Effect of Toasting and Extrusion at Different Shear Levels on Soy Protein Interactions

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The effect of toasting and extrusion at different shear levels on protein interactions in soybean meal was studied by extraction methods using buffers containing urea and dithiothreitol (DTT). It is suggested that noncovalent interactions were the main forces in protein structure formation during the toasting process but are less important during extrusion. After extrusion, both noncovalent interactions and disulfide bonds may be involved during low-shear extrusion. At higher shear levels, other covalent cross-linking reactions may also occur. After extrusion, mainly polypeptides of glycinin were found in the protein fractions obtained after extraction with DTT, especially the acidic polypeptide. In combination with in vitro protein digestibility results, it was concluded that glycinin is less digestible compared with β-conglycinin. It appeared that after toasting and especially after extrusion, an increasing amount of still active trypsin inhibitors could be detected after extraction with DTT, urea, and both urea and DTT, respectively. This suggests that trypsin inhibitors were embedded in the protein matrix and were thereby protected against heat inactivation.

INTRODUCTION

While primarily recognized as an oil crop, soybeans are also a rich source of protein for feeding both animals and man. The two most abundant soybean proteins are glycinin and β-conglycinin. Glycinin is made up of six subunits, each consisting of a basic polypeptide (B) and an acidic polypeptide (A), which are connected by a single disulfide bond. The heterogeneity of the glycinin molecule is expressed in its molecular size, which ranges from 320 to 375 kDa. At least six different acidic and five basic polypeptides were detected in glycinin (Brooks and Morr, 1985) and it has 2 SH groups and 20 S–S bonds per molecule (Fukushima, 1991). β-Conglycinin is a trimeric glycoprotein composed of at least seven different combinations of three subunits, α, α', and β, associated via hydrophobic interactions having a molecular mass of 141–204 kDa (Brooks and Morr, 1985). Two S–S bonds but no SH groups are present in the β-conglycinin molecule (Fukushima, 1991).

In general, the nutritional potential of soybean protein is attained if a certain amount of heat is applied. Heat is necessary to inactivate heat-labile antinutritional factors (ANFs), for example, protease inhibitors, lectins, and antivitamins (Liener, 1994), and to denature the storage proteins to increase their digestibility (Bhattacharya and Hanna, 1988). Some studies reported that heat treatment is not always suitable to achieve complete denaturation of soy proteins, especially the glycinin fraction, which can resist certain loads of heat (Nakamura et al., 1984; Petruccelli and Añón, 1995). During extrusion cooking of soybean meal (SBM), some unique processing features are present,

because the SBM is subjected to high pressure in combination with severe shear forces and high temperatures.

The types of interactions between proteins during extrusion have been examined by extraction of the extruded materials with different solvents. In general, it is assumed that noncovalent interactions are broken by solvents such as urea and sodium dodecyl sulfate (SDS), solubilizing those proteins which were made insoluble by hydrogen bonding or hydrophobic interactions during extrusion or upon cooling after the process. Dithiothreitol (DTT) and sodium sulfite are known to cleave disulfide bonds and are, therefore, able to solubilize large aggregates held together by disulfide bonds after extrusion (Hager, 1984; Arêas, 1992). However, it is possible that insoluble proteins may be solubilized by breaking noncovalent interactions and also by cleaving disulfide bonds. Hager (1984) showed that disulfide bond formation was the most important interaction during extrusion of SBM concentrates, while Ning and Villota (1994) concluded that noncovalent interactions appeared to be the driving force in protein structure formation. The formation of covalent bonds, for example, isopeptide bonds and Maillard products, was suggested by Burgess and Stanley (1976), Stanley (1989), and Horváth and Czukor (1993). However, most studies showed that both disulfide bonds and noncovalent interactions were important in protein structure formation during extrusion and upon cooling (Jeunink and Cheftel, 1979; Mitchell and Arêas, 1992; Prudêncio-Ferreira and Arêas, 1993; Marsman et al., 1995a).

The discrepancy in interpretation of protein interaction mechanisms may be attributed to differences in process temperatures. Next, it should also be mentioned that extrusion trials were performed with different raw materials and different types of extruders.

