Limited Hydrolysis of Soy Proteins with Endo- and Exoproteases

Stephanie Jung[a,*], Coralie Roussel-Philippe[a], Jenny L. Briggs[b], Patricia A. Murphy[a], and Lawrence A. Johnson[a]

[a]Department of Food Science and Human Nutrition, Center for Crops Utilization Research, and [b]Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, Iowa 50011-1061

ABSTRACT: Changes in the native state and functional properties of soy protein achieved by limited proteolysis of soy flour were investigated. Different enzyme-to-substrate ratios (E/S) were used to obtain low (3–5%) and medium (5–10%) degrees of hydrolysis (DH). Six protease preparations (three with predominately exopeptidase activities and three with predominately endopeptidase activities) were evaluated, and their effects on solubility, emulsification capacity, SDS-PAGE profiles, and denaturation enthalpies were characterized. Endoproteases (Multifect® Neutral, Protex™ 6L, and Multifect® P-3000) and exoproteases (Fungal Protease Concentrate, Experimental Fungal Protease #1, and Experimental Fungal Protease #2) yielded similar increases in soy protein solubility. The modifications to the soy peptide profile were similar for the three exoprotease mixtures at a 1% E/S ratio, whereas the extent of hydrolysis with Protex™ 6L was more pronounced than with the two other endoproteases (Multifect® Neutral and Multifect® P-3000). The emulsification capacity of protease-modified soy flour declined regardless of DH and enzyme type (exo- or endoprotease). After hydrolysis to >4% DH, denaturation enthalpies of glycinin and β-conglycinin decreased significantly, whereas hydrolysis to lower DH did not affect these values.

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The consumption of soy products is increasing in Western countries, primarily among those consumers seeking healthier diets. This trend can be attributed to the favorable consumer perception about the high-protein quality and low-fat contents of soy products, as well as the health claim for soy protein approved in 1999 by the U.S. Food and Drug Administration. Improving soy protein functional properties may further increase their use in food products, thus offering consumers a wider choice of soy-containing foods. One approach to improving the functional properties of soy protein is enzymatic hydrolysis. This approach has been widely considered for more than 30 yr and has generated numerous studies (1–4). The effects of enzymatic hydrolysis on soy protein functionality have been shown to vary according to substrate characteristics (e.g., extent of protein denaturation), environmental conditions (e.g., pH), enzyme specificity, and degree of hydrolysis (DH).

The functional properties of soy products are related to the structures of the major globular proteins, β-conglycinin and glycinin. The former is a glycoprotein composed of three subunits, α′, α, and β, whereas the latter is a hexamer of acidic and basic subunit pairs connected by one disulfide bond. The structural and resulting functional changes that may occur when soy protein isolates (SPI) are subjected to different treatments are difficult to foretell because of the structural complexities of glycinin and β-conglycinin (5). It has generally been observed that protein solubility increases after hydrolysis; however, there are contradictions in the literature about the effects of enzyme treatment on other functional characteristics, such as emulsifying and foaming properties.

Processing that involves enzymatic modification normally includes a thermal treatment to inactivate the enzyme activity, which can be problematic. Denaturation temperatures for β-conglycinin and glycinin are approximately 75 and 93°C, respectively (6). Applying a thermal treatment to stop enzymatic activity denatures the substrate protein as well as the enzyme (7) and/or causes new interactions between the hydrolyzed proteins. Lopez *et al.* (8) used acidification and heating for 10 min at 75°C to stop the proteolytic activity of different enzymes on soy protein concentrate (SPC). Accordingly, this treatment reduced the total enthalpy of SPC from 10.4 to 8.8 J/g, and significantly affected surface hydrophobicities of the hydrolysates. Heating SPI to 75 and 98°C significantly decreased β-conglycinin and glycinin enthalpy values as well as free sulfhydryl contents (5).

Pepsin (EC 3.4.23.1), papain (EC 3.4.22.2), bromelain (EC 3.4.22.4), ficin (EC 3.4.22.3), bacterial proteases, or Flavourzyme® (endoprotease) and Alcalase® (endopeptidase) are among the numerous enzymes that have been evaluated for their abilities to modify the functional properties of SPI and SPC. The use of enzymes in food applications continues to grow, and new enzyme preparations are rapidly becoming available on the market because of advances in molecular biology. The availability of these new enzymes justifies the need to characterize their effect on soy protein functionality.

Although numerous publications have described the effects of proteases on SPI and SPC, few attempts have been undertaken to enzymatically modify soy flour (SF) (9–11). The advantages of using flash-desolventized, hexane-defatted SF as the protein source are its cost effectiveness and its relatively high concentration of native-state proteins, a result of mild

*To whom correspondence should be addressed at Department of Food Science and Human Nutrition, Center for Crops Utilization Research, 2312 Food Science Bldg., Iowa State University, Ames, IA 50011-1061.
E-mail: jung@iastate.edu

Present address of third author: Thermo Electron Corp., Madison, WI.

processing conditions. The goal of the present work was to determine the effects of proteolytic enzyme modification of soy proteins with respect to solubility and emulsification capacity for SF hydrolyzed with three endopeptidases and three exopeptidases. Degrees of hydrolysis of less than 10% were employed to avoid the formation of bitter peptides. To reduce the effect of heat on native-state proteins and to understand precise changes caused solely by the enzyme treatment, enzyme activity was not inactivated by thermal treatment.

