meal.

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CHAPTER 6

GENERAL DISCUSSION

Introduction

When this thesis work was initiated in 1998, it was based on the assumption that protein solubilisation from soybean meal is to a certain extent limited by the complexity of the matrix components of the soy cell wall (*1*, *2*). This complexity, in combination with varying forms of heat treatment, is leading to reduced accessibility of the substrate to the enzymes (*3*, *4*). Release of, assumingly, encapsulated protein has been explained by enzymatic disruption of the cell wall matrix, which resulted in a more effective degradation of proteins by the digestive proteases or/and exogenous proteases (*5*, *6*). Later on, Ouhida and coworkers (*7*) reported that incubation of water insoluble polysaccharides in soybean cotyledons with pectinase, cellulase, and xylanase resulted in the release of only low amounts (not more than 5% bound basis) of monosaccharides, mostly uronic acids, xylose, and arabinose. Protein extraction hardly increased after enzymatic incubation. Although recent literature has identified pectinases which are capable of degrading residual pectin structures from okara (soybean milk residue) only a limited effect on protein release was reported (*8*). In the recent work of Rosenthal and co-workers (*9*) and Kasai and co-workers (*10*) proteases were the only enzymes that resulted in a definite and effective increase in protein extraction.

In the mean time the results of this thesis showed that a large proportion of the soy proteins are actually accessible to the proteolytic enzymes but the efficacy of proteolytic extraction is to varying degree affected by peptide aggregation. In this chapter the most important findings of the experiments presented in the preceding chapters are discussed.

Enzymatic Hydrolysis of SBM Proteins

Optimisation of the hydrolysis of soybean meal proteins is not straight-forward. The optimization efforts are complicated by e.g. the variation of the composition of the soybean meals due to e.g. different varieties, growth conditions and different conditions for

heat-treatment (*11*). In addition, several parameters (*12*), including pH, choice of enzyme(s) and their substrate specificity, enzyme dose, and incubation time affect the efficacy of enzymatic processing of soybean proteins. In this thesis only the above mentioned parameters were studied.

For the first experiments (Chapter 2) the hydrolysis conditions (e.g. enzymes, dose and pH) were selected based on previous experience with the enzymes within Novozymes. The experiments were performed with selected proteases and carbohydrases to understand the extent to which the given combinations and dose of the enzymes could degrade differently heated proteins in the SBM matrix. In addition, degradation of purified proteins was studied to understand the effects of the SBM matrix (*13*). For this purpose solubilisation of protein from unheated SBM (USBM) and differently heat-treated SBMs: SBM-H (autoclaved at high humidity), SBM-L (heated at relatively low (15%) humidity), and NN (pelleted at ~85°C) was studied following incubation with combinations of two proteolytic enzymes (Alcalase, Flavourzyme) and two carbohydrase preparations (Energex and Biofeed Plus). Although the water extractability of protein from the different meals varied between 13 and 67%, proteolytic treatment effectively extracted the proteins to a final protein solubilisation of 89 – 94%. An important observation is that the final protein solubilisation level in the different samples differed only to a limited extent. This shows that the proteolytic enzymes could largely compensate for the negative effects of, primarily, dry heat treatment on protein solubility. This means that, although the nutritional implications of the different forms of heat treatment were not considered in this connection, the choice of SBM (high versus low extent of protein denaturation) for different foods and feeds could in some cases become less critical, given that appropriate exogenous protease(s) are selected for the application.

Another important finding is that protein solubilisation was as effective (up to 94%) with proteases alone as it was for the combination of proteases and carbohydrases in spite of a relatively effective solubilisation of soybean pectins by the pectinase preparation used (*13*). This indicates that these 94% of the total soy proteins are not encapsulated, at least not to extents that render them inaccessible to the proteases. When this finding is combined with results from Chapter 4, which show the presence of aggregates in the enzyme-unextractable residues, it becomes evident that only a limited proportion of the proteinaceous material from the enzyme-unextractable material is present in the

form of polypeptides. This implies that the polypeptides are effectively extracted and degraded by the enzymes. However, part of the SBM proteins that are hydrolysed in the process later on become insoluble because the released peptides aggregate and precipitate. At *in vitro* conditions the aggregation process is favored by the neutral pH conditions (*14*) chosen for the hydrolysis. Therefore, larger aggregates are formed that counteract the complete solubilisation of soy proteins. When alkali is used for extraction electrostatic repulsion makes the peptides in the aggregate soluble. Once released from the residues, the aggregated material can be degraded to lower molecular size by proteolytic enzymes.