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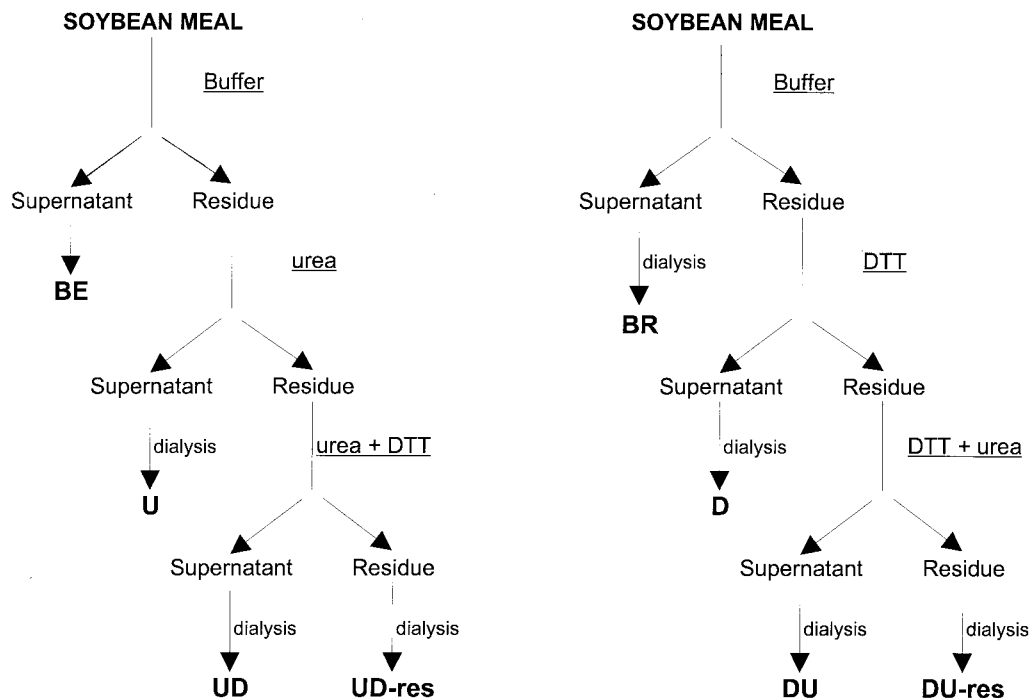
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Figure 1. Extraction procedure used.

While temperature is one important process parameter, the amount of shear forces developed during extrusion should also be considered. It has been shown that development of a certain amount of shear forces can increase the in vitro accessibility of SBM proteins toward hydrolytic enzymes (Marsman et al., 1995b).

In this research, untreated SBM is toasted and extruded at different shear levels. The types of interactions involved in the insoluble protein fractions, after extraction with a phosphate buffer, are studied using buffers containing urea, DTT, or a combination of both solvents. The different protein fractions obtained were also analyzed for trypsin inhibitor activity and for in vitro protein digestibility.

MATERIALS AND METHODS

Materials. Commercial solvent-extracted (fat content < 1%) and toasted (85 °C, 20 min) soybean meal (TSBM) with a protein content (N × 6.25) of 51% was supplied by Cargill, Amsterdam. A part of the solvent-extracted meal was not toasted, but air-dried, yielding untoasted soybean meal (USBM). The protein dispersibility indices (PDI) of TSBM and USBM were 21.2 and 90.8%, respectively.

Extrusion. An Almex Battenfeld single-screw extruder was used. USBM with an initial moisture content of 25% was extruded using torpedo elements with different lengths to vary the shear level during extrusion (Marsman et al., 1995b). These torpedo elements, assembled at the end of the screw, were equipped with 0, 4, and 8 rows of flights and were coded Ex-0, Ex-4, and Ex-8, respectively. The die diameter was 7 mm and the screw-speed 100 rpm. The product temperature at the die was measured manually using a thermocouple and adjusted at 120 °C. Moisturization was performed with a Sunther-Papenmeier mixer. The other process conditions during extrusion are described elsewhere (Marsman et al., 1995b). After extrusion, the extrudates were dried for 2 days at 45 °C and ground to pass a 0.2 mm screen.

Extraction Procedure. USBM, TSBM, and extruded SBM samples (Ex-0, Ex-4, and Ex-8) were used for two sequential extraction procedures using different solvents. A simplified extraction scheme is given in Figure 1. Two batches of 30 g of USBM, TSBM, Ex-0, Ex-4, and Ex-8 were extracted with 400 mL of 0.05 M sodium phosphate buffer (pH 7.0) for 2 h at 20 °C. The extract was centrifuged (20 min, 11000g, 10 °C). The residue was resuspended in 200 mL of buffer solution and centrifuged. The last step was repeated, and the supernatants of each batch were collected. Part of the supernatant (Figure 1, left side) was freeze-dried directly, resulting in the buffer extractable, nondialyzed fraction (BE). The supernatant from the other batch (Figure 1, right side) was dialyzed extensively against demineralized water. The retentate, the part of the material retained after dialysis of the buffer extractable fraction, was freeze-dried, yielding the buffer retentate fraction (BR). The fraction BE−BR is considered to contain SBM components, mainly non-protein nitrogen (NPN) constituents, removed by dialysis.