EXPERIMENTAL PROCEDURES

Protein substrate. A commercial flash-desolventized, hexane-extracted SF, Nutrisoy 7B, was purchased from Archer Daniels Midland Co. (Decatur, IL). The proximate dry-basis composition of the defatted SF was 56.0% protein (Kjeldahl, N × 6.25), 0.83% crude lipid, 7.12% ash, and 36.0% carbohydrate by difference. The moisture content of the material was 5.1%. The protein dispersibility index for the SF, determined by Woodson-Tenent Labs (Des Moines, IA), was 89%.

Enzymes. Six enzyme preparations obtained from Genencor International (Rochester, NY) were evaluated. Multifect® Neutral (EC 3.4.21.62 and EC 3.4.24.38) is a bacterial neutral protease preparation with mainly endopeptidase activities derived from the controlled fermentation of a nongenetically modified strain of *Bacillus amyloliquefaciens*. Protex™ 6L (EC 3.4.21.62) is an alkaline serine endopeptidase derived from a selected strain of *B. licheniformis*. Multifect® P-3000 (EC 3.4.21.62) is a *B. amyloliquefaciens* serine endopeptidase protease expressed in a genetically modified selected strain of asporogenic *B. subtilis.* Fungal Protease Concentrate (EC 3.4.21.14 and/or EC 3.4.24.4), Experimental FP #1, and Experimental FP #2 (EC 3.4.23.18) were derived from *Aspergillus oryzae* strains and contained primarily exopeptidase activities but also some minor endopeptidase activity. Their activities were determined by the supplier and are summarized in Table 1. The percentages of enzymes added for the hydrolysis were based on enzyme/substrate ratios (E/S), defined as grams of enzyme preparation added per gram of soy protein.

Hydrolysate preparation. A 10% (w/w) aqueous dispersion of SF was prepared with 25 g of Nutrisoy 7B at the optimal conditions of pH and temperature for each enzyme (Table 1). Different E/S ratios were used to obtain selected DH ≤10% (Table 2). One hydrolysis was performed per day in a 250-mL jacketed reactor connected to a water bath. The enzymes used in our study had temperature optima in the 50–60°C range and no activity at temperatures <20°C (Table 1). To avoid unwanted modification of functional properties from thermal treatment, we chilled the hydrolysate to 15°C and maintained this temperature during hydrolysate characterization. By monitoring the pH and performing SDS-PAGE electrophoresis (data not shown), we verified that the enzymes had no detectable activity at 15°C during characterization (~4 h). These conditions are not sufficient to deactivate the enzyme; consequently, when the temperature is adjusted to enzyme optimal conditions, protein modification may occur.

For each enzyme treatment, we prepared enzyme-free control samples at exactly the same pH and temperature conditions. Hydrolysis was followed using a pH-stat (718 STAT Titrino; Metrohm, Brinkmann Instruments Inc., Westbury, NY). DH was estimated according to Adler-Nissen (12) as follows:

$$DH = [(V_{NaOH} \times N_{NaOH})/(\alpha \times MP \times h_{tot})] \times 100\% \quad [1]$$

where α is the degree of dissociation of the α-amino groups, MP is the mass of protein (g), and h_{tot} is the total number of peptide bonds in the protein (meq/g protein). The α value, which depends on temperature and pH, was adjusted as a function of the experimental conditions used to performed hydrolysis. The formula used to calculate the α value was

$$\alpha = \frac{10^{pH - pK}}{1 + 10^{pH - pK}} \quad [2]$$

TABLE 1
Temperature and pH Activity Ranges for Enzyme Preparations and Experimental Conditions

Enzyme	Enzyme activity	Range of enzyme activity pH, temperature (°C)	Conditions used pH, temperature (°C)
Endoprotease			
Multifect® Neutral	>1600 AU/g[a]	6.0 to 8.0, 40 to 60	7.0, 55
Protex™ 6L	>580,000 DU/g[b]	7.0 to 10.0, 25 to 70	7.0, 50
Multifect® P-3000	>3,000 GSU/mL[c]	7.0 to 10.0, 20 to 65	7.0, 50
Exoprotease			
Fungal Protease Concentrate	>400,000 HU/g[d]	6.0 to 9.0, 25 to 60	7.5, 50
Experimental Fungal Protease #1	>400,000 HU/g	6.0 to 9.0, 25 to 60	7.0, 50
Experimental Fungal Protease #2	>400,000 HU/g	6.0 to 9.0, 25 to 60	7.0, 55

[a]AU, azo units/g.
[b]DU, delft units. Based on the ability of the protease to cleave *p*-nitroanilide from a synthetic peptide, succinyl-ala-ala-ala-*p*-nitroanilide, resulting in an increase in absorbance at 405 nm.
[c]Genencor subtilisin units. Based on the ability of the protease to cleave *p*-nitroanilide from a synthetic peptide, succinyl-ala-ala-ala-*p*-nitroanilide, resulting in an increase in absorbance at 405 nm.
[d]HU, hemoglobin units. One unit is the activity that will liberate 0.0447 mg of nonprotein nitrogen in 30 min at pH 4.7 and 40°C. All enzyme preparations were acquired from Genencor International (Rochester, NY).

