Enzymatic Hydrolysis and Synthesis of Soy Protein to Improve its Amino Acid Composition and Functional Properties

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ABSTRACT: Soy protein was enzymatically modified and ultrafiltred, and functional properties were evaluated. After enzymatic hydrolysis, hydrolysate (20 g/100 mL) was incubated with chymotrypsin and glycerol at 37 °C. Different methionine methyl-ester concentrations, pHs, and time were tested. Amino acid composition and functional properties of ultrafiltrated fractions (FI>10, 10>FII>3, and 3>FII>1 kDa) were evaluated. Optimum hydrolysis conditions were 12 h and 50 °C, and those of synthesis were 0.07585 g Met/g, pH 7, and 3 h, binding 2.2% to 5% methionine. Fractions under 10 kDa presented 100% solubility and the best clarity. High-methionine fractions had higher foam volume, lower emulsifying capacity and hydrophobicity. Modified hydrolysates have a potential for use in soluble high nutritional products.

Key Words: Enzymatic synthesis, functionality, methionine, soy

Introduction

PROTEIN HYDROLYSATES POSSESS PROPERTIES THAT MAKE them attractive as a protein source in human nutrition. Hydrolysates are used in products for special nutrition, such as diets for elderly and patients with impaired gastrointestinal absorption, hypoallergenic infant formulas, sports nutrition, and weight-control diets, as well as in consumer products for general use (Frøkjaer 1994; Schmidl and others 1994). Peptide-based formulas have been useful because of their high solubility especially under acidic conditions; even during heat treatment, the peptides remain in solution (Frøkjaer 1994). In addition to their solubility in a wide range of pH and other functional properties, such as improvement of texture and water binding capacity (Lin and others 1997), protein hydrolysates are physiologically better than intact proteins because their intestinal absorption appears to be more effective (Ziegler and others 1998).

On the other hand, proteases used in the preparation of protein hydrolysates have the ability to catalyze peptide synthesis under appropriate conditions. Enzymatic synthesis has potential application in the food industry to remove bitterness of protein hydrolysates, to improve amino acids composition, or to modify some functional property (Lozano and Combes 1992).

Soy protein is widely used as the nitrogen source in infant and adult formulas, both in the intact and hydrolyzed form (Henn and Netto 1998; Lahl and Braun 1994; Zhao and others 1997). Although soy hydrolysates present unique functional characteristics, their low methionine content is a limiting factor in the nutritional quality of the final product, especially for infants and patients on medical diets receiving it as the only protein source. In the past, the products were fortified with free methionine in order to improve the nutritional quality, but the fortified products presented undesirable flavors and aroma. Recently, by using enzymatic synthesis, soy protein and other hydrolysates have been modified to improve nutritional quality (Hussein and others 1995; Hajós and others 1996).

Although it has been demonstrated that enzymatic modification of a fraction or isolate soy protein is a suitable route to improve the intact protein nutritional value (Kimura and Arai 1988; Hajós and others 1996), processing to obtain it has not been designed for industrial scale. Obtention steps include pre-extraction, expensive dialysis, and long time thermal incubation for synthesis. Additionally, functional properties of these altered proteins are not known in order to scale up to an industrial level for food formulations.

The objectives of this study were to hydrolyze soy protein, to bind enzymatically methionine to it, to fractionate the hydrolysates by ultrafiltration, and to evaluate their functional properties before and after modification.

Results and Discussion

Characteristics of the enzyme concentrate and soy flour

Enzyme concentrate. The recovery of freeze-dried enzymatic preparation was 26.3 g/kg of pancreatic tissue. It contained 75% protein with trypsin and chymotrypsin activities of 14.6 x 10^{-3} and 5.6 $\times$ 10^{-3} μg of p-nitroanilide/min-mg of the enzyme preparation, respectively. These values are higher than those of purified commercial trypsin (9.5 0^{-3} $\times$ 10^{-3} μg of p-nitroanilide/min-mg of the enzyme) and lower than purified chymotrypsin (9.7 $\times$ 10^{-3} μg of p-nitroanilide/min-mg of the enzyme) used as controls. In addition, the electrophoretic pattern (figure not shown) of the enzyme concentrate presented only 1 weak extra band different to those of purified trypsin and chymotrysin; probably it could be attributable to elastase, according to its molecular weight (25 kDa).