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

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

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

Nitrogen and Protein Content. The nitrogen determination of all the fractions was performed by a semiautomated micro-Kjeldahl method. Protein content was estimated using a conversion factor of N × 6.25.

In Vitro Protein Digestibility. For determination of the in vitro digestibility of the proteins in the obtained fractions, the pH-STAT method was used. A mixture of trypsin, chy-

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motrypsin, and peptidase was added to 1 mg of N/mL of aqueous suspensions of the obtained protein fractions. During hydrolysis at 37 °C, the pH was maintained at pH 8.0 by adding 0.1 M NaOH. The pH-STAT method is described by Pederson and Eggum (1983) and used with minor modifications as described elsewhere (Marsman et al., 1995b).

Trypsin Inhibitor Activity (TIA). TIA was determined using a modified Kakade's method according to Smith et al. (1980). Benzoyl-DL-arginine *p*-nitroanilide hydrochloride was used as the substrate for trypsin. The TIA is expressed as milligrams of inhibited trypsin per gram of protein.

Cysteine Content. The amino acid determination was performed according to the method of Rudemo et al. (1980) with minor modifications. After oxidation of 10 mg of protein with 1 mL of oxidation reagent (3% H_2O_2, 80% formic acid, and 10 mg of phenol/mL) for 16 h at 0 °C (an ice bath placed in a refrigerator), the excess of performic acid and hydrogen peroxide was degraded by adding 168 mg of $Na_2S_2O_5$. After 4 mL of 7.5 M HCl, containing 25 mg of phenol, was added, hydrolysis was performed for 21 h at 110 °C under N_2. The samples were cooled on ice and brought to pH 2.2 with 4.25 mL of 7.5 M NaOH, where the temperature did not exceed 40 °C. After internal standard (norleucine) was added, the sample was diluted to 15 mL with the loading buffer (sodium citrate, pH 2.2). After centrifugation, the samples were analyzed by a Biochrom 20 amino acid analyzer (Pharmacia) using a sodium citrate buffer system (pH 3.2−10).

Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using the Protean II electrophoresis system (Bio-Rad) after reduction of the proteins by β-mercaptoethanol. The method of Laemmli (1970) was followed with minor modifications. A sample, corresponding to 3 mg of protein, was dissolved in 1 mL of buffer solution (62.5 mM Tris-HCl, pH 6.8, 1.25% SDS, 10% glycerol, 0.00125% bromophenol blue, and 5% β-mercaptoethanol). Reduction and solubilization of the proteins were obtained after 3 h of mixing head over tail in Eppendorf cups at 37 °C. Every 90 min the samples were treated in an ultrasonification bath at 60 °C for 15 min. Runs were performed in homogeneous slab gels with a monomer concentration of 12.5% and a cross-linking concentration of 2.6%. Gel slabs were fixed and stained in a solution of methanol, acetic acid, water, and Coomassie Brilliant Blue R-250.

Free Sulfhydryl Groups. Free sulfhydryl content in the protein of USBM, TSBM, Ex-0, Ex-4, and Ex-8 was studied by using the method of Ellman (1959), with some slight modifications. Proteins were suspended in a phosphate buffer (20 mM, pH 8.0) containing 0.5% SDS (Fernandez Diez et al., 1964). After 2 h of stirring, 0.01 M DTNB (buffered with 20 mM phosphate buffer, pH 8.0) was added and the reaction was allowed to proceed for 30 min at room temperature. After centrifugation (10 min, 10000*g*, at room temperature), the extinction was measured at 412 nm. For calculation of the content of free sulfhydryl groups, an extinction coefficient of 13 600 M^{-1} cm^{-1} was used.