Similarities Between *In Vitro* and *In Vivo* Samples

The comparable composition of proteinaceous material from the enzyme-unextracable ileal digesta sample (Chapter 5) (*15*) and that of *in vitro* derived enzyme-unextractable material (Chapter 4) (*16*) is another important discovery of this thesis. In contrast to the *in vitro* residues, which are produced in a batch system, the ileal digesta sample is produced at dynamic conditions in the pig with a complementary range of digestive enzymes optimized through evolution. The nutrients released are continuously absorbed. Consequently one would expect a lower probability of intermolecular interactions (e.g. hydrophobic) between peptides released, interactions that eventually lead to aggregation behavior. However, the results clearly show that peptide aggregation is also occurring in the pigs.

As stated above, a surprisingly comparable composition of the enzyme-unextractable material of the enzyme-unextractable *in vitro* and *in vivo* residues is observed. Proteins constitute about 15-20% and carbohydrates about 50-60% of both residues. For both conditions an effective proteolytic extraction/degradation of protein from the meals was shown by the low abundance of "intact" polypeptides in the residues. However, complete extraction of protein from SBMs seems extremely hard to reach. The primary proportion of the enzyme unextractable proteinaceous material was composed of peptides of relatively low molecular weight (≤ 1200 Da), which had aggregated and precipitated during protein hydrolysis. These findings were rather surprising considering the different conditions for protein hydrolysis *in vitro* and *in vivo* and the different proteases concerned. Consequently, it was an inspiration to study the effects of

a selection of commercial proteases on the proteins of the intact ileal digesta sample, which contains soluble and insoluble components.

Effects of Exogenous Proteases on Composition of Ileal Digesta Proteins

The proportion of residual material from the original soybean meal that ends up in the ileal digesta sample after gastro-intestinal passage has only been in contact with proteolytic enzymes from the digestive tract of the pig (primarily pepsin and pancreatin). Therefore, it was of relevance to study the potential further solubilisation/degradation of the digesta proteins by commercial proteases, including exo- and endo enzymes. For this purpose an ileal digesta sample collected from pigs fed a single meal of Soy 44 was obtained (*15*). The complete digesta sample contained 2.19% (w/v) protein) and dry matter comprised 16% (w/w) of the digesta. The carbohydrate content was 62% (w/w) of DM and cellulose comprised about 50% of this (*15*). For hydrolysis different commercial proteases were selected and incubated (pH 7; 40°C; 5h) with the digesta sample according to Table 1. All enzymes were dosed at high concentrations. After incubation the effects of the proteases on protein solubilisation and and digestible protein were determined (Table 1).

Further solubilisation of protein from the digesta sample by the different proteases was generally difficult to obtain for the different treatments. The solubilisation was negatively affected by papain and PTN P (reduced to 93.6%) and also for Novo Pro D a minor negative effect (98.2%) is seen. Only Pronase and Flavourzyme had a limited (non-significant) positive effect (101.2 and 101.0%, respectively, relative to blank). The negative effects of PTN P and papain were also reflected on the level of digestible protein, as determined by gel permeation chromatography (peptide material below 1500 Da). The amount of digestible protein was significantly reduced to about 90% and 92%, respectively. Digestible protein was significantly positively affected by Pronase (104.7%), and numerically positively affected by Alcalase (104.2%) and Flavourzyme (103.2%).