TABLE 2
Final Degrees of Hydrolysis (DH) (%) of Modified Soy Flours for Different Enzyme/Substrate Ratios (E/S)[a]

Endoprotease	E/S (%)	DH (%)	Exoprotease	E/S (%)	DH (%)
Multifect® Neutral	1	3.4[b]	Fungal Protease Concentrate	1	4.0[b]
	2	5.3[c]		3	6.0[c]
	5	8.3[d]		5	7.8[d]
Protex 6L™	0.1	2.6[b]	Experimental Fungal Protease #1	0.5	4.6[b]
	0.5	4.9[c]		1	6.1[c]
	1	5.7[c]		3	8.5[d]
	2	7.6[d]			
Multifect® P-3000	0.1	2.6[b]	Experimental Fungal Protease #2	1	5.7[b]
	0.5	4.3[c]			
	1	5.0[c]			
	2	6.5[d]			

[a]Controls showed an apparent average DH value of 0.72 (average of all the controls). This value had the roman superscript "a" for statistical analysis. Values for each enzyme followed by the same roman superscript were not significantly different ($P < 0.05$).

The pK and h_{tot} values for soy protein were 7.1 and 7.8, respectively (12). At a minimum, all hydrolysis treatments were performed in duplicate.

Characterization of functional properties. Solubility was determined by using 10% aqueous dispersions of the samples (w/w) adjusted to pH 7.0 with 1 N HCl and stirred at 15°C for 45 min. The samples were then centrifuged at 10,000 × g for 20 min at 15°C. The protein contents (N × 6.25) of the supernatants and initial dispersions were determined by using the semiautomated Kjeldahl method and a Tecator Kjeltec digestion system (Tecator, Hoganas, Sweden). Protein solubility was expressed as the ratio of soluble protein to total protein. Determinations were conducted in duplicate. The emulsification capacity (EC) was determined as follows: Soybean oil dyed with ~4 ppm of Sudan Red 7B (Sigma, St. Louis, MO) was continuously added at the rate of 37 mL/min into 25 mL of a 2% (pH 7) dispersion of soy hydrolysate with a Bamix wand mixer (ESGE AG Model 120; ESGE, Mettlen, Switzerland) until phase inversion was reached, as identified by the abrupt decrease in homogeneity and loss of viscosity. The weight of oil needed to reach phase inversion was measured, and EC was expressed as grams of oil emulsified/gram of soy protein. EC was determined three times for each sample.

For rheological determinations, the samples were freeze-dried after proteolytic treatment. Rheological flow behavior was measured by using a 10% (gram protein/gram water) dispersion. The rheological behavior of the sample was measured by using a RS-150 Rheo Stress Rheometer (Haake, Karlsruhe, Germany). Shear was applied with a 60-mm 2° titanium cone over the range of 10–500/s. The experimental flow curves were modeled using the power law model: $\tau = K\dot{\gamma}^n$, where τ was the shear stress (mPa); K, the viscosity coefficient; $\dot{\gamma}$, the shear rate (s^{-1}); and n, the flow index. Samples were tested a minimum of three times.

DSC. DSC measurements to quantify thermal properties and estimate the extents of protein denaturation were performed by using an Exstar 6000, Seiko SII calorimeter (Seiko Instruments Inc., Torrance, CA). The enthalpies for thermal denaturation of the two major soy proteins (ΔH expressed in J/g protein) were determined. Twenty to 25 mg of 10% soy protein hydrolysate dispersion (w/w) prepared in water was adjusted to pH 7.0 and hermetically sealed in aluminum pans. An empty pan served as a reference. The samples were heated from 25 to 120°C at 10°C/min. All assays were replicated at least four times.

SDS-PAGE. SDS-PAGE was performed by using a discontinuous buffer system on 13% slab gels. Gels were run in mini-slabs (mini Protean III Model; Bio-Rad Laboratories, Hercules, CA). Protein samples were diluted in a solution containing 125 mM THAM [2-amino-2 (hydroxymethyl-1,3-propanediol)], 5.0 M urea, 2% β-mercaptoethanol, 0.20% SDS, 20% glycerol, and 0.01% bromophenol blue, pH 6.8. Ten micrograms of protein sample, 5 μg of a M.W. marker (Sigma M3913), or 5 μg of purified β-conglycinin was loaded into the lanes. Gel electrophoresis was carried out at a constant 120 V. The gels were stained following the procedure described by Neuhoff *et al.* (13).

Statistical analysis. Data were analyzed by appropriate statistical methods to determine the level of significance, with $P < 0.05$ using the general linear models of the Statistical Analysis System (version 6.0; SAS Institute Inc., Cary, NC). *T*-tests were performed to compare means.