RESULTS AND DISCUSSION

Yields and Protein Content. Yields and protein contents of the fractions obtained are given in Table 1. The recovery for the different fractions ranged from 96 to 100%. After extraction with urea, DTT, and a combination of both reagents, mainly proteins have been extracted, especially in the heat-treated samples (69−89%). The high amount of dialyzable material from each starting material (7−20%) is the result of the high content of saccharose, stachyose, and raffinose in SBM (Schols et al., 1993). In the residues, the protein content varied from 4% in USBM to values exceeding 50% in Ex-8. In these fractions also cell wall components such as pectin, cellulose, and hemicellulose will be present (Huisman et al., 1996).

Buffer Extractable Nitrogen. In Figures 2 and 3, the nitrogen distributions among the BR, U, UD, and

Table 1. Yields (Grams) and Protein Content (Percent) of the Fractions Obtained after Sequential Extraction with Buffer, Urea, DTT, and Urea and DTT

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

[a] The yield is expressed on as is basis. [b] The protein content is expressed as weight percentage (as is basis) of each fraction.
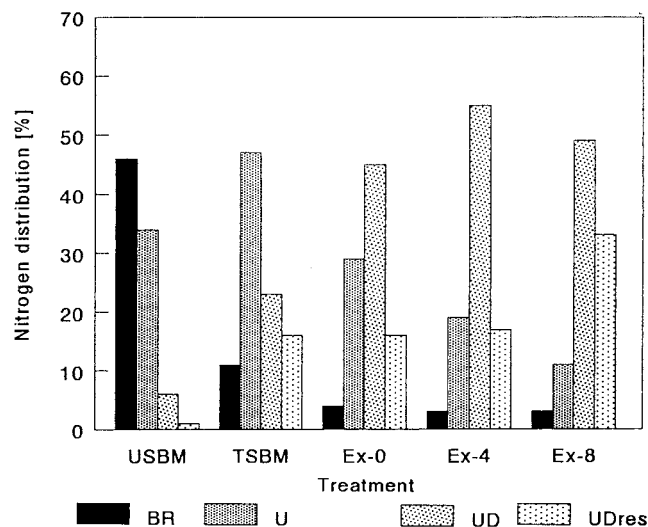

Figure 2. Nitrogen distribution (percent of total nitrogen) after extraction with urea and both urea and DTT.
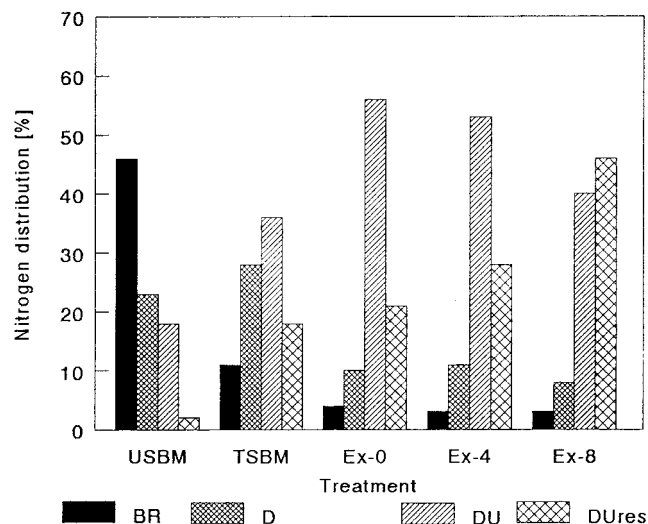

Figure 3. Nitrogen distribution (percent of total nitrogen) after extraction with DTT and both DTT and urea.

UD$_{res}$ fractions and the BR, D, DU, and DU$_{res}$ fractions are given, respectively. For a good comparison, the BR fraction is presented in both figures.

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

Heat treatment had a marked effect on protein solubility in the BR fractions. In USBM, 46% of the nitrogen could be solubilized with the sodium phosphate buffer, but this amount decreased to 11% after toasting, whereas after extrusion even lower levels were found (Figure 2). Among the extruded samples, increasing shear levels did not change the amount of protein found in the BR fractions. The sharp decrease in buffer soluble nitrogen as a result of toasting or extrusion is due to denaturation and aggregation of the native proteins in USBM, whereby extrusion had more impact on nitrogen solubility compared with toasting, which was also shown in previous research by the differences in NSI in potassium hydroxide and PDI values between these meals (Marsman et al., 1995b).