The enzyme concentrate with high proteolytic activity was prepared in order to reduce costs. Additionally in Mexico, the porcine pancreas is an enzyme source completely underutilized. Recently other authors (Henn and Netto 1998; Hettiarachchy and Kalapathy 1997) using commercial pancretin to hydrolyse soy protein isolates obtained good results respect to protein functionality.

Soy flour. Before hydrolysis, defatted soy flour contained 46.3% (N $\times$ 6.25) protein, 6.2% ash, and 9.5% moisture. After grinding, soy flour presented a particle size distribution similar to commercial flours. An advantage of using defatted soy flour as starting material is its cost, since it is one of the less expensive soy protein products.

Soy protein hydrolysis

The effect of temperature on proteolytic activity was evident from the proteolysis curves. Soluble peptide concentrations were higher at 50 °C (data not shown). Higher temperatures (as 55 °C) induce denaturation of trypsin (Mullally and others 1995), and therefore peptide production decreases. Stability of pancreatic enzymes at 50 °C for 24 h was demonstrated by the increased degree of hydrolysis (DH) values. Value of pH=8.0 was a pre-established parameter because it is the best for pancreatic enzyme activities (for hydrolysis) as well as for soy protein solubility (Kim and others 1990).

Enzymatic hydrolysis of soy protein did not proceed very fast in the 1st 6 h of reaction (DH = 13.2%), but after 12 h 41% of hydrolysis was obtained. Degree of hydrolysis is not the best parameter to anticipate the optimum hydrolysis time, but this last one depends on the amount of material included into the hydrolysis fraction of interest for a food application. For instance, for a hypoallergenic protein source, a fraction under 3000 Da will have the lowest antigenicity and allergenicity (van Beresteijn and others 1994; Ena and others 1995). Indeed, a high degree of hydrolysis may result in many free amino acids (released from the terminal amino acid residues), but the number of hydrolyzed peptide bonds in the longer peptide chain would be low, leaving a number of antigenic epitopes intact.

Enzymatic synthesis

Table 1A shows concentrations (μmole/mL) of bound methionine at pH 6 and 7 after 3, 6, and 9 h reaction. The best time for methionine incorporation was 6 h at both pHs, with the higher binding at pH 7 after 3 and 6 h. After 9 h reaction, methionine incorporation was reduced probably because chymotrypsin was denatured or its predominant activity switched to the hydrolytic mode. The chosen conditions were pH 7 and 3 h because 0.079 μmole/mL corresponds to 21.2% methionine bound to the protein which is almost 10 times higher than recommended for human nutrition (FAO/WHO 1991). Therefore, lower amounts of added methionine methyl ester (Met-ME) were tested.

Optimization of added Met-ME to the reaction at pH 7 for 3 h was done. According to the results in Table 1B, the lowest amount of Met-ME added (0.0569 g/g hydrolysate) was enough to obtain a protein with bound Met (2.33%) enough to fulfill the amino acid requirements for adults but not that of infants. On the other hand, addition of 0.1138 g Met-ME to the reaction increased bound Met to 5%, a higher value than that recommended for infants (FAO/WHO 1991). Because approximately 55% of the added methionine (as Met-ME) was bound to the hydrolysate, addition of 0.076 g Met-ME/g hydrolysate (corresponding to 0.057 g of Met) was chosen in order to achieve binding of 3.14% Met. This is enough to fulfill the infant requirements for sulfur amino acids (4.2%), considering that soy protein contains 1% to 2% cysteine (Bressani 1981; del Valle 1981). The data of final methionine incorporation into the ultrafiltration (UF) fractions are discussed below.

