Functional Properties of Soy Protein Hydrolysates from a Continuous Ultrafiltration Reactor

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Soy protein hydrolysates produced in a continuous ultrafiltration reactor with a 10000 molecular weight cutoff membrane consisted of four major fractions, the largest being approximately 2400 in molecular weight. The type and specificity of the protease used and overall reactor activity affected functional properties. At high enzyme activity, the hydrolysate was completely soluble and clear over the entire pH range. Enzyme decay during operation caused a shift in molecular weight distribution toward the larger fractions. In addition, the hydrolysate became less soluble, especially at pH 4.6, and highly turbid, except at pH 7. Moisture sorption at intermediate water activities was much higher than the unmodified soy isolate, but foaming and emulsification properties were poor.

Once the nutritional value and safety of a proteinaceous food product or new protein ingredient have been established, its functional properties become of paramount interest (Richardson, 1977). Modification of the protein by enzymatic or chemical means can usually improve certain functional properties. Enzymatic modification using specific proteases has several advantages over acid or alkali hydrolysis (Kinsella, 1976), among them milder process conditions and lower salt content in the final neutralized hydrolyzate.

Conventional batch protein hydrolysis has several disadvantages such as (i) high cost of using large quantities of enzymes, (ii) difficulty in controlling the extent of reaction that can result in nonhomogeneous products consisting of fractions of varying molecular weight, (iii) low yields, and (iv) the need to inactivate enzymes by pH or heat treatment at the end of the reaction, which adds to the processing costs (Cheryan, 1986).

To overcome some of these problems, a continuous enzymatic process using semipermeable membranes was developed (Cheryan and Deeslie, 1983, 1984). The major advantages of the membrane reactor over traditional batch reactors are (a) more efficient continuous processes could be used, (b) productivity per unit enzyme used was vastly superior since the enzyme was recycled and reused, and (c) the molecular weight distribution of fractions in the hydrolysate could be controlled within limits by the appropriate membrane. This last feature is of particular importance since it appears that the functional properties of a protein or its hydrolysate are governed by its molecular size (Adler-Nissen, 1986).

This paper reports a study of selected functional properties of a soy protein hydrolysate produced in a continuous ultrafiltration (UF) reactor. Special attention was given to the interrelationships among functional properties (such as solubility, clarity, water binding, foaming, emulsification, and flavor, primarily bitterness) and performance of the bioreactor as reflected by changes in substrate conversion and molecular weight distribution of hydrolysate fractions.

MATERIALS AND METHODS

Production of UF Reactor Hydrolysate. The hydrolysate was produced in a continuous membrane reactor as described previously by Cheryan and Deeslie (1983, 1984) and Deeslie and Cheryan (1981a,b, 1982). A commercial soy protein isolate (Promine-D; Central Soya Co., Fort Wayne, IN) was the substrate. It assayed 4.4% ash, 0.2% crude fiber, and 93.3% protein (N × 6.25), of which 2.8% was nonprotein nitrogen (Deeslie, 1980). It was made up in distilled water to the required concentration, preheated at 100 °C for 30 min, and prefiltered before hydrolysis.

Several proteases were screened for these experiments (Deeslie and Cheryan, 1981a,b). Pronase, a mixture of exo- and endo-peptidases from *Streptomyces griseus* (Calbiochem-Behring, La Jolla, CA) was selected because of its high proteolytic activity and nonspecificity, which was essential for long-term operation of the reactor. However, it is expensive, and thus Alcalase (type 0.6L; Novo Industri A/S, Bagsvaerd, Denmark) was also used for some of the experiments.

The membrane was in the form of hollow fibers (H1P10; Amicon Corp., Lexington, MA). It was made of polysulfone with a molecular weight (MW) cutoff of 10000. The permeate from the reactor (containing the hydrolysate) was lyophilized and stored refrigerated until used for analysis. Further details on the operation of continuous ultrafiltration reactors are available elsewhere (Cheryan, 1986).