Table 1. Effects of different protease preparations on the level of solubilised and "digestible" protein in ileal digesta sample from pigs

Enzymes[1]	Organism	Dosage [2]	Sol P (%)	CV	Dig P (%)	CV
Blank	-		100.0 [a]	2.8	100.0 [a]	2.6
Alcalase (2,4 L)[a]	*Bacillus licheniformis*	2.5%	99.6 [a]	2.1	104.2 [a]	2.6
Flavourzyme[a]	*Aspergillus oryzae*	5.0%	101.0 [a]	1.7	103.2 [a]	2.1
PTN P (pancreatin)[a]	Porcine pancreas	4.8%	93.6 [b]	3.6	90.9 [c]	4.1
Pronase[b]	*Streptomyces griseus*	2 mg/mL	101.2 [a]	3.4	104.7 [b]	3.3
Novo Cor ABL[a]	*Rhizomucor* orgamism	10%	100.2 [a]	3.6	101.1 [a]	3.6
Novo Pro D[a]	*Bacillus clausii*	0.5 mg/g	98.2 [a]	2.9	99.9 [a]	4.8
Papain[a]	*Carica papaya*	0.5 mg/mL	93.6 [a]	3.3	92.4 [c]	2.6

[1]Enzyme manufacturers: [a] Novozymes A/S; [b] Roche Molecular Biochemicals
[2] Enzyme dosage. Percentages (v/w) should be read as: volume of enzyme preparation relative to protein content in digesta sample
***Sol P (solubilised protein):** Calculated from the protein content (Dumas combustion method; N x 6.25) of the different fractions. Effects of protease treatments were calculated relative to the level of solubilised protein in the blank digesta sample (set to 100). Positive protein solubilising effects are seen as numbers higher than 100, for negative effects numbers are lower than 100.*
***Dig P (digestible protein):** Determined using the molecular size distribution (Superdex 30 peptide column, 214 nm) of soluble protein material in the different protease treated digesta samples. Digestible protein is defined as the relative change to the proportion of soluble protein with a molecular size smaller than 1500 Da compared to blank.*
Samples with different letters are significantly different.

In conclusion, the results show that primarily for the PTN P and papain treatments the proportion of digestible protein (peptide material below 1500 Da) is reduced compared to the blank (i.e. intestinal conditions). Based on the results of the previous chapters it is highly likely that peptide aggregation is occurring during incubation as seen by the simultaneous reduction of protein solubilisation. Considering that pancreatic enzymes had already been in contact with the digesta sample in the animals it was, in this respect, interesting to observe that protein solubilisation and digestibility were still so negatively affected by PTN P, which is a preparation of pancreatin complex from porcine pancreas. The results imply that aggregation inducing behavior of protease preparations should be considered as part of the performance evaluation of novel enzyme candidates for animal feed. In future studies it would be interesting to study differences in aggregation behavior of different proteases *in vivo* by including them with the soybean meal prior to ingestion and final collection of the ileal digesta.

Effects of Cell Wall Polysaccharides on Protein/Peptide Aggregation

Throughout the experiments of this thesis (Chapters 2, 3, and 5) it has been speculated that the cell wall polysaccharides (e.g. cellulose or other cell wall components of the

SBM matrix), which are present during hydrolysis of the SBM proteins, might affect the level of protein/peptide aggregation. A preliminary study on this topic (unpublished data) was conducted using microcrystalline cellulose as a rather simplistic model for enzyme-unextractable cell wall polysaccharides. For this purpose soy protein isolate (5 g) and cellulose (1.2 g) were suspended in water and mixed for 1h at 40°C. The suspensions were hydrolyzed at pH 7 using Alcalase (2.5%) and Flavourzyme (5%) (essentially according to Chapter 3). After incubation the soluble materials were separated from the solids by centrifugation and analyzed after freeze drying of the residues. The extents of solubilisation of protein are presented in Table 2.

Table 2. Solubilisation of protein from soy protein isolate after incubation with/without proteases and cellulose

Treatments	Sol P (%)	CV
Isolate	53.9	3.13
Isolate + cellulose	64.9	3.19
Isolate + proteases	96.6	0.09
Isolate + cellulose + proteases	96.0	0.03

Sol P (solubilised protein): Calculated from the protein content (Dumas combustion method; N x 6.25) of the different fractions.