The final product of our synthesis contained similar amount of methionine as that obtained by Hussein and others (1995) and Hajós and others (1996) for milk proteins and soy albumins. However, our reaction time was 5.3 times shorter than those reported by these workers. Furthermore, we were able to accomplish the same results utilizing 6 to 3 times less reactive material (as Met-ME). This possibly could be due to the inclusion of glycerol in the reaction mixture, which can act as a water activity depressor (Lozano and Combes 1992).

Ultrafiltration fractions

Recovery. Ultradiafiltration resulted in an increase of protein content in all of the fractions because of remotion of soluble carbohydrates or oligosaccharides with low molecular weights, as shown in Table 2. After UF fractionation, protein in hydrolysis fractions increased to 64% to 73% and to 65% to 84% in the fractions before and after synthesis, respectively. In spite of the final high protein concentration, total protein recovery was 52%. Heating and centrifugation after hydrolysis resulted in an initial loss of 11.4%. Ultrafiltration of hydrolysate removed 24% of the protein as low molecular weight peptides. Additionally, in the enzymatic synthesis, the hydrolysis reaction proceeded down to production of peptides under 1 kDa, which were further lost after ultrafiltration (12.7%). Part of the lost peptides could be recovered if it were possible to find a commercial preparative UF membrane of 500 Da MWCO; smaller peptides can not be added to the usable fraction for special nutrition beverages because in addition to amino acid debalancing (data not shown), they contain a high concentration of free amino acids and induce high osmolarity of the final product.

Molecular weight profiles. Each UF fraction presented only 1 large peak with molecular weight within the expected range. UF fractions ranged from 12 to 45 kDa for FI, from 2.7 to 10.2 kDa for FII, and from 0.8 to 3 kDa for FIII. The molecular weight of enriched fractions ranged from 10 to 44 kDa for FI-E, 2.6 to 9.6 kDa for FII-E, and 0.8 to 2.1 kDa for FIII-E. Diafiltration with 2 volumes of water was enough to completely remove lower molecular weight peptides of each fraction. In a similar UF system Deeslie and Cheryan (1991) obtained basically the same profile in permeates from 5 and 10 kDa MWCO membranes, probably because either they used 1 cycle or only 1 volume of water for diafiltration.

Control over the molecular weight range of peptides is very important in products, such as hypoallergenic infant formulas. Typically 2 modification approaches are follow: extensive and moderate hydrolysis (Blecker and others 1997). In the 1st case, development of bitter taste and high osmolarity are expected, while less extensive hydrolysis may result in increased residual antigenicity

Table 1—Methionine content (μmole Met/mL) covalently bound to the modified soy proteins hydrolysate.

1A. Effect of pH and reaction time at constant concentration of Met-ME (0.4552 g/g hydrolysate).

	Time (h)		
pH	3	6	9
6.0	0.01698	0.06305	0.06288
7.0	0.07858	0.11587	0.01734

1B. Effect of addition of Met-ME at constant pH and time (pH 7, 3 h reaction)

	Bound Met-ME	
ME-Met added (g/g hydrolysate)	μmole/mL	(%)
0.2276	0.04676	12.594 ± 0.33
0.1138	0.01874	5.048 ± 0.16
0.0569	0.00864	2.328 ± 0.51

Table 2—Protein concentration and protein recovery (as related to the starting protein content) in UF fractions before and after methionine modification.

Product or Fraction		Protein recovery (%)	Protein concentration (%)
Soy flour		100.0	45.92
Soy hydrolysate[a]		88.60	59.10
FI		41.52	73.17
FII		14.15	64.41
FIII		8.99	66.02
	Total of fractions	64.66	
FI-E		20.46	65.49
FII-E		12.41	84.44
FIII-E		19.06	84.29
	Total of fractions	51.93	

[a]Before UF.

Table 3—Comparison of suggested (FAO/WHO, 1991) patterns of amino acid requirements with the composition of soy flour (SF) and the ultrafiltrated fractions (g/100g of protein)[1].