RESULTS AND DISCUSSION

Extent of enzymatic modification. Protein hydrolysis in SF with different types (exo- and endoproteases) and amounts of enzymes was monitored by following DH over 3 h of hydrolysis (Fig. 1). Increasing the E/S ratio increased the initial hydrolysis rate and final DH (Table 2). Different E/S ratios were selected for each enzyme such that the final DH was <10%. As expected, all enzyme treatments caused sharp increases in DH over the first 30 min of reaction, and rates slowed thereafter. This trend was consistent with results obtained by other researchers using various proteases and substrates (1,4,8). The shape of the hydrolysis curve, particularly the decrease in the hydrolysis rate, is not yet fully understood. Alder-Nissen (12) emphasized that this behavior cannot be attributed to substrate exhaustion. Constantinides and Adu-Amankwa (14) suggested several explanations including a depletion of the

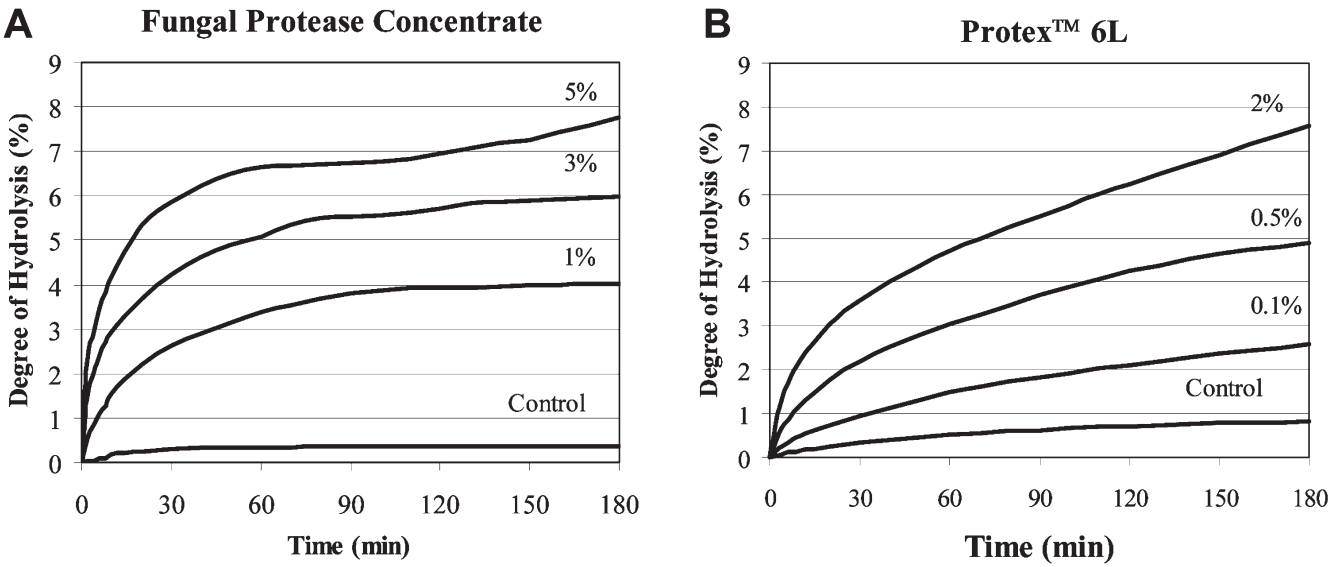

FIG. 1. Degree of hydrolysis (DH) as a function of time for various enzyme-to-substrate (E/S) ratios using (A) Fungal Protease Concentrate and (B) Protex™ 6L. The curves represent the averages of two replications.

peptide bonds suitable for the enzyme, product inhibition, and/or enzyme inactivation.

The pH of the Nutrisoy 7B control sample decreased slightly over time, which was attributed to increased protein solubilization. The corresponding DH values for the control samples were termed "apparent" and were not related to proteolytic action of the enzymes. The mean apparent DH for the controls was 0.72 (SD 0.22). A decrease in pH from 8.5 to 8.0 for a soy flake dispersion (1:10 or 1:15 flake-to-water ratio) after 1 h of stirring at 20°C was also reported by Wu *et al.* (15).

After adding a 1% E/S ratio of protease, the highest DH value was achieved with Experimental Fungal Protease #1, followed by Experimental Fungal Protease #2 and Protex™ 6L, Multifect P-3000, Fungal Protease Concentrate, and Multifect Neutral. At a 1% E/S, there was no significant difference between the average DH values obtained for the predominately exopeptidase enzymes and the predominately endopeptidase enzymes.

Physicochemical properties. DSC thermograms of the soy flour showed two endothermic transitions with peak temperatures at 75 and 93°C, which corresponded to the denaturation temperatures for the major soy proteins, β-conglycinin and glycinin, respectively (results not shown). Slight variations around our measured denaturation temperatures have been reported in the literature. These variations may be due to genotypical differences and/or differences in processing, i.e., temperature applied and the presence of other components, such as salt, to prepare the soy protein substrate (6,8). After hydrolysis, peak temperatures were similar at low DH, regardless of the enzyme preparation method. For >6% DH, the peak temperature decreased slightly to 70 and 89°C for β-conglycinin and glycinin, respectively (results not shown). A similar shift of denaturation temperature to a lower temperature was reported by Lopez *et al.* (8) after hydrolysis of SPC at DH ranging from 5 to 15%.