Of the protein isolate 53.9 % was water-soluble after 16h incubation at pH 7. In total, 96 % of the protein was solubilised in the presence of proteases (16h) and cellulose. When cellulose was excluded 96.6 % of the protein isolate was solubilised. Therefore, the results did not indicate a noteworthy effect of the cellulose on the efficacy of protein solubilisation. Nevertheless, the results indicated that the cellulose is affecting the distribution of protein between the soluble and insoluble fractions during incubation/enzymatic extraction of the protein isolate. Without cellulose inclusion the enzymatic solubilization of protein was ~43% (96.6 – 53.9%). Inclusion of cellulose increased the solubilization of the isolate protein in water by 11%, thereby reducing the apparent solubilising effects of the proteases to ~32% of total protein. Accordingly, the soy protein isolate was more soluble in the presence of cellulose than in its absence. There is no obvious explanation for this observation, but the effect was consistent. Recently, Kasai and co-workers (*10*) reported the composition of an remaining inner-shell

of a body complex of soybean seeds after enzymatic degradation. The carbohydrate composition differed from the enzyme-unextractable residues (Chapter 2) by being rich in mannose. The amino acid composition was almost similar to the ones of the enzyme-unextractable residues and the authors suggested that the indigestible proteins found in our study could be part of the remaining inner shell of the soybean seed body complex.

The protein composition of the enzyme-extractable and enzyme-unextractable fractions from the 'isolate + protease' and the 'isolate + cellulose + protease' treatments (Table 2) were analysed by SDS-PAGE. In previous experiments (*16*) the efficacy of the SDS sample buffer used for extraction of enzyme-unextractable protein from SBM residues was found to be 90-95%.

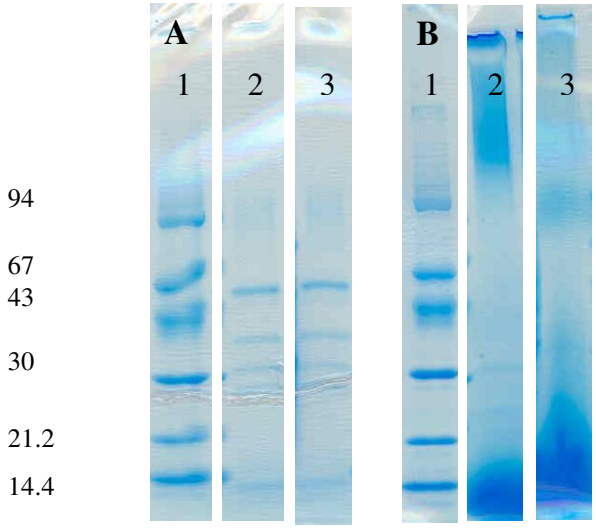

Figure 1. SDS-PAGE analysis. (A), Enzyme-extractable protein material; (B), Enzyme-unextractable residues.
Lanes: 1, Molecular weight marker (from top: 94, 67, 43, 30, 21.2, 14.4 kDa); 2, 'Isolate + cellulose + proteases'; 3, 'Isolate + proteases'.

For the enzyme-extractable fractions (Figure 1A lanes 2 and 3) five distinct protein bands with molecular sizes ranging from 12-65 kDa were observed, both with and without cellulose. This shows that, even though proteolysis resulted in extensive degradation of the protein, (partly degraded) polypeptides were still present in the enzyme-extractable fraction. A comparable distribution pattern of protein bands was observed for the two conditions.

For the enzyme-unextractable fractions extracted by sample buffer (Figure 1B, lanes 2 and 3) no electrophoretically recognizable protein bands were present indicating an effective extraction and degradation of the major soy proteins from the isolate by the pro-

teases. Consequently, the enzyme-unextractable protein material is likely to be composed of aggregated peptides which are extracted by the SDS-sample buffer (*15*, *16*).

In Figure 2, the molecular size distribution of proteinaceous material extracted by dilute alkali from the enzyme-unextractable residues of the 'isolate + cellulose + proteases' and the 'isolate + proteases' treatments is given. In earlier experiments the efficacy of protein extraction by this treatment was ~85% (*16*).

