Enzymatic Extractability of Soybean Meal Proteins and Carbohydrates: Heat and Humidity Effects

Morten Fischer,[‡,†] Lene V. Kofod,[‡] Henk A. Schols,[§] Sander R. Piersma,[†,||] Harry Gruppen,[†,§] and Alphons G. J. Voragen*[,†,§]

Centre for Protein Technology TNO-WU, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, Novozymes A/S, Bagsvaerd, Denmark, Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Wageningen, The Netherlands, and Department of Protein Technology, TNO Nutrition and Food Research, Zeist, The Netherlands

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 with 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 soybean meal (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 characterization 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 side chains, 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*−*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 the literature (*7*, *11*−*15*). Purified native proteins may show some resistance toward 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 that published on 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 than those in the toasted and

* To whom correspondence should be addressed. Phone: +31 (0) 317 48 3209. Fax: +31 (0) 317 48 4893. E-mail: office@chem.fdsci.wag-ur.nl.

† Centre for Protein Technology TNO-WU.

‡ Novozymes A/S.

§ Wageningen University.

|| TNO Nutrition and Food Research.

10.1021/jf010061w CCC: $20.00
Published on Web 08/29/2001

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 (360−2000 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 SBM and SBMs heat-treated at different humidities. High concentrations of commerial 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 characterized 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 × 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 of 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 of water). Equilibration was allowed for 3 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 × 6.25) of 49% (w/w), was conditioned at 68 °C for 10 min, and pelletized (3 mm × 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 × 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 flow rate 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, St. Louis, MO) in the same buffer. The eluate was collected and washed by diafiltration using a 400-mL ultrafiltration cell (Amicon, CA) 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 × 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 flow rate 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) 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, St. Louis, MO) 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 of 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 of 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 of 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 the most protein from the meals in subsequent experiments. Meal (200 g) was mixed with 1800 mL of 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, 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 com-

binations 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; 15000g; 4 °C). Supernatants were discarded. Residues were washed 3 times with 500 mL of ice-cold deionized 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 those of the first hydrolysis. After hydrolysis, the pH was adjusted to 7, and supernatants and residues were separated by centrifugation (20 min; 15000g; 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 hydrolyze 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 been previously examined in triplicate. The enzymatic protein extraction (EP) has a standard deviation of less than 1%, and the standard deviations of EDM and EC are 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 × 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 × 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). Each sample (10 mg) was hydrolyzed in 100 μL of 6 M HCl for 16 h and subsequently derivatized by PITC. Separation was performed using a C18 reversed-phase column (21 mm × 220 mm) at a flow rate of 300 μL/min. The absorbance of the eluate was monitored at 254 nm. Amino acid standard H (Pierce, IL) 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, Hercules, CA) using 10−20% pre-cast gels (Bio-Rad). Gels were processed at 200 V, 20 mA for 50 min, stained by Coomassie Brilliant Blue overnight, and then destained with 30% methanol and 10% acetic acid in deionized water according to the instructions of the manufacturer. Gels were scanned using a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

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). The resistance of these two proteins is supported by Hessing et al. (*17*) and by Armor 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. 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