Analytical Methods. Moisture and ash content were determined by standard methods (AOAC, 1970). Protein content is expressed as total nitrogen (N) × 6.25. Nitrogen was determined by the direct Nesslerization procedure of Rao and Whitney (1960). Molecular weight distribution of hydrolysate fractions was determined by gel permeation chromatography using Sephadex G-15 and G-50 as recommended by Pharmacia (1975). The eluant was 0.067 M phosphate buffer, pH 7.4 (Schwartz and Bodansky, 1966). Freeze-dried hydrolysate (78 mg) was dissolved in 10 mL of the same phosphate buffer and drawn into the 1-mL injection loop of a Chromatronix injector, which was placed in-line between the elution buffer pump and the gel column. The eluant from the column passed through a flow-through cell placed in a Beckman DBG recording spectrophotometer (Beckman Instruments, Inc.) set at either 280- or 215-nm wavelength. For recording at 215 nm, a 1:5 or 1:2 dilution of the above was made with the above phosphate buffer, and 1 mL was injected into the column.

Eluant samples were pooled and subjected to nitrogen analyses to determine the relative distribution of each fraction separated on the gel by a technique described by Steinhart and Kirchgessner (1973).

Functional Properties. *Solubility.* Solubility of the soy isolate and hydrolysates was determined by a protein dispersibility index (PDI) method (AOCS, 1970), modified to accommodate smaller sample size. A 1% aqueous solution was blended for 10 min, with either 2 N HCl or 2

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N NaOH to adjust the pH. After holding for 15 min and readjusting the pH if necessary, the sample was centrifuged at 1400*g* for 10 min. The nitrogen content of the supernatant was determined. Solubility is expressed as the percent of the total nitrogen of the original sample that was present in the supernatant.

Clarity. This was evaluated with 1% w/v aqueous suspensions of the unmodified soy isolate or its hydrolysates in deionized distilled water. The pH was adjusted to 3.0, 4.6, or 7.0 with 2 N HCl or 2 N NaOH. Clarity was evaluated objectively by turbidity measurements, i.e., the optical density at 660-nm wavelength of the samples. Deionized distilled water was used as the blank.

Foaming Properties. The method suggested by Puski (1975) was used in this functionality test. A 1% aqueous suspension was prepared in a Waring Blendor as described earlier for the solubility tests. The blender contents were immediately transferred to a 250-mL graduated cylinder, and the foam volume was noted. The cylinder was placed in a 25 °C water bath for 30 min and residual foam volume measured. Foam stability is expressed as the percent loss of foam volume.

Moisture Sorption. The method of Lang et al. (1981) using minidesiccators was used to determine the water adsorption isotherm of hydrolysates and Promine-D.

Flavor. The primary purpose was to determine bitterness of the hydrolysates. Samples were made up to 1% w/v in tap water and adjusted to pH 7 with 2 N HCl or 2 N NaOH for evaluation. Fifteen individuals were screened to determine their threshold for bitterness detection with quinine dihydrochloride as the reference bitter compound (Ricks et al., 1978) at 0.0001–0.0008% w/v concentrations. The method of constant stimuli (Kling and Riggs, 1971) was used to screen individual judges. A JND (just noticeable difference) was determined for each judge. Six panelists with the lowest JND's (lowest threshold) for bitterness were selected for training to use the method of magnitude estimation (Kling and Riggs, 1971; Moskowitz, 1974). Soy isolate, hydrolysates, and known bitter samples (the reference solution, assigned a bitterness score of 10) were evaluated. Judges were assigned test samples relative to this score (i.e., if a sample was twice as bitter, it received a score of 20). Panelists were also trained in perception of common flavors associated with soybeans such as "beany" or "cereal-like" and were able to differentiate bitterness from these more typical flavors.

For statistical analysis, panels were conducted in a randomized complete block design, with four replicate observations per sample per judge. Analysis of variance and LSD tests were used to determine significant difference between samples.

