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Aggregation of Peptides during Hydrolysis as a Cause of Reduced Enzymatic Extractability of Soybean Meal Proteins

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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*−*5*) the observed resistance to enzymatic extraction could not be ascribed to the 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 latter two 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,

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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 + 5 mM β-mercaptoethanol; detergent Triton X-100 (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 of 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; 12000*g*; 25 °C).

For extraction at pH 2 the residues (150 mg) were suspended in 1200 μL of 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; 12000*g*; 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 of 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; 12000*g*; 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 desiccator 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 standard deviations 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; 12000*g*; 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 × 6.25) of the final residues was determined by a semiautomated 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 autoanalyzer (Skalar, Breda, The Netherlands). Protein contents are the means of three determinations.

Carbohydrate Composition. *Neutral Carbohydrates.* 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 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) on a CE Instruments GC 8000 TOP (ThermoQuest Italia, Milan, Italy). It was operated at 200 °C and equipped with a flame ionization detector (ThermoQuest Italia) set at 270 °C.

Uronic Acids. The uronic acid content was determined using an automated *m*-hydroxybiphenyl assay (*9*). For the procedure, 96% (w/w) H_2SO_4 was used containing 0.0125 M sodium tetraborate 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). 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 × 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) 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 analysis 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 nonlinearity of the data. Analyses were performed using SPSS software (SPSS version 8.0, SPSS Inc., Chicago, IL). The significance level was 5%.

SDS-PAGE. SDS-PAGE was performed on a Protean system (Bio-Rad, Hercules, CA) using 10−20% Tris-HCl and 10−20% Tris-Tricine precast gels (Bio-Rad). According to the manufacturer, the gels have optimized separation ranges of 10−100 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 deionized 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).

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 (Bio-Rad). The gel was processed according to the instructions of the manufacturer. Blotting was performed using a Mini Trans-Blot Cell (Bio-Rad) and Hybond-P, a PVDF transfer membrane (Amersham Pharmacia Biotech). 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 Jose, CA).

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 × 300 mm) connected to an ÄKTA purifier system (all equipment was 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 plus 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 + 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 × 300 mm) connected to a SMART-system (Amersham Pharmacia Biotech).

Table 1. Extractability of Protein from the Enzyme-Unextractable Solids of Differently Heated Soybean Meals by the Various Extractants

	extractability of protein[a] (%)					
	urea	urea + β-ME	Triton	pH 12 (10 min)	pH 2 (10 min)	pH 12 (1 h)
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

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

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 × 250 mm) from Vydac (Hesperia, CA) connected to an HPLC system from Spectra Physics (San Jose, CA). 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 Jose, CA). 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) according to the instructions of the manufacturer. MALDI-ToF MS spectra were recorded on a PerSeptive Voyager DE-RP (PerSeptive Biosystems, Framingham, MA) 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 of matrix in 1 mL of 50% (v/v) aqueous acetonitrile containing 0.3% (v/v) TFA. The 2,5-dihydroxybenzoic acid was dissolved in 1 mL of distilled water containing 0.3% (v/v) TFA. Samples were prepared using the dried droplet method by mixing 9 μL of matrix solution with 1 μL of sample solution. Aliquots of 1 μL 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, 2466.7, and 3660.2 Da, respectively), and bovine insulin (5734.6 Da) 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**.

On the basis of the extractability of protein from the four enzymatic residues by either urea, urea plus β-mercaptoethanol, detergent Triton X-100, 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 the 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 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**.

To understand whether alkaline 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 initial 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. 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