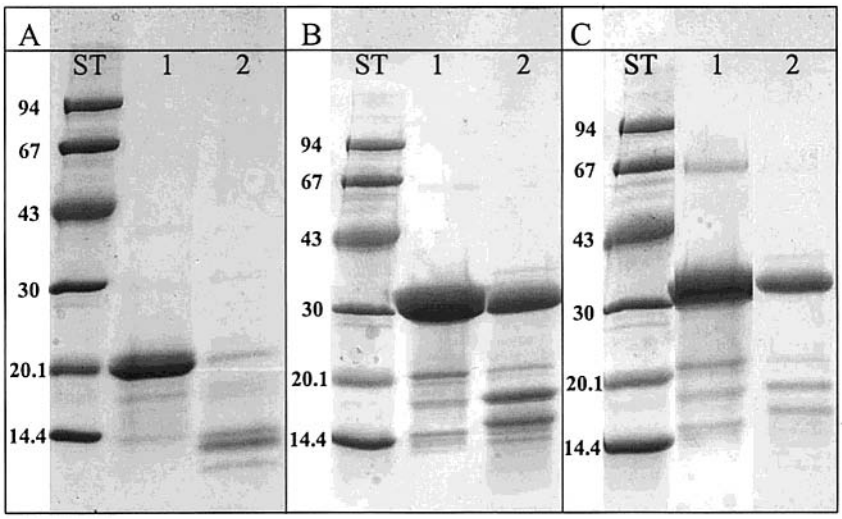

Figure 1. SDS−PAGE gels showing native KSTI (A), unheated lectin (B), and low humidity heat-treated lectin (C). The contents of the lanes are: ST, molecular weight standard (94, 67, 43, 30, 20.1, and 14.4 kDa); 1, protein solution before hydrolysis; 2, enzymatic hydrolysate (without separation).

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 × 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.

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 × 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.

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 the proteins of 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). 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 EPs 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

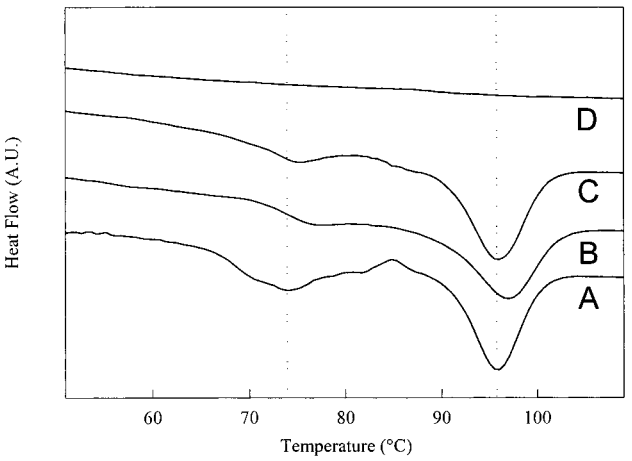

Figure 2. DSC-thermograms of soybean meal suspensions (50 mg protein/mL) in 35 mM potassium phosphate buffer containing 0.1 M NaCl (pH 7.6), $I = 0.2$ M; A, USBM; B, NN; C, SBM-L; D, SBM-H. Scanning rate was 1.2 K min^{-1}.

(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. The 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. 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 areas 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%).

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

[a] All data are based on six determinations. 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 (*7*). [c] Calculated from Kjeldahl N × 6.25. Expressed as % of dry matter. [d] The sum of asparagine + aspartic acid. [e] The sum of glutamine + glutamic acid. [f] Fully destroyed during hydrolysis.

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% → 100%) was never reached. Therefore, the composition of the enzyme unextractable residues was analyzed in more detail.

Composition of Enzyme-Unextractable Material. *Protein.* The amino acid compositions 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 the 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, and 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.

Generally, the increased hydrophobic character of the enzymatic residues suggests that the residues were enriched in hydrophobic amino acids or hydrophobic

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)

[a] All data are based on triple determinations. 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).

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 compositions of the meals and enzyme unextractable solids were analyzed as seen in Table 4. The meals were generally rich in Glc, Gal, UA, Ara and contained low levels of Man, Rha, and Fuc. Compared to the contents of 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 proportions of Glc. The proportions of cellulosic and noncellulosic glucose were determined for the meal and the 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*", on the basis of 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, cellulose, which is a major component of the residues) may explain the incomplete enzymatic extractability of protein from the meals. This is a topic of future investigation.

ACKNOWLEDGMENT

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

LITERATURE CITED

(1) Marsman, G. J. P.; Gruppen, H.; Mul, A. J.; Voragen, A. G. J. In-vitro accessibility of untreated, toasted, and extruded soybean meals for proteases and carbohydrases. *J. Agric. Food Chem*. **1997**, *45*, 4088−4095.