RESULTS AND DISCUSSION

Previous studies on the optimization of the ultrafiltration reactor (Cheryan and Deeslie, 1983; Deeslie and Cheryan, 1981b) showed that substrate conversion (X) is a function of substrate concentration (S_0'), enzyme concentration (E), reaction volume (V), and flux (J). These independent variables were correlated with conversion by an expression (eq 1) derived from the Michaelis–Menten

$$X + \frac{K_m X}{S_0'(1 - X)} = k_2 \tau \qquad (1)$$

kinetic model, where X = fractional conversion = P'/S_0', S_0' = substrate (protein) concentration corrected for nonprotein nitrogen, P' = product (hydrolysate) concentration corrected for nonprotein nitrogen in the substrate, K_m = Michaelis constant, k_2 = reaction rate constant, and

Table I. Amino Acid Composition (% w/w) of Pronase UF Reactor Hydrolysates at Various Operating Times

		hydrolysate at reactor operating times, h		
amino acid	Promine-D	1–7	42–60	86–96
Asp	13.10	12.14	12.12	13.85
Thr	3.96	4.17	4.33	3.58
Ser	7.98	7.55	7.44	7.55
Glu	19.05	17.12	20.45	20.61
Gly	7.51	7.16	7.74	7.62
Ala	6.66	6.60	5.61	5.79
Val	4.13	7.12	5.27	3.77
Met	0.99	0.92	1.02	0.99
Ile	2.97	4.48	3.41	2.81
Leu	8.33	8.28	8.39	7.68
Tyr	2.73	2.77	2.87	3.08
Phe	3.47	3.40	3.78	3.83
His	1.51	1.49	0.71	1.88
Lys	5.02	4.91	4.72	5.51
Arg	6.69	6.67	5.76	5.21
Pro	5.83	5.17	6.31	6.18

τ = modified space time = $EV/S_0'J$.

The space time (τ) correlates better with the performance of the ultrafiltration reactor compared to the conventionally used residence time (V/J); it is also a more convenient parameter since all operating variables affecting reactor performance are grouped together in one term. There is a gradual stepwise decrease in enzyme activity during UF reactor operation (Deeslie and Cheryan, 1982) due to thermal inactivation, loss of mineral activators, and/or leakage of enzyme fractions through the membranes. Thus, as will be seen later, changes in reactor activity due to changes in space time result in different effects than changes in activity due to enzyme decay.

Composition. Proximate analysis of the UF reactor hydrolysate from over 30 runs under varying operating conditions averaged, on a dry basis, 91.5% protein (N × 6.25) and 9.1% ash (Deeslie, 1980). No measurable quantities of fat or carbohydrate were found in the UF reactor product. Compared to the substrate (soy isolate), which was 93.3% protein and 4.4% ash, the higher ash content of the hydrolysate is due primarily to the sodium hydroxide added to the reactor to keep the pH constant at pH 8.0 (Deeslie and Cheryan, 1981a).

The amino acid profile of the hydrolysate was not significantly different from that of the substrate (Table I). In contrast, Kirchgessner and Steinhart (1974) observed during batch hydrolysis of soy protein isolate that the sequence of amino acids within different molecular weight fractions of the hydrolysate changed at different digestion times. In the present study, however, unhydrolyzed protein is continuously recycled back to the reaction vessel until its molecular size was sufficiently reduced to allow passage through the membrane. Thus close similarity between the amino acid profiles of the substrate and hydrolysate is expected.

Molecular Weight Distribution. *a. Effect of Space Time.* Figure 1 shows the effect of space time on the molecular weight distribution of fractions in the hydrolysate. Four separate peaks can be seen in each chromatogram, although the relative proportions of the peaks changed with space time. At the highest space time, τ = 39.94, the peaks were at approximately 2400, 1400, 1000, and 275 molecular weight (MW). As space time decreased, the lower molecular weight fractions decreased, and the relative amounts of higher molecular weight fractions increased. The highest MW fractions are not as rapidly hydrolyzed as the lower MW fractions. Higher conversions due to higher space times would result in more hydrolysis of larger molecules, resulting in a decrease in the relative