The relationship between denaturation enthalpy values for glycinin and β-conglycinin and DH are illustrated in Figure 2 when using Fungal Protease Concentrate, Protex 6L, and Multifect Neutral. The average β-conglycinin enthalpy value for the control samples was 2.2 J/g. Values of 0.08 and 7.53 J/g have been reported for SPI and purified β-conglycinin, respectively (16). For <4% DH, the β-conglycinin enthalpy values for the controls and the hydrolysates were not statistically different. For >4% DH, enthalpy decreased. The average enthalpy for glycinin denaturation in the control samples was about 8 J/g. Lakemond *et al.* (17) reported enthalpy values at pH 7.6 for different ionic strengths in the range of 7 to 17 J/g for glycinin. For controls, the enthalpy values of glycinin were approximately four times greater than those of the β-conglycinin. This was attributed to the disulfide bonds of the glycinin, which stabilize protein conformation against thermal denaturation (6). The changes in denaturation enthalpies for glycinin caused by hydrolysis followed the same trend we observed for β-conglycinin. After hydrolysis to >4% DH, the denaturation enthalpy decreased significantly compared with the control. The decline in enthalpy values among β-conglycinin and glycinin as well as the decline in denaturation temperatures with increasing DH can be attributed to modifications of the hydrophobic and/or hydrophilic properties of the molecule and the degree of aggregation, which resulted in greater sensitivities of the proteins to heat denaturation.

To determine the extent of peptide profile changes caused by hydrolysis, SDS-PAGE analyses of the hydrolysates obtained at a 1% E/S ratio were performed (Fig. 3). The unhydrolyzed control showed a typical electrophoretic pattern for soy protein flour: the first band being lipoxygenase; the next three bands being the α′, α, and β subunits of β-conglycinin;

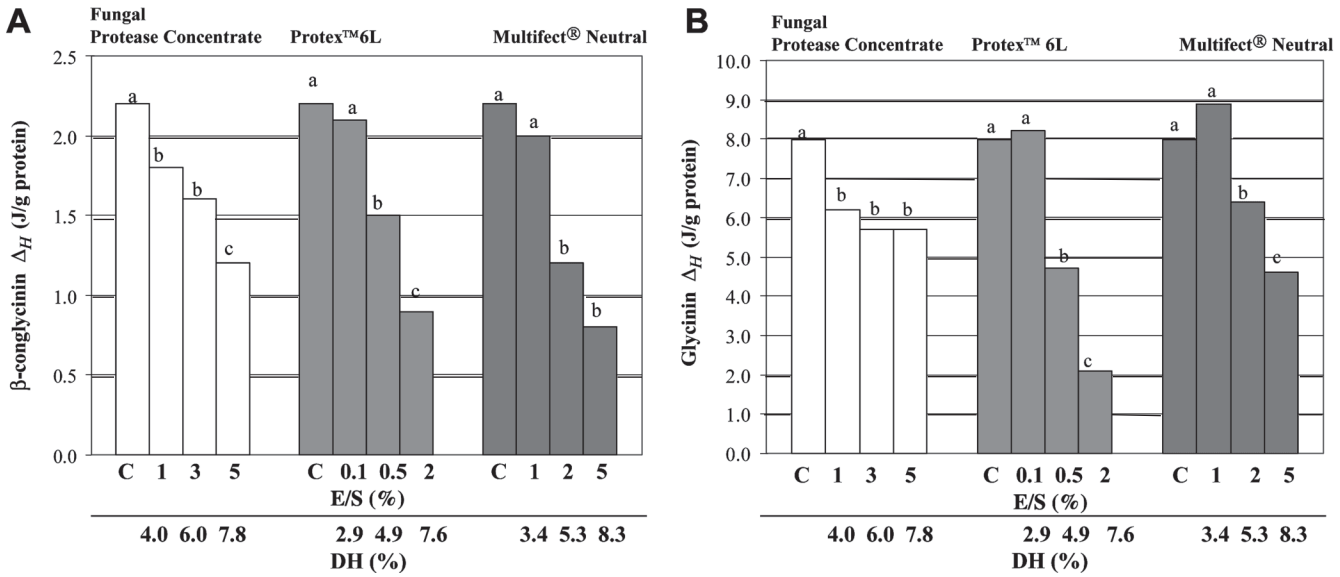

FIG. 2. Denaturation enthalpies (J/g protein) for (A) β-conglycinin and (B) glycinin as a function of the E/S ratios for exoprotease Fungal Protease Concentrate and endoproteases Protex™ 6L and Multifect® Neutral.