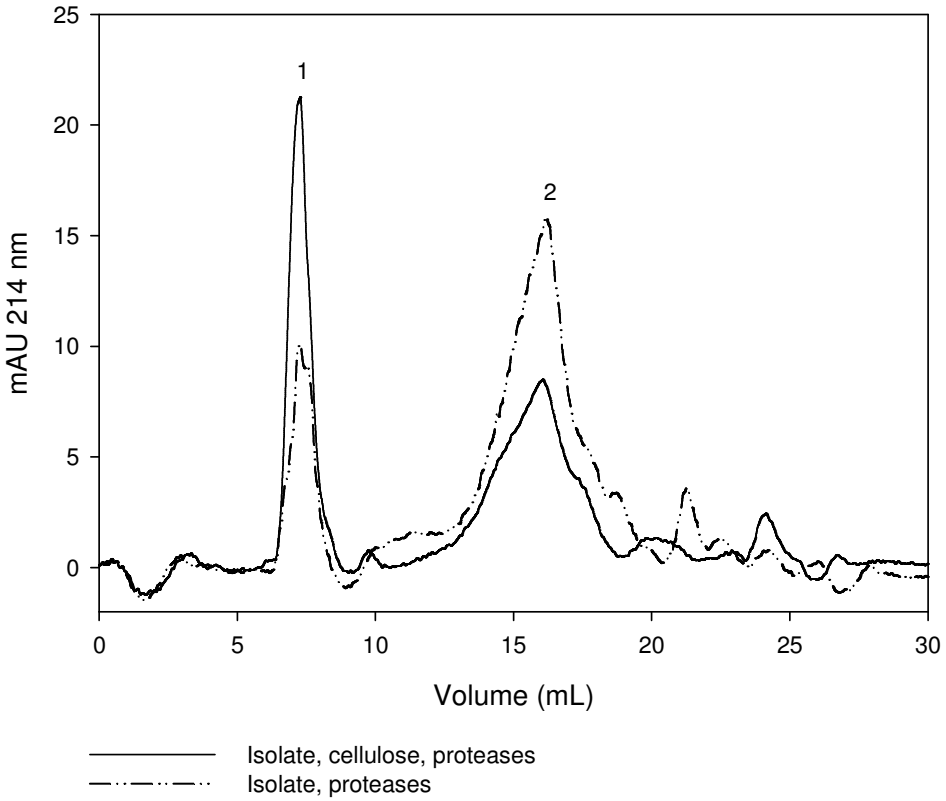

Figure 2. Gel permeation chromatogram (Superdex 75) showing the absorbance (214 nm) for the alkaline supernatants (S_{ext}). Samples: 'isolate, cellulose, proteases' and 'isolate, proteases'. Prior to analysis the supernatants were diluted to same protein content [V_0 = 7.7 mL and V_t = 15 mL].

For both curves two primary peaks (peaks 1 and 2) were seen. Peak 1, eluting around V_0 of the column, contained protein material > 70 kDa. Peak 2 eluting between ~13-19 mL retention volume) contained proteinaceous material with apparent molecular sizes ≤14 kDa. When the relative proportions of the two peaks was considered for the two conditions a higher proportion, ~50% of total peak area, of peak 1 was found with cellulose inclusion compared to ~25% without cellulose. The different proportions of the two major peaks between the two treatments show that cellulose inclusion affected the molecu-

lar size distribution of the alkali extractable proportion of proteinaceous material in the two enzyme-unextractable residues. Comparable curves were recorded for the two treatments at 280 nm indicating that the increased size of the peak (214 nm) at the void volume was not (partly) due to solubilisation of cellulose.

Since the protein solubilisation level (Table 3) was about the same (~96%) in these two treatments the different size of the aggregates did not seem to translate into a reduced solubilisation for the sample containing cellulose – only the molecular size distribution was affected. The effective solubilisation and degradation of protein from the isolate is in line with the generally effective degradation of, unheated as well as heat-treated purified glycinin and β-conglycinin by Alcalase and Flavourzyme presented in Chapter 2 (*13*).

In conclusion, the experiment indicated that the size of the aggregates formed during protein hydrolysis increases in the presence of cellulose. Although this affected the apparent molecular size distribution (or aggregate size) in the enzyme-unextractable protein material for the conditions tested, it did not lead to a reduced efficacy of protein solubilisation from the protein isolate. This topic clearly warrants further investigations. In future it would be interesting to investigate the effects of different types of cellulose and/or cell wall material and different hydrolysis conditions followed by a detailed analysis of peptides from the enzyme-unextractable residues.

Concluding Remarks

The results of this thesis have highlighted that the importance of peptide aggregation upon enzymatic hydrolysis of proteins as a limiting factor for enzymatic accessibility is not limited to *in vitro* situations, but is also occuring in the digestive system of animals.