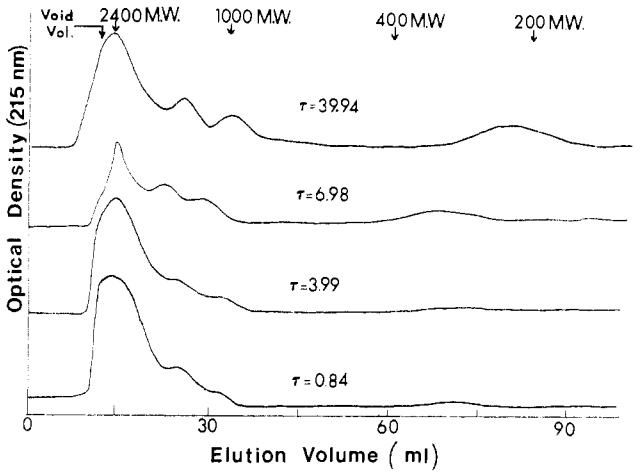

Figure 1. Molecular weight distribution of Pronase UF reactor hydrolysate on Sephadex G-15. Effect of space time (τ). Samples were taken during the initial stages of reactor operation.

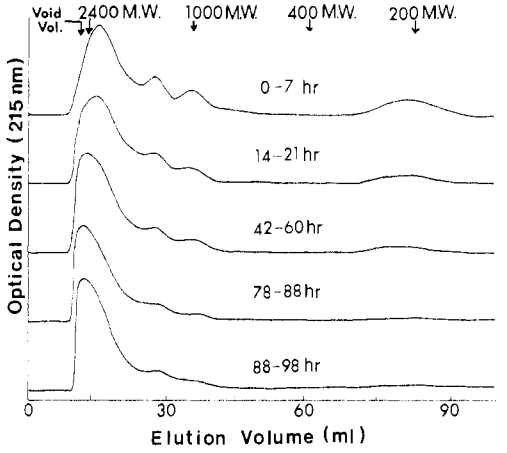

Figure 2. Effect of operating time on molecular weight distribution (Sephadex G-15) of Pronase UF reactor hydrolysate. Space time during run was 39.94 min.

quantity of the fraction in the 2400 peak.

b. Effect of Operating Time. Figure 2 shows similar effects of operating time at a fixed space time of $\tau = 39.94$ on the MW distribution of UF reactor hydrolysate fractions. The decrease in enzyme activity causes a reduction in the lower MW peak until it almost disappeared, while the middle MW peaks became smaller and the highest MW peak became relatively larger.

Solubility Characteristics. Figure 3 shows solubility profiles of preheated and unheated soy isolate and the UF reactor hydrolysate take during the initial stages of reactor operation. The UF reactor hydrolysate was completely soluble at all pH values. In addition, the hydrolysates were easily and completely dispersible over the entire pH range, making them simple to incorporate into formulated liquid food products compared to other soy protein products that are fairly difficult to disperse.

It is also apparent that molecular size alone is not a sufficient criterion for solubility, even at these low molecular weights. The effect of reactor operating time on solubility is shown in Figure 4. There are significant losses in solubility of the hydrolysate after 42 and 86 h of reactor operation, with the decrease especially pronounced at pH 4.5. Data presented earlier (Figure 2) showed that longer operating times resulted in a drop in substrate conversion and a shift in MW distribution to larger peptides. However, all fractions permeating the membrane throughout the run were less than 2500 molecular weight, which was confirmed on other Sephadex gels also (Deeslie, 1980). The

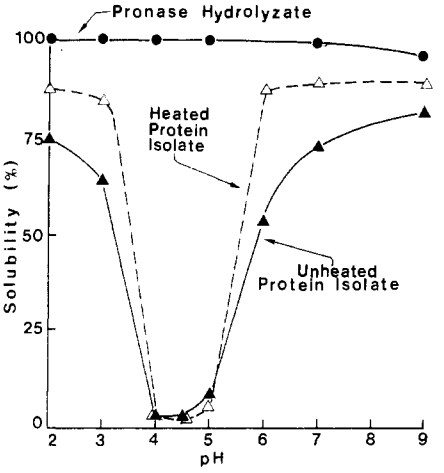

Figure 3. Solubility profiles of unmodified soy isolate and Pronase UF reactor hydrolysate. Least significant difference (LSD) at the 1% level was 10.4.