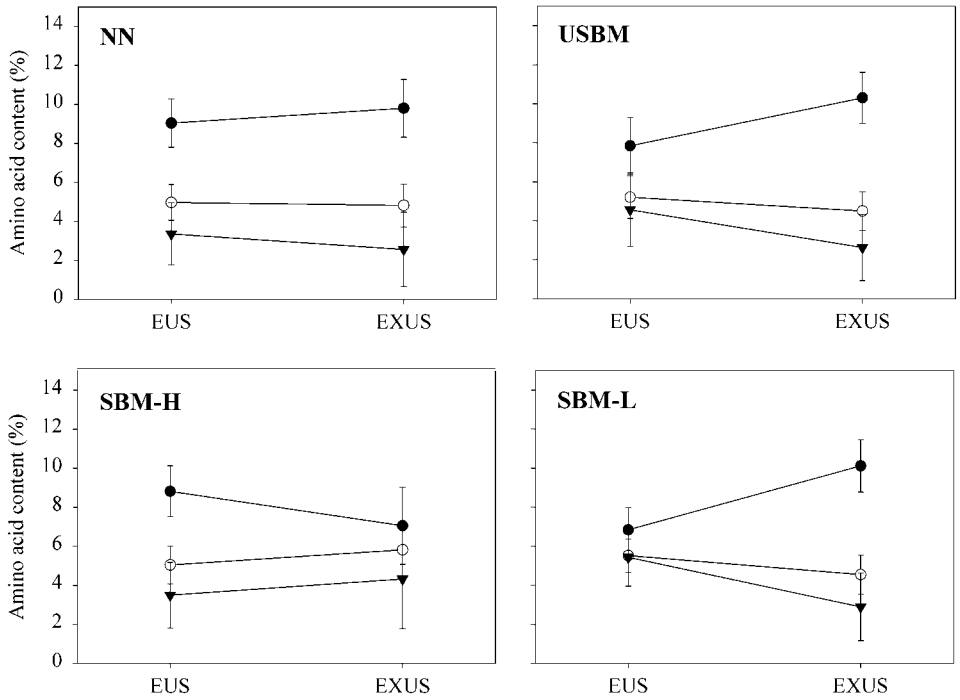

Figure 1. Average molar percentage (± standard error) for each category of amino acids for the NN, USBM, SBM-H, and SBM-L residues: (●) hydrophobic amino acids; (○) neutral amino acids; (▼) hydrophilic amino acids.

Table 2. Protein Content and Amino Acid Composition (Molar Percent) 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

[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] Calculated from Kjeldahl N × 6.25. Expressed as percent of dry matter. [c] Sum of asparagine + aspartic acid. [d] Sum of glutamine + glutamic acid. [e] Fully destroyed during hydrolysis.

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.

Table 3. Yield of Dry Matter and Carbohydrates after Enzymatic Hydrolysis and Extraction with pH 12 (1 h) and Molar Carbohydrate Compositions of Enzyme-Unextractable Solids (EUS) and Extractant-Unextractable Solids (EXUS)[a]

				carbohydrates (molar %)							
	yield[b]	C[c] (%)	C[d] (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

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

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**. 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 a 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%, whereas the xylose content remained steady at 8−14%. It has

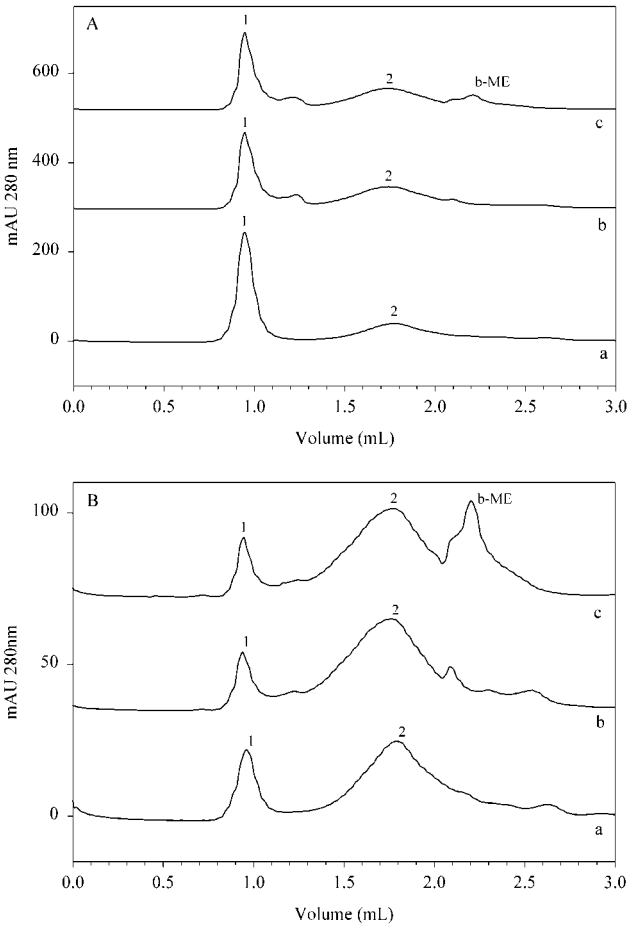

Figure 2. Gel permeation chromatograms (Superdex 75) of pH 12 (1 h) extracts of SBM-H (A) and USBM (B). Extracts were diluted and eluted in three different buffers: (a) 35 mM potassium phosphate buffer containing 0.1 M NaCl, pH 7.6; (b) buffer a + 6 M urea, pH 7.6; (c) buffer a + 6 M urea + 5 mM β-mercaptoethanol,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