	Infant	Pre-school child	SF	FI	FII	FIII	FI-E	FII-E	FIII-E
His	2.60^{b}	1.90^{ab}	2.64^{b}	2.04^{ab}	2.43^{ab}	2.86^{b}	1.54^{a}	1.94^{ab}	2.47^{ab}
Thr	4.30^{bc}	3.40^{abc}	3.95^{abc}	2.61^{ab}	3.48^{abc}	4.78^{c}	3.80^{abc}	2.46^{a}	4.85^{c}
Phe+Tyr	7.20^{bc}	6.30^{b}	9.92^{d}	8.44^{cd}	8.38^{cd}	6.99^{bc}	6.36^{b}	3.45^{a}	5.80^{b}
Met	4.20^{d}	2.50^{c}	1.77^{ab}	1.42^{ab}	1.53^{ab}	1.17^{a}	4.98^{d}	2.24^{bc}	4.42^{d}
Val	5.50^{bc}	3.50^{a}	5.61^{bc}	5.32^{abc}	5.16^{abc}	4.95^{abc}	5.95^{bc}	4.13^{ab}	6.53^{c}
Ile	4.60^{abc}	2.80^{a}	5.31^{bc}	6.23^{c}	4.52^{abc}	4.42^{abc}	5.74^{bc}	4.25^{ab}	6.13^{bc}
Leu	9.30^{c}	6.60^{ab}	6.99^{ab}	9.45^{c}	7.63^{bc}	6.74^{ab}	8.42^{bc}	5.03^{a}	7.47^{bc}
Lys	6.60^{ab}	5.80^{ab}	6.56^{ab}	7.85^{c}	7.36^{bc}	6.73^{ab}	4.62^{a}	5.34^{ab}	6.27^{ab}

[1]Values in the same row with different superscripts are significantly ($p < 0.05$) different.

and a higher allergenicity, as has been proven by different commercial 'hypoallergenic' formulas (van Beresteijn and others 1994; Ena and others 1995; Halken and others 1993; Hoffman and Sampson 1997). After removal of high molecular weight mass proteins and free amino acids, the FIII and FIII-E fractions had the best molecular weight profiles for hypoallergenic infant formulas.

Amino acid composition. Table 3 shows the essential amino acid profiles of the native soy protein (soy flour) and the UF fractions obtained from hydrolysate before and after methionine enrichment. Suggested amino acid requirements (FAO/WHO 1991) for infant and preschool children (recommendation for all of the age groups different to infants) are included in the table. Cys was not determined in this study. Hence, the values of Met alone were used for the sulfur amino acid requirements.

Fractions obtained from hydrolysate before methionine enrichment contained the same ($p > 0.05$) essential amino acid concentrations than those of the soy flour with two exceptions: Phe + Tyr was lower ($p < 0.05$) for FIII and Leu and Lys were higher ($p > 0.05$) for FI. In general, amino acid recommendations for human could be supplied by soy protein alone except by sulfur amino acids for infants. Although Cys was not measured in this study, soy protein contains 1% to 2% Cys (Bressani 1981; del Valle 1981), which when added to the Met values reported in Table 3 could be enough to meet the sulfur amino acid requirements for preschool children. The 2nd limiting amino acid in soy protein is Leu. The Leu content in FI and FI-E would meet the requirement for infants, while FII, FIII, and FIII-E would meet the requirement for preschool children

The requirement for sulfur-containing amino acids for infants and preschool children is 4.2% and 2.5% (FAO/WHO 1991). Fractions FI-E and FIII-E had enough methionine (Table 3) to meet the infant requirement and FII-E met the requirement for children and adults. Moreover, amino acid composition of FIII-E, the proposed fraction to use in a hypoallergenic infant formula, was not different ($p < 0.05$) than that of the recommendation for infants, even if Cys is not considered for comparison. Although we have no information on nutritional quality, Kimura and Arai (1988) and Hajós and others (1996) reported that enzymatically modified soy oligopeptides were utilized more efficiently than soy protein isolates or amino acid mixtures with similar composition.