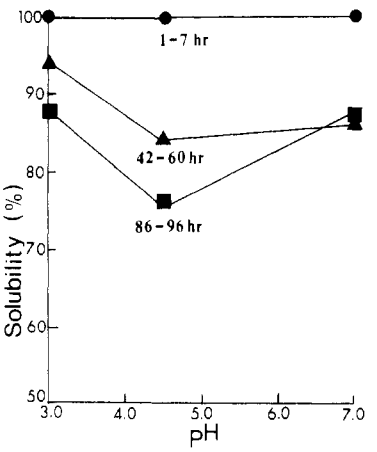

Figure 4. Effect of operating time on solubility profile of hydrolysate. LSD betweeen treatments at the 1% level was 15.3.

Table II. Effect of Space Time, Conversion, and pH on Turbidity of Pronase Hydrolysates (Samples Taken during the Initial 3–7 h of Operation)

space time, min	conversion, %	turbidity[a] pH 3.0	pH 4.6	pH 7.0
39.94	94	0.052	0.015	0.005
1.50	77	0.085	0.015	
0.75	70	0.120	0.025	0.010
0.42	67	0.125	0.015	0.075

[a] Optical density readings at 660 nm. Values above 0.1 appear turbid.

change in solubility could be due to specific changes in the distribution and arrangement of amino acids in the fractions obtained at longer operating times, resulting from changes in activity and specificity of the enzyme during reactor operation.

The UF reactor hydrolysate with Pronase as the enzyme was extremely stable in the presence of calcium. A 1% suspension of the hydrolysate was 99% soluble in 0.03 M calcium chloride at pH 7 compared to the isolate that was only 8.5% soluble. Calcium tolerance would be important in formulated foods, such as imitation dairy products, where calcium is added to improve nutritional quality and where a stable suspension or solution is desirable.

Clarity and Turbidity. Clarity is a desirable property for fortification of sparkling clear beverages; clarity at acidic pH is essential for carbonated beverages (Kinsella, 1979). Table II shows the effect of substrate conversion