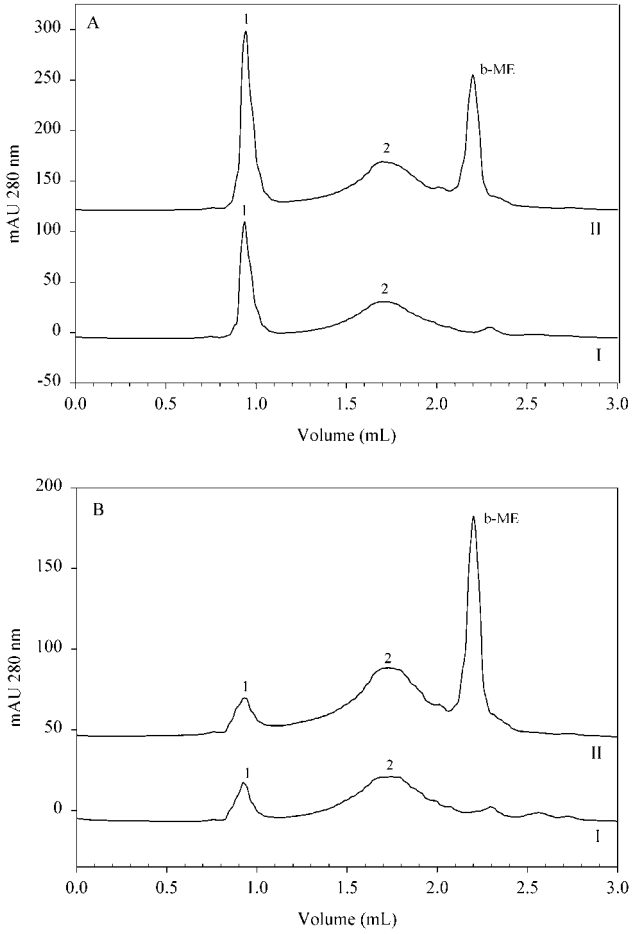

Figure 3. Gel permeation chromatograms (Superdex 75) of SBM-H (A) and USBM (B) extracts. The urea and urea + β-mercaptoethanol extracts are denoted by I and II, respectively. Extracts were diluted and eluted with 35 mM potassium phosphate buffer containing 0.1 M NaCl and 6 M urea, pH 7.6. Eluting peaks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

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. 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 a retention volume of 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 (**Figure 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 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. 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

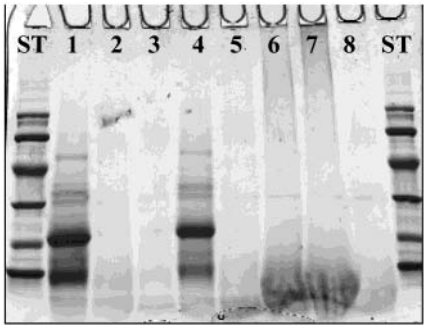

Figure 4. SDS-PAGE gels showing pH 2 and 12 extracts of the USBM, NN, SBM-H, and SBM-L residues: (lanes 1–4) pH 2; (lanes 5–8) pH 12. ST (starting from above): molecular markers 97, 67, 43, 30, 21, and 14 kDa.

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**. 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 cannot 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 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*−*17*) from soy glycinin. The protein of the 14 kDa 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 toward 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.