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

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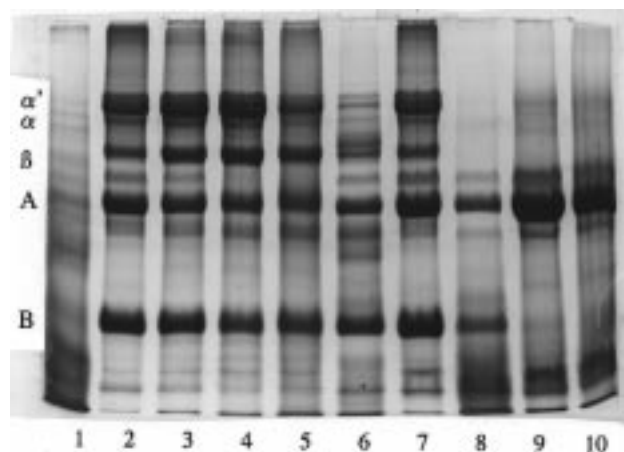

Figure 4. Soy protein composition of the fractions obtained after urea and DTT extraction: lanes 1–5, USBM, TSBM, Ex-0, Ex-4, and Ex-8 extracted with urea; lanes 6–10, USBM, TSBM, Ex-0, Ex-4, and Ex-8 extracted with DTT.

formation of larger insoluble aggregates in which disulfide bonds are involved (Yamauchi et al., 1991).

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

DTT Extractable Proteins. Extraction of the buffer residue with the buffer containing DTT (D fractions) showed that, in general, less protein was extracted compared with the urea extraction (Figure 3). For USBM, 79% of all the nitrogen was extracted with a buffer containing DTT (10% NPN + 46% BR + 23% D). In TSBM, 28% of the proteins could be solubilized with the DTT reagent, and this amount decreased to 10% after extrusion. There was only a limited shear level effect (Figure 3).

Hager (1984) defined four *states* of protein: (1) protein soluble in "simple" buffers; (2) protein insoluble due to noncovalent forces; (3) protein insoluble due to disulfide covalent bonds; and (4) protein insoluble due to a combination of both disulfide bonds and noncovalent interactions. In this scheme, it was not taken into account that some aggregated proteins may be solubilized by breaking noncovalent interactions but also by cleaving disulfide bonds. This means that the same proteins may be found in states 2 *and* 3, resulting in double counts. In this research, with these defined states of protein, for USBM double counts were calculated. If in USBM 56% of the nitrogen is extracted by the sodium phosphate buffer, 34% by a buffer containing 8 M urea, and 23% by a buffer containing 0.01 M DTT, it can be calculated that with a nitrogen yield of 113% at least 13% of the nitrogen in USBM could be solubilized by breaking noncovalent interactions as well as cleaving disulfide bonds. This number could be slightly higher or lower considering analysis errors. Because
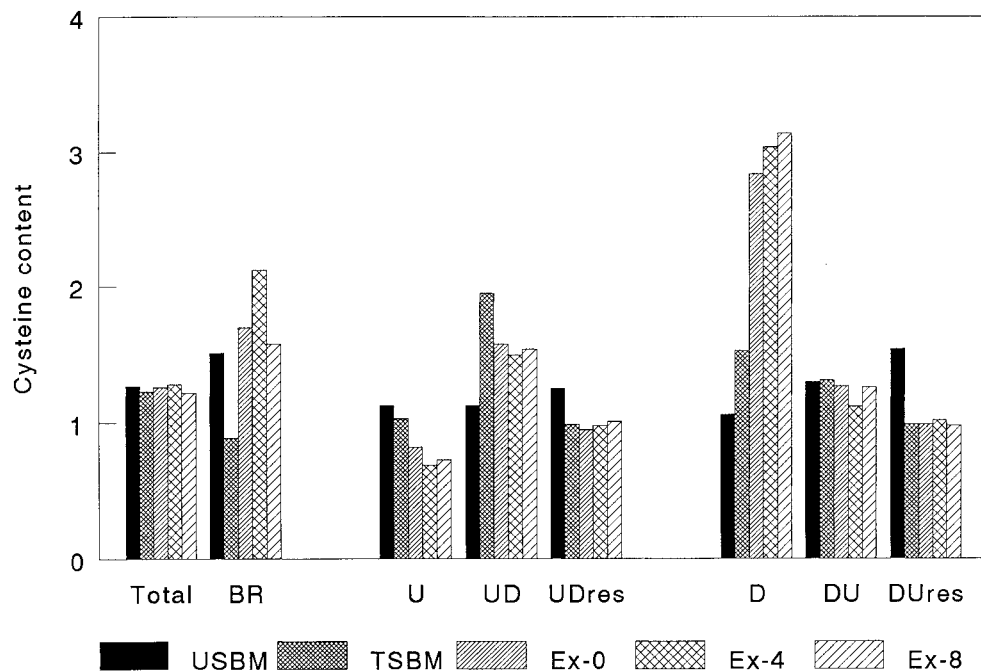
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Figure 5. Cysteine content (percent of protein in the fraction) after extraction with DTT, urea, and a combination of urea and DTT.

of the low yields of the buffer extractable fractions, it is not possible to calculate double counts in TSBM and the extruded samples, but most likely they will also occur in these materials.