then several bands for the glycinin subunits, two for the acid subunits (A_3, and A_{1a}, A_{1b}, A_2, A_4, as indicated by the letter "A" on the gel and in the text below), and finally one for the basic subunits (B). The common modifications that occurred with the use of endo- and exoproteases were (i) disappearance of the lipoxygenase band; (ii) disappearance of the α′ band of the β-conglycinin with simultaneous decreases in the intensities of A subunits of the glycinin, which were more or less pronounced depending on the enzyme used; and (iii) formation of peptides with M.W. <6,500 kDa, which were observed as increased intensity of the band at the bottom of the gel. The electrophoretic patterns of the hydrolysates obtained with the exopeptidase Fungal Protease Concentrate were similar to the peptide profiles of Experimental Fungal Protease #1 and Experimental Fungal Protease #2. Among the endoproteases, the extent of modifications at a 1% E/S ratio increased in the order Multifect Neutral, Multifect P-3000, and Protex 6L. For Protex 6L hydrolysate, the acidic subunits of glycinin disappeared, and the intensity of the α band of the β-conglycinin was considerably decreased. The greater degradation of the peptides with Protex 6L agreed with our DSC results, showing that the highest loss of denaturation enthalpy was achieved by using this enzyme. Since equal amounts of protein were loaded in each lane of the SDS-PAGE gels, the sustained intensities of specific bands indicated that these subunits were more resistant to enzymatic hydrolysis due to the amino acid composition of the peptides or the location of these peptides in the protein structure. The decreased intensity of the A band was more substantial for the endoproteases than for the exoproteases. This effect was expected among the glycinin peptides because the acidic fraction contains more acidic amino acids and should be preferentially attacked by the endoproteases (18).

Several peptides appeared in the 36–20 kDa area for the hydrolysates obtained with the exoproteases. A new peptide with ~60 kDa M.W. was observed in all of the hydrolysates (Fig. 3). For the endoproteases, the band intensity of this peptide was less pronounced than for the exoproteases, especially for the hydrolysate obtained with Protex 6L, where a band with very low intensity was observed. Since peptide degradation was more extensive with Protex 6L, the low density of the 60-kDa band may be explained by faster degradation of this intermediate product with Protex 6L compared with the other enzymes. Figure 4 shows the progressive changes in the peptide profile that occurred with an increasing DH by Multifect P-3000. At 2.6% DH, the peptide profiles of the hydrolysates were similar to the control except for the loss of the lipoxygenase band and the existence of low-M.W. pep-

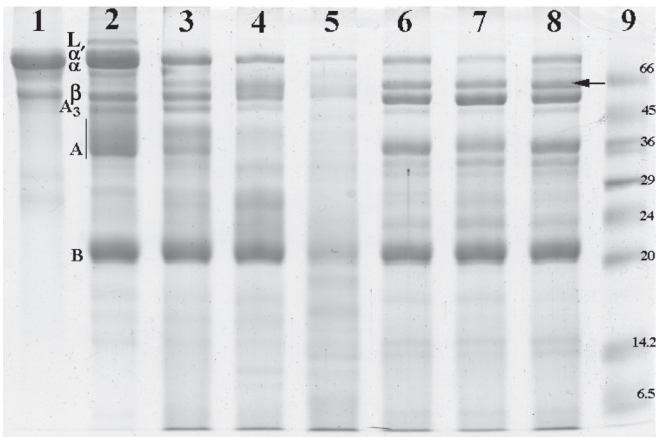

FIG. 3. SDS-PAGE of soy flour control and 1% E/S ratio hydrolysates. 1. β-conglycinin; 2. Control; 3. Multifect® Neutral; 4. Multifect® P-3000; 5. Protex™ 6L; 6. Fungal Protease Concentrate; 7. Experimental Fungal Protease #1; 8. Experimental Fungal Protease #2; and 9. M.W. marker; L, lipoxygenase; A_3, A_3 acidic glycinin subunit; A: A_{1a}, A_{1b}, A_2, A_4 acidic glycinin subunits; B: basic glycinin subunit.

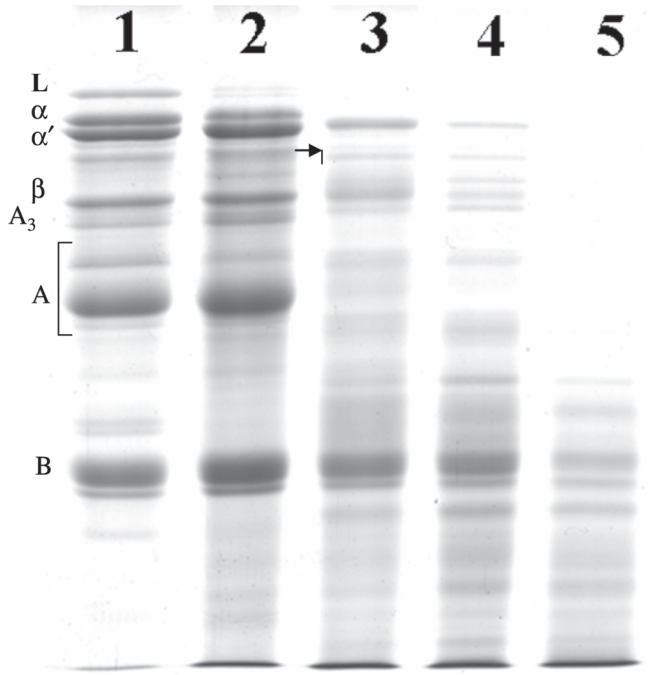

FIG. 4. SDS-PAGE of Multifect® P-3000 hydrolysates as function of the degree of hydrolysis. 1, Control; 2, 2.6% DH hydrolysate; 3, 4.3% DH hydrolysate; 4, 5.0% DH hydrolysate; and 5, 6.5% DH hydrolysate. For abbreviation see Figure 1.