Enzymatic hydrolysis of plant proteins is generally perceived as a way of increasing protein solubilisation. The soluble fraction of an extensive hydrolysate produced from a rapeseed protein isolate by Alcalase and Flavourzyme was characterized by Vioque and co-workers (*17*). The final hydrolysate had a degree of hydrolysis of 60%. The hydrolysate was almost 100% soluble between pH values 2.5 and 7. The peptide characteristics of sunflower protein hydrolysates prepared using Alcalase and Flavourzyme was studied by Villanueva and co-workers (*18*). The combined use of the two proteases

generated the highest degree of hydrolysis (54%) and the highest solubility (90%) between pH 2.5 and 7. In addition, Conde and co-workers (*19*) reported that solubility of sunflower protein hydrolysates increased with increasing degree of hydrolysis (DH) over the range from DH 0 to 46%.

In none of the above-mentioned papers on hydrolysis of rapeseed and sunflower aggregation behavior of the intensively hydrolysed degradation products were reported. However, the results of this thesis provide clear evidence that peptide aggregation plays a larger role as a limiting factor for effective proteolytic solubilisation of soy proteins than anticipated when the project was initiated. During hydrolysis of proteins *in vitro* and *in vivo* the structure of the solubilised soy proteins is altered, and hydrophobic groups become exposed, which might result in aggregate formation. *In vitro*, during extensive hydrolysis of bovine whey proteins with Alcalase and limited hydrolysis and limited hydrolysis with *Bacillus licheniformis* protease BLP (*20*, *21*) these aggregates can form gel networks.

For soy proteins it has been shown that different, mainly alkaline and neutral proteinases of microbial origin can induce coagulation of proteins in soy milk (*22*). Inouye and co-workers (*23*, *24*) reported that subtilisin Carlsberg induces aggregation during the hydrolysis of soy protein isolates. *In vivo* the experimental settings are more or less given, but *in vitro* it may be possible to identify the conditions that are less favorable for aggregate formation by controlling the parameters known to influence this behavior e.g. degree of hydrolysis, pH and ionic strength. This may, however, may not be uncomplicated. Kuipers and co-workers (*14*) reported that aggregation (cold gelation) behavior of soy proteins is not simply a balance of repulsive electrostatic and attractive hydrophobic interaction, but is more complex. Possibly, specific electrostatic attractions play a role, making the aggregation mechanism less predictable and difficult to control e.g. on an industrial scale. A PhD project on this topic is currently ongoing at the Laboratory of Food Chemistry at Wageningen University.

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Summary

Despite all the research performed soybean proteins during the last decades, and despite a widespread use of exogenous enzymes in food and feeds to enhance protein digestion/utilization, the composition of proteinaceous enzyme-unextractable residues has never gained much attention. Consequently, there is a lack of knowledge about the structure of the different soy polymers resisting enzymatic digestion. Therefore, the aim of this thesis was to broaden the knowledge about factors affecting the efficacy of enzymatic extraction of protein and carbohydrates from soybean meals (SBM). Also, the composition of protein in enzyme-unextractable soybean meal fractions produced *in vitro* and *in vivo* were investigated because this knowledge contributes to a better understanding of the characteristics of this protein fraction.

In Chapter 1, background information is given about SBM and its application in foods and animal feed. The processing applied to soybeans for the production of defatted SBM is presented. The protein composition of SBM is discussed with focus on the proteolytic degradation of the major soy proteins following different heat treatments. Carbohydrates, in particular the cell wall polysaccharides of soy, and carbohydrate degrading enzymes of relevance are also discussed

In Chapter 2, one unheated and three heat-treated soybean meals were produced with the purpose of studying the incomplete enzymatic extractability of proteins and carbohydrates of thermally treated soybean meals. To obtain enzyme resistant material, the meals were extracted by a repeated hydrolysis procedure using excessive concentrations of different combinations of commercial protease and carbohydrase preparations. For all soybean meals enzymatic treatment extracted most of the protein (89 - 94%). Addition of carbohydrase preparations did not improve protein extraction. Based on the conditions for heat treatment the enzymatic residues can be divided into two groups: '*high humidity*' and '*unheated + low humidity*'. High-humidity heat treatment led to a more effective enzymatic extraction. Nevertheless, in all cases the protein extraction from the meals was incomplete. In addition, the major proteins present in soybean meal, glycinin, β-conglycinin, and protease inhibitors were purified and subjected to enzymatic degradation using the same conditions. Taking into account the more efficient degradation of purified soy proteins, it is clear that the soybean matrix affects the enzymatic extraction of protein from the meals. It is speculated that interactions between protein and other

components e.g. the cellulose, a major constituent of the enzyme-unextractable residues, may reduce the efficacy of protein extraction from the meals.