The cysteine content of each fraction (percent) is given in Figure 5. A slight increase in cysteine was found in the D fractions after toasting compared with USBM. However, cysteine contribution tripled after extrusion compared with USBM. This suggests that an increasing part of the proteins in these fractions was obtained by breaking disulfide bridges rather than breaking noncovalent interactions. The amount of free sulfhydryl (SH) groups decreased from 4.0 nmol/mg of protein in USBM to 1.8, 1.7, 1.7, and 1.1 nmol/mg of protein in TSBM, Ex-0, Ex-4, and Ex-8, respectively (no further results shown). This also indicates that formation of extra disulfide bonds may be formed upon heat treatment. These results are not in line with the results of Hager (1984), who reported an increase in free SH groups after extrusion of soy concentrate but also stated that intermolecular disulfide bonding is an important factor contributing to extrudate structure at low-temperature extrusion.

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

In general, it is stated that after heating (80 °C) of β-conglycinin and glycinin in buffered solutions, the B polypeptides and β subunits are located in the precipitate as a B−β complex (Damodaran and Kinsella, 1982), in which both noncovalent and disulfide bonds were important in maintaining network structures (Utsumi and Kinsella, 1985). The existence of this complex also depends on the ionic strength of the buffer used (Nakamura et al., 1986). The A polypeptide and α, α′ subunits are found in the supernatant (Wolf, 1993).

However, most of these studies were performed with protein concentrations up to 12% (w/v). In this research, extrusion was performed at an initial moisture content of 25% at relatively high shear forces, which means that dissociation and aggregation occurred under different environmental conditions. Nevertheless, the high content of the A polypeptide in the DTT extracts of the extruded meals (Figure 4, lanes 8−10) suggests a complex consisting of only A polypeptides held together by disulfide bonds (AA complex). The presence of A polypeptides coincides with the higher total cysteine content found in these D fractions (Figure 5). It is known that two-thirds of the disulfide bonds of glycinin are contributed by the A polypeptides and the residual part by B polypeptides (Wolf, 1993). The fact that DTT alone was not able to solubilize complexes in which also noncovalent interactions are involved may explain that only limited amounts of the B polypeptide and β subunits were found in these fractions. While it has been suggested that at low protein concentrations α and α′ subunits may form a complex with the AA complex (Yamauchi et al., 1991), in this study after extrusion, limited amounts of the α′ and α subunits were found in the D fractions (lanes 8−10). Probably, at high shear levels also α, α′ subunits are participating in the B−β complex and remain insoluble after DTT extraction.

Urea plus DTT Extractable Protein and the Remaining Residue. The amounts of protein in the UD and DU fractions are shown in Figures 2 and 3, respectively. In USBM, almost all of the urea and DTT insoluble proteins were extracted (Figures 2 and 3, respectively). In TSBM, also, relatively high amounts of protein were extracted, but the highest protein yields (45−55%) were obtained in Ex-0 and Ex-4. However, at high shear levels (Ex-8) protein yields started to decrease again. It can also be seen that, especially for TSBM and Ex-0, more proteins were extracted from the residue after DTT extraction (Figure 3) compared with the amount of proteins extracted from the residue after urea extraction (Figure 2). This confirms the hypothesis that DTT alone was only able to cleave disulfide bonds
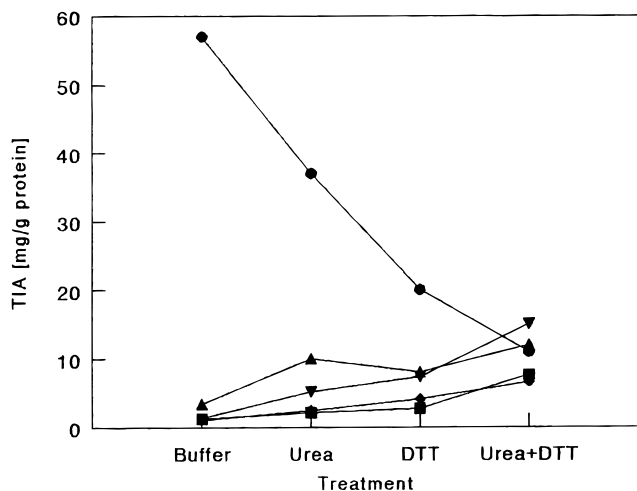
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Figure 6. TIA (milligrams per gram of protein) in the USBM (●), TSBM (■), Ex-0 (▲), Ex-4 (▼), and Ex-8 (◆) blanks after treatment with buffer, urea, DTT, and a combination of urea and DTT.