(2) Liener, I. E. Implications of antinutritional components in soybean foods. *Crit. Rev. Food Sci. Nutr*. **1994**, *34*, 31−67.

(3) Voragen, A. G. J.; Beldman, G.; Schols, H. A. Chemistry and enzymology of pectins. In *Advanced Dietary Fibre Technology*; McCleary B. V., Prosky, L., Eds.; Blackwell Science: Oxford, UK, 2000; pp 379−396.

(4) Huisman, M. M. H.; Schols, H. A.; Voragen, A. G. J.; Wolf, W. J. Cell wall polysaccharides from soybean (*Glycine max*.) meal. Isolation and characterisation. *Carbohydr. Polym*. **1998**, *37*, 87−95.

(5) Carpita, N. C.; Gibeaut, D. M. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J*. **1993**, *3*, 1−30.

(6) Camire, M. E. Protein functionality modification by extrusion cooking. *J. Am. Oil Chem. Soc*. **1991**, *68*, 200−205.

(7) Henn, R. L.; Netto, F. M. Biochemical-characterization and enzymatic-hydrolysis of different commercial soybean protein isolates. *J. Agric. Food Chem*. **1998**, *46*, 3009−3015.

(8) Marsman, G. J. P.; Gruppen, H.; de Groot, J.; Voragen, A. G. J. Effect of toasting and extrusion at different shear levels on soy protein interactions. *J. Agric. Food Chem*. **1998**, *46*, 2770−2777.

(9) Araba, M.; Dale, N. M. Evaluation of protein solubility as an indicator of overprocessing soybean meal. *Poult. Sci*. **1990**, *69*, 76−83.