In Chapter 3, the effects of time, pH and dose of two commercial protease preparations s were studied with the purpose of obtaining a high solubilisation of protein and/or high degree of hydrolysis from soybean meal by these two preparations. Protein solubilisation was favored by a relatively high pH (7.5) reflecting the pH optimum of the endoproteinase and emphasizing the importance of this type of enzyme to reach a final high level of protein solubilisation. Experiments indicated that the efficacy of the hydrolysis is to some extent negatively affected by degradation products formed in the protein digests. The ratio of the endo/exo proteases in the enzyme mixture was found to be an important parameter, which could affect both protein solubilisation and degree of hydrolysis in both a negative or positive direction. The presented data illustrate that effective protein solubilisation is not solely a matter of selecting efficient endo- and exoproteases. The enzymes can be overdosed and the ratio between endo- and exo-enzymes is equally important depending on the need for high protein solubilisation or high DH in the final hydrolysed protein product. Therefore, a combined objective of high protein solubilisation and high degree of hydrolysis in a single hydrolysis product may be conflicting. Two models were developed with the purpose of predicting protein solubilisation and degree of hydrolysis as function of the dosage of the proteases. The protein solubilisation model was tested and results indicated a slight improvement of protein solubilisation using the prediction of the model. Although further optimization is needed, it is clear that such models have a potential in commercial production of hydrolysed vegetable proteins.

The reasons for the limitations of the enzymes to enable complete solubilisation of soy proteins were further studied in chapters 4 and 5 by analyzing the fraction of the soy proteins, which resisted enzymatic extraction. In Chapter 4, different extractants were screened for their ability to extract proteins from enzyme-unextractable residues of different heat-treated soybean meals with the purpose of analyzing the size and composition of enzyme-unextractable proteins. The largest solubilising effects were obtained with urea, urea plus β-mercaptoethanol, and dilute alkali; the latter extracted up to 87% of the enzyme-unextractable protein. Gel permeation chromatography indicated that a large proportion of the urea-extracted enzyme-unextractable material was of high apparent molecular weight. However, the combined results from gel electrophoresis, LC-MS, and MALDI-ToF MS showed that the extracted protein material was composed of ag-

gregated peptides. The largest aggregates were observed in the enzyme-unextractable residues originating from meals heat-treated at high humidity. Extracted aggregates were fully degraded upon subsequent proteolytic treatment. It was concluded that part of the extracted and hydrolyzed proteins assumingly become insoluble during hydrolysis because part of the peptides released by the proteases are obviously prone to aggregation. Aggregation of peptides is favored by the neutral pH conditions that exist during hydrolysis of the original SBMs.

In Chapter 5, the molecular size and composition of proteinaceous material extracted from the insoluble components of a digesta sample obtained from pigs fed a single soybean meal was analyzed. Gel permeation chromatography indicated that the alkali-extractable fraction of the proteinaceous material from the residue was of relatively high apparent molecular weight. However, the combined results from gel electrophoresis, RPLC-MS, and MALDI-ToF MS showed that the extracted protein material was in fact, to a high extent, composed of aggregated peptides. To our knowledge this has not previously been described. Aggregates extracted by dilute alkali were fully degraded upon subsequent proteolytic treatment. N-terminal sequencing of selected protein bands from SDS-PAGE gels indicated the presence of partly degraded β-conglycinin α-subunits in the residue. The results indicated a high efficiency of the digestive enzymes of the pig for extraction/degradation of SBM proteins. Naturally, a large proportion of the soluble protein material is readily absorbed by the animal or stays in the soluble fraction of the ileal sample. However, during digestion insoluble peptide aggregates are formed when the soy proteins are hydrolysed in the stomach or the ileal part of the pig's gastrointestinal tract and these aggregates become inaccessible to the digestive enzymes of the animals.