tides at the bottom of the gel. Increasing the DH led to progressive disappearance of the β-conglycinin subunits and the A_3 and A glycinin subunits, as well as decreased intensity of the polypeptide B band. The peptide profile evolution for increasing DH with Multifect P-3000 also illustrated that the 60-kDa peptide was an intermediate product of degradation that was further degraded over time. This observation reinforced the idea that the splitting of the peptide bonds was probably a result of simultaneous reactions in which peptides resulting from the initial attack of the proteases acted as substrates for further degradation to smaller peptides (12).

Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, and exopeptidases hydrolyze amino acid(s) from either the N- or C-terminus. Therefore, we expected that hydrolysis with the endo- and exoproteases would lead to different changes in the peptide profiles. The rate of decrease in peptide band intensity and the pattern of the appearance of peptides with low M.W. were different for endo- vs. exoproteases. However, the peptide profiles showed similarities between the hydrolysates obtained with the endo- and exoproteases.

Solubility. Solubility is a prerequisite for many other functional properties, such as gelling, foaming, and emulsifying, and depends on numerous factors. The control sample, which was the mean of the controls obtained for each enzyme, had 74% (SD 2.3) protein solubility at pH 7.0. Increased solubility was observed after hydrolysis, except for samples treated at 1% E/S Fungal Protease Concentrate, 0.1% E/S Protex 6L, and 2% Multifect P-3000 (Table 3). Increased E/S did not considerably affect protein solubility of the hydrolysates. For example, increasing the E/S ratio from 0.5 to 2% Protex 6L increased the solubility from 82 to 85%. Qi *et al.* (1) reported solubility changes after hydrolysis that were consistent with our results, namely, hydrolysis of SPI with pancreatin between 7 and 17% DH increased the solubility at pH 7.0 from ~85 to ~90%. In our study, increased solubility caused by protease treatment was low compared with the results reported after treating SPC with Alcalase at 15% DH and Pomiferin at 10% DH; solubility at pH 7.0 increased from 10% for the control to 50% (8). Similarly, an increase from 10 to approximately 50% was observed by Don *et al.* (19) after enzymatic modification of SPC with fungal and bacterial proteases at 10% DH. This difference in solubility increase may be explained by substrate characteristics. Both Lopez *et al.* (8) and Don *et al.* (19) used SPC, whereas we used soy flour. Because of the process used to obtain SPC, one would expect it to contain fewer native-state proteins than does flour.

The protein native state is of primary importance when enzyme modifications are expected because it will determine the ease with which the enzyme can find cleavage sites. The accessibility of the cleavage site may affect the size of the peptide that is produced during hydrolysis. With substrates containing native proteins, intermediate products that may be

TABLE 3
Solubilities at pH 7 and Emulsification Capacities of the Hydrolysates as a Function of the E/S Ratio[a]

Endoprotease	E/S (%)	Solubility (%)	Emulsification capacity (g oil/g protein)	Exoprotease	E/S (%)	Solubility (%)	Emulsification capacity (g oil/g protein)
Multifect® Neutral	1	80^{b}	327^{b}	Fungal Protease Concentrate	1	75^{a}	$218^{b,c}$
	2	84^{c}	356^{b}		3	81^{b}	274^{b}
	5	86^{c}	322^{b}		5	82^{b}	140^{c}
Protex™ 6L	0.1	76^{a}	430^{b}	Experimental Fungal Protease #1	0.5	83^{b}	265^{b}
	0.5	82^{b}	$332^{b,c}$		1	83^{b}	284^{b}
	2	85^{c}	316^{c}		3	87^{b}	268^{b}
Multifect® P-3000	0.1	82^{b}	399^{b}	Experimental Fungal Protease #2	1	85^{b}	265^{b}
	0.5	83^{b}	344^{b}				
	1	84^{b}	388^{b}				
	2	73^{a}	346^{b}				

[a]The solubility and emulsification capacity of the control were 74% (SD 2.3) and 560 g oil/g protein (SD 78). These values had the roman superscript "a" for statistical analysis. Values for each enzyme followed by the same roman superscript were not significantly different ($P < 0.05$). For abbreviations see Table 2.

formed would be used rapidly as substrate by the enzyme because of the native protein structural conformation. With substrates containing denatured proteins, there may be competition between the denatured substrate and intermediate product formed during hydrolysis.