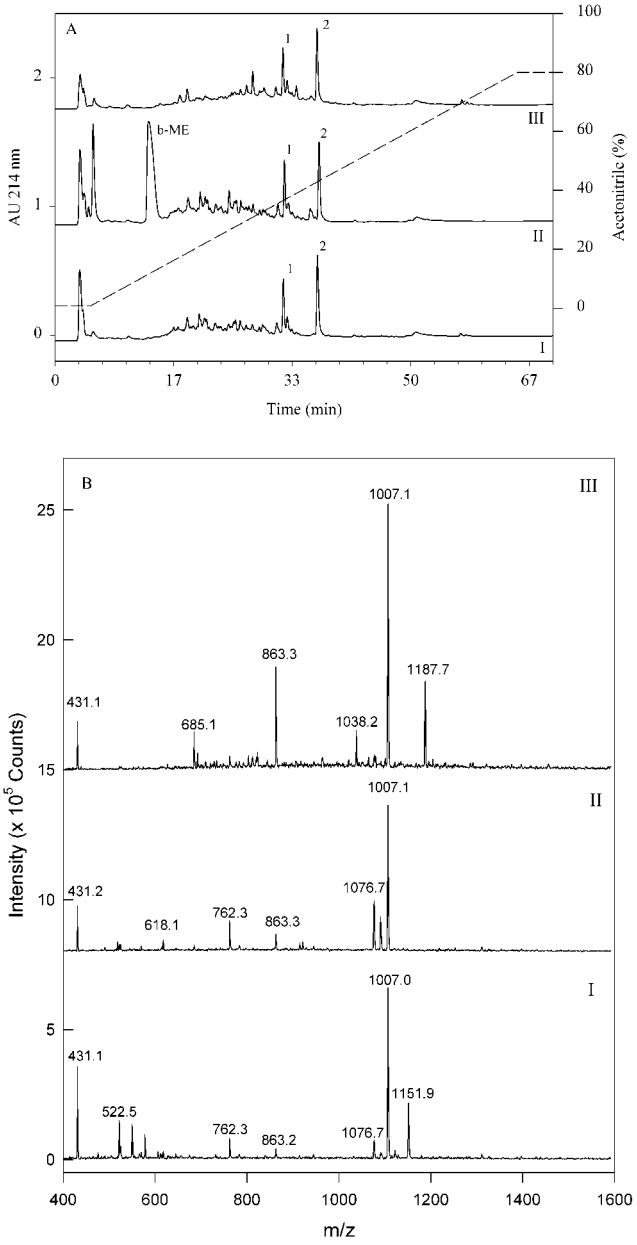

Figure 5. LC-MS reversed phase chromatograms for USBM after extraction by urea (I), urea + β-ME (II), and pH 12 (1 h) (III): (A) peaks are denoted b-ME (for the β-mercaptoethanol peak) and 1 and 2 (for the peaks eluting at 36 and 42% acetonitrile concentrations, respectively); (B) recorded mass spectrum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

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 5A**, curves I−III. 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, although 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

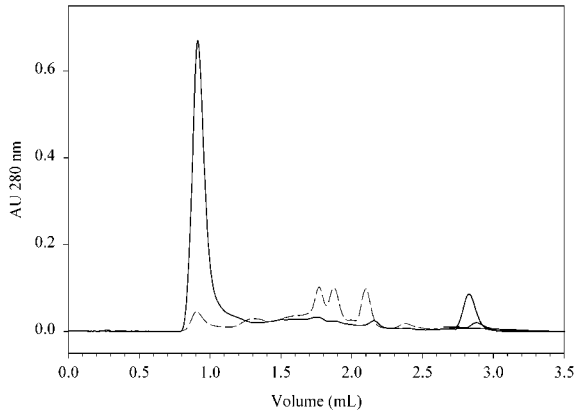

Figure 6. Gel permeation chromatogram (Superdex 75) of the pH 12 extract of the SBM-H residue and the enzymatic hydrolysate resulting after incubation with protease preparations: (solid line) pH 12 extract; (dashed line) hydrolysate (V_0 = 0.9 mL and V_t = 1.9 mL). Samples were eluted with 35 mM potassium phosphate buffer containing 0.1 M NaCl, pH 7.6.

distinguished for all three extractants. Mass spectra were acquired from 400 to 1800 Da over the whole chromatogram.

In **Figure 5B**, curve I, an example of a mass spectum of the USBM residue after extraction with urea is shown for peak 1 of **Figure 5A**. 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 (1 h) 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 could masses >2000 Da be distinguished, indicating that the material released from the residues by these extractants was composed of peptides.

The instrumental conditions applied are comparable to those in ref *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 pH 12 (10 min) was examined. The resulting chromatograms (280 nm) of the pH 12 extract and the resulting hydrolysate are shown in **Figure 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 solubilization due to electrostatic repulsion (*22*), in line with the strong effect of alkaline extractants for protein extraction. Once extracted, the proteinaceous material can be hydrolyzed 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.

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, Flavourzyme; 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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