Functional properties

Solubility. Solubility profiles of soy protein hydrolysate, UF fractions (before and after modification), soy flour, and soy isolate at pH 4 to 10 were compared (Figure 1). The whole hydrolysate and all the fractions under 10 kDa were 100% soluble at all pHs (4 to 10). The solubility of FI was greater than that of FI-E ($p < 0.05$), both increased with increasing pH values and were higher ($p < 0.05$) than that of soy flour in the range tested. The solubility of FI was higher ($p < 0.05$) than that of soy isolate at pH 4 to 8, and the 1st one solubilized fully (100%) at pH 7 to 10, while the 2nd one did at pH 9 and 10. FI-E solubility values were higher ($p < 0.05$) at pH 4 to 7, comparable at pH 8, and lower at pH 9 to 10 than those of soy isolate.

Improvement in protein solubility due to partial hydrolysis by different proteases has been reported (Hettiarachchy and Kalapathy 1997; Deeslie and Cheryan 1988), while no data have been reported about hydrolysates after enzymatic synthesis. Our results demonstrate still that only the solubility of the fraction above 10 kDa (FI-E) was adversely affected at pH 7 to 10, and it was better than that of soy protein isolate used in some foods, such as infant formula, prepared at pH 6.5 to 7. In addition, all the fractions under 10 kDa can be used to increase the application of soy proteins, incorporating them into low pH beverages and enteral or parenteral diets.

Clarity. Turbidity profiles, expressed as optical density (OD), of commercial soy isolate, hydrolysate, and the UF fractions at various pH values are shown in Table 4. Hydrolysis decreased turbidity respect to that of soy protein, especially for fractions under 10 kDa (FII and FIII), which can be considered clear at all the tested pHs because their optical density was under 0.1 at 660 nm (Deeslie and Cheryan 1988). Turbidity of FI was higher because it contains the less hydrolyzed polypeptides, and increased turbidity was obtained with decreasing pH. Fractions under 10 kDa could be used for any kind of beverages, especially those carbonated ones where clarity at acidic pH is an essential requirement (Kinsella 1979).

On the other hand, bound methionine of modified hydrolysates increased turbidity without any effects of pH for FI-E. For

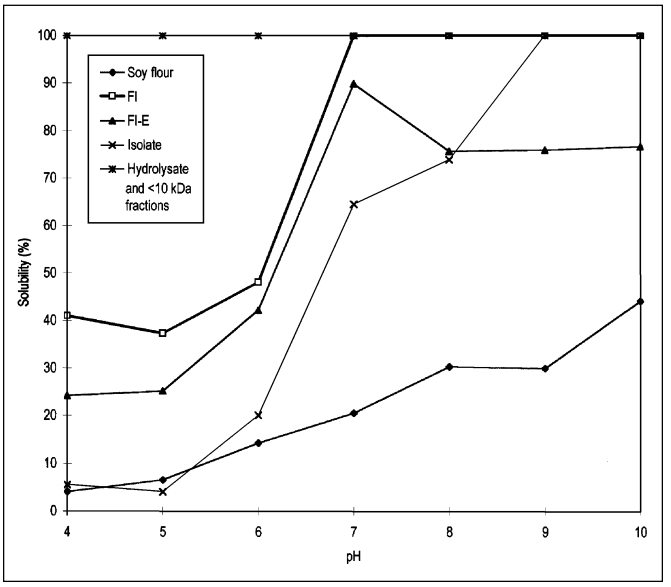

Fig. 1—Solubility profiles of soy flour, comercial soy protein isolate, hydrolysate and UF fractions.