In Chapter 6, the most important results from the previous chapters are discussed. Special attention is given to the possible role of cell wall polysaccharides in aggregate formation.

In conclusion, the results of this thesis show that the importance of peptide aggregation upon enzymatic hydrolysis of proteins as a limiting factor for enzymatic accessibility is not limited to *in vitro* laboratory situations, but aggregation of peptides is also occurring in the digestive system of animals.

Curriculum Vitae

Morten Fischer was born on November 14th 1968 in Copenhagen. In 1991 he began studies of agricultural science at The Royal Veterinary and Agricultural University (RVAU), Copenhagen. Due to a large interest in applied enzyme science the experimental work for, and writing of, the master thesis was performed at Novozymes A/S (former Novo Nordisk A/S) in Department of Food Functionality. He graduated in 1997 with a specialisation in plant biology and plant biochemistry. From 1997 he worked, primarily with molecular biology, at Department of Plant Biology, in the laboratory of Professor Birger Lindberg Møller. In Spring 1998 he joined the Feed Applications Department at Novozymes working on a so-called International Industrial Ph.D. Fellowship, administered by the Danish Academy of Technical Sciences and supported The Danish Ministry of Economic and Business Affairs. In connection with the project research collaboration was established with the Laboratory of Food Chemistry at Wageningen University, The Netherlands, headed by Professor Fons Voragen. The project was dealing with aspects of protein and carbohydrate science in relation to the application of enzymes. It also involved the Centre for Protein Technology, led by Professor Harry Gruppen and the carbohydrate group, led by Dr. Henk Schols. A fifty-fifty time-split between the company research facilities in Bagsvaerd, Denmark and Wageningen University was agreed upon for the four years time frame of the project. The experiments performed during this period are presented in this thesis. Since January 2002 Morten has been working as research scientist and project manager in the Feed Applications department of Novozymes dealing with *in vitro* application development enzymes for animal feed.

List of Publications

Papers

Fischer,M.; Kofod,L.V.; Schols,H.A.; Piersma,S.R.; Gruppen,H.; Voragen,A.G.J. Enzymatic extractability of soybean meal proteins and carbohydrates: heat and humidity effects. *J Agric Food Chem* **2001**, *49*, 4463-4469.

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Wittstock,U.; Fischer,M.; Svendsen,I.; Halkier,B.A. Cloning and characterization of two cDNAs encoding sulfatases in the Roman snail, Helix pomatia. *IUBMB life* **2000**, *49*, 71-76.

Patents

Lassen,S.F.; Sjoholm,C.; Ostergaard,P.R.; Fischer,M. WO2005123911-A2. New polypeptide having protease activity, useful in detergents or in animal feed or animal feed additives, in increasing digestible or soluble protein in animal feed, or in increasing the degree of hydrolysis of proteins in animal diets. Novozymes A/S.

Svendsen,A.; Kaasgaard,S.; Borch,K.; Fischer,M.; Pettersson,D.; Gregory,P.C. WO2005115445-A1. New protease derived from Nocardiopsis sp., NRRL 18262, useful for treating digestive disorders, pancreatic insufficiency, pancreatitis, cystic fibrosis, diabetes type I and/or diabetes type II. Novozymes A/S.

Lassen,S.F.; Sjoholm,C.; Ostergaard,P.R.; Andersen,C.; Fischer,M.; Sjoholm,C.; Appelman,B.; Fish,E.J. WO2004111220-A1. New polypeptide having protease activity and 78 degrees C melting temperature, useful for improving nutritional value of animal feed, for increasing digestible or soluble protein in animal diet or as detergent component. Novozymes A/S.

Kofod,L.V.; Fischer,M.; Nielsen,P.M.; Pommer,K. WO9818343-A. Production of a food flavouring agent from plant protein and insoluble plant material - useful for imparting a variety of flavour notes, e.g. a roasted character, a smoked and pork like note, or a vegetable flavour. Novozymes A/S.

Kofod,L.V.; Fischer,M.; Nielsen,P.M.; Pommer,K. WO9818342-A. Production of a food flavouring agent by hydrolysis of plant proteins and carbohydrates - useful for imparting a variety of flavour notes, in the same manner as conventional hydrolysed vegetable protein (HVP). Novozymes A/S.