(10) Araba, M.; Dale, N. M. Evaluation of protein solubility as an indicator of underprocessing of soybean meal. *Poult. Sci.* **1990**, *69*, 1749−1752.
(11) Clatterbuck, K. L.; Kehrberg, N. L.; Marable, N. L. Solubility and in vitro digestibility of soy flours, concentrates, and isolates. *J. Food Sci.* **1980**, *45*, 931−935.
(12) Bernardi-Don, L. S.; Pilosof, A. M. R.; Batholomai, G. B. Enzymatic modification of soy protein concentrates by fungal and bacterial proteases. *J. Am. Oil Chem. Soc.* **1991**, *68*, 102−105.
(13) Kim, S. Y.; Park, P. S. W.; Rhee, K. C. Functional properties of proteolytic enzyme modified soy protein isolate. *J. Agric. Food Chem.* **1990**, *38*, 651−656.
(14) Lopez, L. M. I.; Brullo, A.; Natalucci, C. L.; Caffini, N. O.; Sorgentini, D. A.; Wagner, J. R. Thermal behavior, solubility and structural properties of soy concentrate hydrolyzed by new plant proteases. *J. Food Biochem.* **1998**, *22*, 125−141.
(15) Lynch, C. J.; Rha, C. K.; Catsimpoolas, N. Tryptic hydrolysis of glycinin and its subunits. *J. Sci. Food Agric.* **1977**, *28*, 971−979.
(16) Shutov, A. D.; Pineda, J.; Senyuk, V. I.; Reva, V. A.; Vaintraub, I. A. Action of trypsin on glycinin: Mixed-type proteolysis and its kinetics; molecular mass of glycinin T. *Eur. J. Biochem.* **1991**, *199*, 539−544.
(17) Hessing, M.; Bleeker, H.; Biert, M.; Vlooswijk, H. A. A.; Hamer, R. J. Enzymatic hydrolysis of soya products and analysis of antinutritional factors. *Meded. - Fac. Landbouwkd. Toegepaste Biol. Wet. (Univ. Gent)* **1994**, *59*, 2257−2262.
(18) Plumb, G. W.; Mills, E. N. C.; Tatton, M. J.; D'Ursel, C. C. M.; Lambert, N.; Morgan, M. R. A. Effect of thermal and proteolytic processing on glycinin, the 11S globulin of soy (*Glycine max*): a study utilizing monoclonal and polyclonal antibodies. *J. Agric. Food Chem.* **1994**, *42*, 834−840.
(19) Vaintraub, I. A.; Yattara, H. B. Proteolysis of Kunitz soybean trypsin inhibitor, influence on its activity. *J. Agric. Food Chem.* **1995**, *43*, 862−866.
(20) Chiang, W. D.; Shih, C. J.; Chu, Y. H. Functional properties of soy protein hydrolysate produced from a continuous membrane reactor system. *Food Chem.* **1999**, *65*, 189−194.
(21) Lee, J. Y.; Lee, H. D.; Lee, C. H. Characterization of hydrolysates produced by mild-acid treatment and enzymatic hydrolysis of defatted soybean flour. *Food Res. Int.* **2001**, *34*, 217−222.
(22) Lakemond, C. M. M.; de Jongh, H. H. J.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Soy glycinin: influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *J. Agric. Food Chem.* **2000**, *48*, 1985−1990.
(23) Thanh, V. H.; Shibasaki, K. Major proteins of soybean seeds. A straightforward fractionation and their characterization. *J. Agric. Food Chem.* **1976**, *24*, 1117−1121.
(24) Gordon, J. A.; Blumberg, S.; Lis, H.; Sharon, N. Purification of soybean agglutinin by affinity chromatography on sepharose *N*-ϵ-aminocaproyl β-D-galacto-pyranosylamine. *FEBS Lett.* **1972**, *24*, 193−196.
(25) AOAC. *Official Methods of Analysis*, 13th ed.; Association of Official Analytical Chemists: Washington, DC, 1988.
(26) Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total nonstarch polysaccharides by gas−liquid chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109*, 937−942.
(27) Thibault, J. F. An automated method for the determination of pectic substances. *Lebensm.-Wiss. Technol.* **1979**, *12*, 247−251.
(28) Armour, J. C.; Perera, R. L. C.; Buchan, W. C.; Grant, G. Protease inhibitors and lectins in soya beans and effects of aqueous heat-treatment. *J. Sci. Food Agric.* **1998**, *78*, 225−231.
(29) Mohammed, Z. H.; Hill, S. E.; Mitchell, J. R. Covalent cross-linking in heated protein systems. *J. Food Sci.* **2000**, *65*, 221−226.
(30) Marsman, G. J. P.; Gruppen, H.; Van der Poel, A. F. B.; Huisman, J.; Saini, H. S. Effect of extrusion on the in vitro digestibility of toasted and untoasted soybean meal. In *Recent Advances of Research in Antinutritional Factors in Legume Seeds. Proceedings of the Second International Workshop*; Wageningen Press: Wageningen, The Netherlands, 1993; 461−465.
(31) Fry, S. C. *The growing plant cell wall: Chemical and metabolic analysis*; Longmann Scientific and Technical: New York, 1988.
(32) Hermansson, A. M. Physicochemical aspects soy proteins structure formation. *J. Texture Stud.* **1978**, *9*, 33−58.
(33) Renkema, J. M. S.; Lakemond, C. M. M.; de Jongh, H. H. J.; Gruppen, H.; van Vliet, T. The effect of pH on heat denaturation and gel forming properties of soy proteins. *J. Biotechnol.* **2000**, *79*, 223−230.
(34) Creighton, T. E. *Proteins. Structures and Molecular Properties*, Second ed.; W. H. Freeman and Company: New York, 1996.

Received for review January 16, 2001. Revised manuscript received May 22, 2001. Accepted June 15, 2001. This research was financially supported by Danish Academy of Technical Sciences, (Lyngby, Denmark).

JF010061W