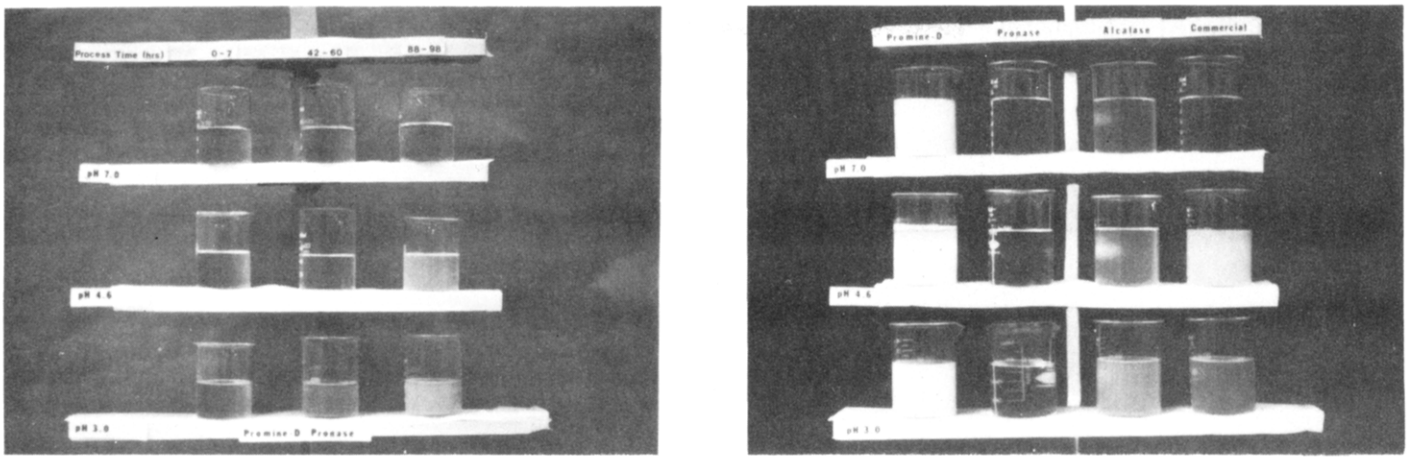

Figure 5. Clarity of UF reactor hydrolysates (1% protein w/v). Left: Effect of pH and operating time using Pronase. Turbidity values are given in Table III. Right: Comparison of soy isolate (Promine-D), UF reactor hydrolysates using Pronase or Alcalase and commercial hydrolysate.

Table III. Effect of Operating Time and pH on Turbidity of Pronase Hydrolysates (Samples Taken during a Run with Space Time of 39.94 min)

operating time, h	conversion, %	turbidity[a] pH 3.0	pH 4.6	pH 7.0
0–7	94	0.052	0.015	0.005
42–60	84	0.132	0.035	0.007
88–98	67	0.480	0.135	0.005

[a] Optical density readings at 660 nm.

(as a function of space time) on the turbidity of hydrolysate solutions at various pH values (solutions appear turbid only above an optical density of 0.1 at 660 nm). Lower space time resulted in lower conversion and corresponding higher turbidity. At each level of conversion, turbidity increased with a decrease in pH, which indicates possible polymerization or formation of small aggregates (Wolf and Cowan, 1971).

A decrease in enzyme activity as a result of decay had slightly different effects on turbidity (Table III). Higher turbidity values were observed at pH 4.6 and 3.0, but not at pH 7. Figure 5 is a more dramatic representation of the physical significance and importance of these effects as determinates of functionality of hydrolysates.

The relationship between clarity and solubility during long-term operation is interesting. Hydrolysates obtained during the initial 7 h of the process are completely soluble (Figure 4) and clear (Figure 5) at all pH values. However, after 88 h of operation, solubility decreased the most at pH 4.5, but turbidity increased the most at pH 3.0. In addition, turbidity data show no large effect of operating time at pH 7, but solubility drops significantly. At pH 7, the hydrolysate possesses a net negative charge, which together with a particular conformation of the peptide chain may have prevented aggregation. The same did not happen at lower pH perhaps due to a neutralization of the negative charges, resulting in greater turbidity. The generally poor correlation between solubility and turbidity could also be an artifact of the test procedures, since solubility was measured by a high-shear, short-time method that may not have allowed the protein/peptides to come to complete equilibrium. The NSI test, which is a low-shear, long-time method, may have given better correlations.

Figure 5 also shows a comparison of the clarity of UF reactor hydrolysates, the soy isolate used as feed to the reactor, and a commercial hydrolysate prepared by the batch method using pepsin (Gunther's D100 WA; A. E. Staley Manufacturing Co., Decatur, IL). Pronase hydrolysates contained a greater amount of lower molecular weight hydrolysates and amino acids, as shown earlier (Deeslie and Cheryan, 1981a), while the Alcalase reactor hydrolysate showed one molecular weight fraction of 2500 molecular weight, slightly larger than that of Pronase. This difference between hydrolysates of Pronase and Alcalase is due to the lack of exo-peptidase activity in Alcalase, while Pronase contains both exo- and endo-peptidase activity. The commercial sample showed one major peak at the void volume of the Sephadex G-15 gel. Molecular weight distribution of this commercial product is in the range of 2500–14 000 (Gunther, 1979). Thus, in general, larger molecular weight fractions showed reduced clarity at acid pH values.