Figure 7. In vitro protein digestibility (percent) in the USBM (●), TSBM (■), Ex-0 (▲), Ex-4 (▼), and Ex-8 (◆) blanks after treatment with buffer, urea, DTT, and a combination of urea and DTT.

on the outface of the protein aggregates. Protein composition, as studied by SDS−PAGE, showed for the UD and DU fractions of TSBM, Ex-0, Ex-4, and Ex-8 typical soybean protein patterns. However, it should be noted that results were overshadowed by a high amount of insoluble complexes which remained in the stacking gel even if SDS−PAGE with the presence of urea in the gels was used (results not shown). The decrease in urea and DTT extractable proteins at high shear forces was accompanied by an increase in yields of proteins remaining insoluble after urea and DTT extraction (UD$_{res}$ and DU$_{res}$ in Figures 2 and 3, respectively). It was expected that the yield of UD$_{res}$ should be similar to that of DU$_{res}$. It is not known why this is not the case in this study.

From the high yields of U, UD, D, and DU after extrusion, it is concluded that both disulfide bonds and noncovalent interactions were involved during low-shear extrusion (Ex-0) as found by Jeunink and Cheftel (1979) and Mitchell and Arêas (1992). However, at higher shear levels (Ex-4 and Ex-8) the high yields of UD$_{res}$ and DU$_{res}$ suggest that also covalent cross-linking reactions become more important. The latter was also found by Horváth and Czukor (1993) after extrusion of full-fat soy flours at temperatures exceeding 140 °C.

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

In USBM, the TIA decreased from 57 mg/g of protein in the buffer-treated fraction to 37, 20, and 11 mg/g of protein after treatment with urea, DTT, and the combination of both reagents, respectively (Figure 6). Much lower TIAs were found in the buffer-treated blanks of the toasted and extruded SBM samples. However, when treated with urea, DTT, and the combination of both reagents, an increase in TIA could be noticed, especially after extrusion (Figure 6). Two possible
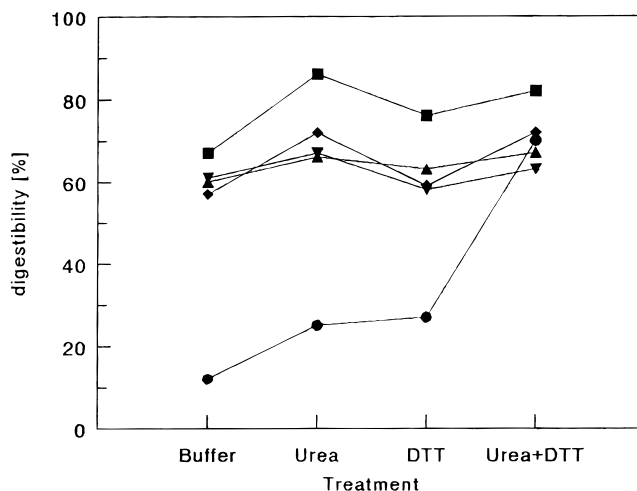
reasons may explain this increase: (1) The extraction procedure in the TIA determination procedure was not effective in extracting all trypsin inhibitors. Both breaking noncovalent interactions and cleaving disulfide bonds were necessary to extract higher amounts of trypsin inhibitor. (2) Thermomechanical treatments, such as toasting and extrusion, can effectively decrease TIA levels, but at the same time it is possible that still active trypsin inhibitors can be embedded in or bound to the protein matrix or other components and thereby be protected against inactivation. This result was also seen by others. If dehulled SBM was boiled for 30 min, this heating process reduced the TIA from 53 to 1 mg/ g. When these boiled SBMs were incubated with crude enzyme preparations of *Rhizopus oligosporus*, the TIA increased significantly (up to 35 mg/g). An increase in TIA was not seen if raw SBM was incubated with these enzyme preparations (Wang et al., 1972). Also, Delobez et al. (1971) reported the release of trypsin inhibitors from heat-treated soybeans by peptic digestion of by acid treatment, indicating that these bound trypsin inhibitors will be set free by gastric digestion.