Protein solubility is related to the proportion and distribution of surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interactions with water. Increased solubility may also be related to the formation of small peptides, as was observed by Ortiz and Añón (20) after treating an SPI with bromelain. The formation of small peptides is probably one of the reasons for the increased solubility observed in our study, as was revealed by the SDS-PAGE data. The hydrolysate peptide profile observations and our solubility results showed that (i) increased solubility can be obtained with minor modifications in the SDS-PAGE profile (see the result obtained with Multifect P-3000 at 2.6% DH), and (ii) extensive changes in the peptide profile do not confer a substantial increase in solubility (see the results obtained with Protex 6L). The comparison between the solubility values and denaturation enthalpy values suggested that (i) even without a significant loss of the native state, solubility increased and (ii) increased protein denaturation was not proportional to increased solubility.

To determine whether a relationship existed between viscosity and increased solubility among our hydrolysates, we determined the apparent viscosity profile as a function of shear rate applied to a control, 2 and 4% DH hydrolysates obtained with Multifect Neutral (Fig. 5). For the three samples, increased shear rate was accompanied by decreased viscosity, which can be attributed to rearrangement of the molecules in an ordered state and/or to destruction of the original structure (21). The apparent viscosities observed at a 500 s^{-1} shear rate

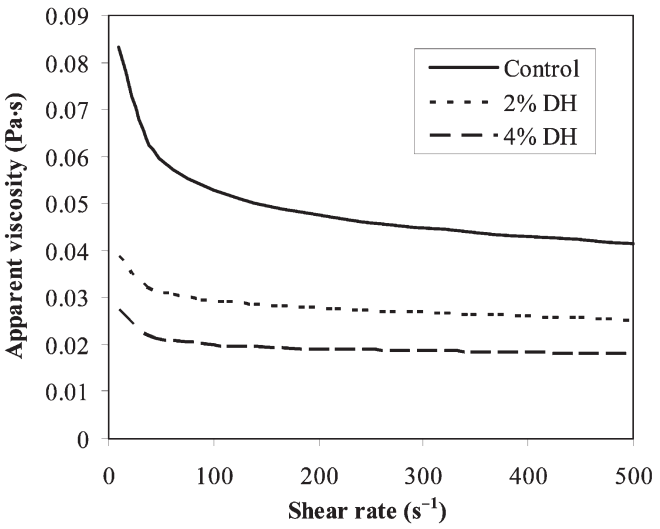

FIG. 5. Apparent viscosities of control and Multifect® Neutral hydrolysates obtained after hydrolysis 2 and 4% DH. The curves represent the averages of two replicates. For abbreviation see Figure 1.

were 0.025 and 0.018 Pa·s for the 2 and 4% DH samples, respectively, and 0.041 Pa·s for the control. This decreased viscosity, which agreed with the decreased viscosity of enzyme-modified SPI reported by Puski (22), can be related to the appearance of low-M.W. peptides as observed in the SDS-PAGE gels and to probable protein conformation changes. Decreased viscosity after enzymatic treatment may be useful in improving spray-drying feed rates of soy protein or increasing opportunities for their use in nonfood products such as wood adhesives.

Emulsification capacity. Proteins are one of the most important groups of emulsifiers used in the food industry. They affect the size of oil droplets that determine the appearance and textural characteristics of food products such as salad dressings and mayonnaises. Depending on their physicochemical characteristics, proteins will be more or less effective in stabilizing oil droplets against coalescence and reducing oil–water interfacial tension. The average EC value obtained for the controls was 560 g oil/g protein (SD 78). Compared with the control samples, EC was lower for the hydrolysates obtained with both endoproteases and exoproteases, regardless of DH (Table 3). Even for the low DH (2.6% for Multifect P-3000 and Protex 6L), the hydrolysates had significantly lower EC than the control sample.

Surface hydrophobicity and emulsifying properties have often been related to explain the emulsifying properties of proteins (23). The decrease in surface hydrophobicity was closely related to the decrease in emulsifying properties of casein after pancreatic hydrolysis in the study of Mahmoud *et al.* (24), and increased hydrophobicity was related to an increased emulsifying activity index in the study of Qi *et al.* (1). However, surface hydrophobicity has not always been a good indicator of emulsifying properties. The use of papain to modify soy protein increased the hydrophobicity to 40.1 compared with 8.1 for the controls, but this increase did not improve the emulsification activity or the emulsification stability of the hydrolysates (2). The comparison of two SPI showed that the highest EC could not be related to the highest surface hydrophobicity in the study of Rickert *et al.* (16). These authors suggested that high surface hydrophobicity–low EC results could be explained by poor solubilities of the samples, which lost the ability to interact at the oil–water interface. A change in surface hydrophobicities may explain our decrease in EC; however, more investigations need to be done to verify this hypothesis.

Our study demonstrated that enzymatic treatment to achieve <4% DH decreased the EC of soy protein flour hydrolysate while causing only minor modifications to the SDS-PAGE profile and no change in the native states of glycinin and β-conglycinin. This decrease in EC was maintained upon further hydrolysis, where peptide profiles were greatly modified and denaturation enthalpy values declined. The protein modification that took place during hydrolysis may have improved the ability of the protein to move quickly to the interface but yielded conformations that failed to reduce interfacial tension at the oil–water interface.

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