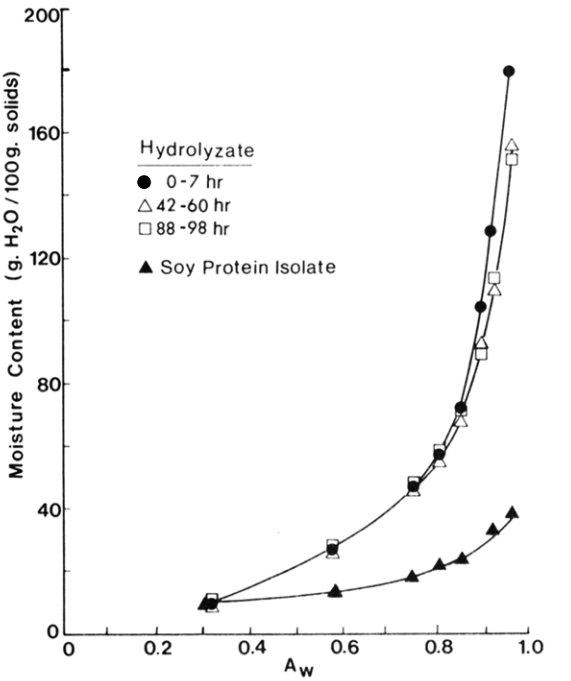

Figure 6. Moisture sorption isotherms at 20 °C of Pronase hydrolysate at various operating times. Data are means of two replicates.

Moisture Sorption. Figure 6 shows water adsorption isotherms for samples of UF reactor hydrolysates taken at various reactor operating times. Compared with the isotherm for the unmodified soy isolate, there is a substantial increase in moisture sorption at water activities of 0.59–0.95. Hydrolysis alters the conformation of the protein and produces short-chain polypeptides, greatly

Table IV. Foaming Properties of Pronase UF Reactor Hydrolysates, Soy Isolate, and Commerical Soy Hydrolysate

pH	operating time, h: 1–7 (X = 94%)	42–60 (X = 83%)	88–98 (X = 58%)	soy isolate (Promine-D) (X = 0)	commerical (Gunther D100WA)
	A. Initial Foam Volume (mL of Foam/100 mL of 1% Aqueous Solution)[a]				
7.0	170	220	206	129	192
4.6	83	194	175	124	195
3.0	8	206	201	127	193
	B. Foam Volume after 30 min (mL)[b]				
7.0	19	7	13	28	109
4.6	4	5	5	48	130
3.0	0	0	8	32	120

[a] Least significant difference (LSD) at 1% level, 18.8. [b] Least significant difference (LSD) at 1% level, 2.4. X is percent conversion of substrate.

Table V. Sensory Evaluation for Bitterness of Pronase UF Reactor Hydrolysates (1% Protein w/v, pH 7) (Samples Taken during Reactor Operation at a Space Time of 39.94 min; Reference Sample (Quinine Dihydrochloride) Assigned a Score of 10)

sample	conversion, %	mean score
soy protein isolate	0	4.0[a]
hydrolysate at 1–7 h	94	4.6[a]
hydrolysate at 42–60 h	83	3.2[a]
hydrolysate at 88–98 h	59	3.9[a]

[a] Analysis of variance: F(calcd) = 0.4770; F(tabld) = 3.25. Thus, there was no significant difference between treatment means.

increasing the availability of polar binding sites, resulting in greater water sorption. Soy proteins contain a relatively large amount of ionized polar amino acids such as glutamic and aspartic acids (Table I), which can bind almost 3 times as much water as that of nonionized polar groups (Chou and Morr, 1979). In the native protein, some of these groups are buried and thus are not available for water binding. In addition, at intermediate water activity, peptide linkages aid in water binding after polar side chains have been saturated (Chou and Morr, 1979). These results are in general agreement with those of Beuchat et al. (1975) and Puski (1975). The greatly improved water-binding capacity of UF reactor hydrolysates is of potential significance in the manufacture of intermediate moisture foods.