The in vitro protein digestibility in the USBM blank showed a sharp increase from 12% in the buffer fraction to 70% after treatment with both urea and DTT. TSBM treated with urea, DTT, and both reagents showed a clear increase in digestibility values, while also in the extruded samples digestibility values were slightly increased after treatment with urea and a combination of both solvents (Figure 7). From the results of Figures 6 and 7 it can be concluded that the increase in digestibility in the USBM blank may partially be explained by the sharp decrease in TIA. However, it should be considered that also native glycinin and β-conglycinin will be denatured by urea and DTT, which makes them more accessible for enzyme attack. In the absence of trypsin inhibitors, Rothenbuhler and Kinsella (1986) also found sharp increasing pH-STAT values after treatment of the native glycinin with urea and DTT.

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

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Table 2. TIA (Milligrams per Gram of Protein) in the Fractions Obtained after Sequential Extraction with Buffer (BR), Urea (U), DTT (D), Urea and DTT (UD, DU), and the Residues (UD$_{res}$, DU$_{res}$)

fraction	USBM	TSBM	Ex-0	Ex-4	Ex-8
starting material	54	12	7	3	2
BR	138	14	50	8	5
U	7	7	48	22	10
UD	tr[a]	47	46	39	13
UD$_{res}$	—[b]	14	2	2	1
D	2	24	91	56	54
DU	1	8	13	6	5
DU$_{res}$	—[b]	17	2	2	1

[a] Traces, <1 mg/g of protein. [b] Not determined.

Table 3. In Vitro Protein Digestibility[a] (Percent) and Relative in Vitro Protein Digestibility[b] (Percent of the Corresponding Blank) in the Fractions Obtained after Sequential Extraction with Buffer (BR), Urea (U), DTT (D), Urea and DTT (UD, DU), and the Residues (UD$_{res}$, DU$_{res}$)

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

[a] The measured in vitro protein digestibility. [b] The measured in vitro protein digestibility as a percentage of the corresponding blank. [c] Not determined.

solvent effect should be taken into account when the in vitro protein digestibility in the fractions obtained is determined.

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

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

The solvent effect and the high level of TIA in some of the fractions make it complicated to draw any conclusions from the results obtained from the in vitro protein digestibility of the fractions. For that reason, also the relative in vitro protein digestibilities are given in Table 3. In this case the digestibility values were compared with the corresponding blanks. A number <100% suggests a fraction that has a lower digestibility than the corresponding blank, whereas a number >100% suggests a fraction that has a digestibility higher than the corresponding blank.

In the DTT-extracted fractions of the heat-treated samples, low relative protein digestibilities were found compared with the corresponding blanks, whereas extraction with urea showed the opposite effect and resulted in protein fractions with higher relative digestibilities compared with the corresponding blanks. From these results, it can be concluded that proteins extractable by breaking noncovalent interactions are more accessible for enzyme attack compared with proteins which are extractable after cleaving of disulfide bonds. More evidence for this conclusion was found by the fact that if the urea and DTT insoluble proteins were extracted with a combination of both urea and DTT, proteins extracted from the DTT insoluble residue were more digestible compared with proteins extracted from the urea insoluble residue. SDS–PAGE showed that after extrusion mainly subunits of glycinin were found in the DTT fractions. These polypeptides, especially the A polypeptide, showed a high resistance against the proteases used in the pH-STAT method. This can be explained not only by the high level of disulfide bonds but also by the two free sulfhydryl groups in glycinin. Sulfhydryl–disulfide intrachange in the subunits or sulfhydryl–disulfide interchange among subunits may occur during processing, resulting in a complex protein aggregate where also noncovalent interactions and other covalent cross-linking reactions are playing a role. From these results, it is stated that glycinin is less digestible compared with β-conglycinin. The same conclusion was drawn by other workers (Kim et al., 1990; Aufrère et al., 1994) and in previous research in which the in vitro accessibility of heat-treated soybean meal toward hydrolytic enzyme preparations was studied (Marsman et al., 1997).

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

Effect of Toasting and Extrusion on Soy Protein Interactions

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