Foaming. Table IV shows foaming properties (foam volume, foam stability). Hydrolysis increased the foam volume at pH 7 but decreased it at pH 4.6 and 3.0 during the initial 7 h of reactor operation. With an increase in process time, foam volume of hydrolysate is much greater than that of the soy isolate at all pH values and is comparable to the commercial hydrolysate. However, the foam stability of the UF reactor hydrolysates was much poorer. The relatively small peptides of less than MW 2400 in the UF reactor hydrolysate were apparently capable of forming films at the air–water interface to entrap air, but the films were not strong enough to maintain their integrity and the foams collapsed. The commercial "whipping protein" hydrolysate was prepared by a limited hydrolysis and thus contained larger peptides, resulting in stable foams. Emulsification properties of the UF reactor hydrolysates were also poor compared to the soy isolate and the commercial hydrolysate [not shown here; see Deeslie (1980)] due no doubt to the same reasons.

Flavor. Table V shows results of the sensory evaluation for bitterness of Promine-D/Pronase UF reactor hydrolysates at various operational times. It appears that these products were perceived by the panel as being less than half as bitter as the reference solution, which itself was at a barely detectable level of bitterness. Although substrate conversion decreased during long-term operation, there was no significant change in bitterness of the hydrolyzates.

Table VI. Sensory Evaluation for Bitterness (Samples Resuspended to 1% Protein (w/v, pH 7); UF Reactor Samples Taken in the First 4–10 h of Operation)

sample	mean score[a]
soy protein isolate	2.43 (a)
Pronase UF reactor hydrolysate	6.02 (a)
commerical hydrolysate	12.42 (b)
Alcalase UF reactor hydrolysate	18.62 (c)

[a] Scores with the same letter are not significantly different from each other at the 1% level of significance

Table VI is a comparison of the bitterness level of UF reactor products and the commerical product. The Pronase UF reactor product and the soy isolate were significantly less bitter than the Alcalase UF reactor product and the commercial hydrolysate. As mentioned earlier, Alcalase does not possess exo-peptidase activity (Novo, 1977), which may have been responsible for the bitterness.

These results are particularly interesting since bitterness is most frequently associated with low molecular weight peptides. The weight-averaged mean molecular weight of the UF reactor hydrolysates is approximately 2000. Arai et al. (1970) claimed that a molecular size of 2400 or less is sufficient to promote bitterness, but Fujimaki et al. (1968) found that most bitter peptides of soy protein hydrolysates were MW 1500 or less.

Ney (1971) suggested that bitterness of peptides is caused by a combination of high proportion of hydrophobic amino acids and low molecular weight. Thus, the bitterness of protein hydrolysates can be predicted by calculation of the Q value of the protein, which presents the average hydrophobicity of the side chains of the amino acids involved (Belitz and Wieser, 1985). Q values can be calculated from Tanford's free energy data for individual amino acids. If the Q value is less than 1300 cal/mol, no bitterness should be observed. Q values above 1400, on the other hand, would result in bitterness, according to Ney (1971).

For the Promine-D/Pronase UF reactor hydrolysate, the Q value was 1016. Thus, our hydrolysate meets the criterion for nonbitterness on this basis. Our results agree with those of Alder-Nissen (1986) that bitterness increases with limited proteolysis but decreases upon extensive hydrolysis.

SUMMARY AND CONCLUSIONS

The molecular weight distribution of soy protein hydrolysate fractions produced in a continuous ultrafiltration reactor is a function not only of the pore size distribution of the membrane but also of the nature and activity of the enzyme. With the Promine-D/Pronase system using a

10 000 molecular weight cutoff membrane, four major fractions were observed, the largest being approximately 2400 in molecular weight. Lower reactor activity, due to either changes in space time or decay during operation, caused a shift in molecular weight distribution toward the larger fractions. At high reactor activity, the hydrolysates showed good solubility and clarity and greatly increased sorption at intermediate water activities. Decay in enzyme activity during operation caused the Pronase UF reactor hydrolysate to become less soluble, especially at pH 4.6, and highly turbid, except at pH 7. Foaming and emulsification properties were poor. The properties of the UF reactor hydrolysates indicate good potential for their use for specialized infant formulas, in protein fortification of sparkling clear acidic beverages, and in intermediate moisture foods where water binding is of importance.

ACKNOWLEDGMENT

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Registry No. Pronase, 9036-06-0; promine D, 51434-41-4; water, 7732-18-5.

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