Table 4—Turbidity (as optical density at 660 nm) of soy protein isolate, hydrolysate and UF fractions, at variuos pH values[1].

pH	Isolate	Hydrolysate	FI	FII	FIII	FI-E	FII-E	FIII-E
4.0	1.314^{j}	1.141^{h}	1.308^{j}	0.089^{b}	0.025^{ab}	1.655^{l}	0.194^{c}	0.475^{e}
5.5	1.360^{k}	1.034^{g}	1.283^{i}	0.035^{b}	0.009^{a}	1.643^{l}	0.073^{b}	0.271^{d}
7.0	0.637^{f}	0.323^{d}	0.479^{e}	0.018^{a}	0.008^{a}	1.645^{l}	0.055^{b}	0.075^{b}

[1]Data followed by different superscripts are significantly ($p < 0.05$) different.

FIII-E with a high methionine level as in FI-E, turbidity increased with decreasing in pH, and FII-E with less bound methionine, had only turbidity at pH 4.

A good correlation between turbidity and solubility was obtained respect to hydrolysis, but it was not so clear cut for synthesis. In addition to pH and molecular size of the fractions, the level of the bound methionine appears to influence turbidity, probably due to an increase in the tendency to aggregation by hydrophobic interaction.

Foaming properties. At pH 4 and 7, the foaming properties of soy protein were improved by enzymatic hydrolysis (Table 5). However, stability of the foam decreased with decreasing molecular weight of the fraction, especially at pH 7. By contrast, synthesis presented an opposite effect, where the higher molecular weight fraction (FI-E) presented the poorest foaming properties (except stability at pH 7). Comparing hydrolysates before and after methionine enrichment, the foam volume was better for enriched hydrolysate fractions and the stability was better for FI and FII fractions of hydrolysis before enrichment, especially at pH 4.

In addition, the foaming properties of the whole hydrolysate and their FI and FII fractions were comparable to those of albumin and better than those of soy isolate, except for stability of the FII's foams at pH 7.

The same effect of hydrolysis and fractionation by UF on foaming properties of soy protein was reported before (Deeslie and Cheryan 1991), using 2 levels of conversion in hydrolysis and bigger molecular weight cut-off in UF. Lieske and Konrad (1996) reported that the foam stability was higher near the isoelectric point of whey hydrolysate, and lower values were obtained at increased degree of hydrolysis. In our study, bound methionine shifted the isoelectric point of peptides, inducing an overall loss in foam stability at pH 4, with respect to those of the hydrolysate fractions.

Surface hydrophobicity (S_o). Values for soy flour, hydrolysate, UF fractions, and bovine serum albumin (BSA), are shown in Fig. 2. According to some authors (Mahmoud and others 1992), enzymatic hydrolysis decreases hydrophobicity, while according to others (Hettiarachchy and Kalapathy 1997) it increases. Our whole hydrolysate had a comparable S_o value to that of soy protein. Apparently, more than the hydrolysis itself, it is the nature of the hydrolyzed protein and molecular weight size of the peptides what determines hydrophobicity; thus the UF fraction above 10 kDa (FI) presented the highest S_o value, and the smaller molecular weight fraction the lower one.

Synthesis decreased S_o value of FI-E compared with the hydrolysis fraction FI. This might be due to folding of peptide chains favored by methionine binding, thus effectively preventing hydrophobic groups from being surface exposed.

Emulsifying activity. Figure 3 shows the emulsifying activity index (EAI) of commercial soy isolate, hydrolysate and UF fractions. The overall effect of enzymatic hydrolysis was to increase ($p < 0.5$) the EAI of soy proteins (data not shown). In agreement with the S_o values, the high molecular weight fraction (FI) from hydrolysis presented the highest ($p < 0.05$) EAI and FIII the lowest. In addition, EAI for FI was comparable ($p > 0.05$) to that of isolate soy protein and higher ($p < 0.05$) than that of albumin used as a standard for comparison (date not shown).

A high correlation between emulsifying properties and hydrophobicity has been reported by other authors for different proteins (Nakai and others 1980; Li-Chan and others 1984; Mahmoud and others 1992). It has been found that a milder hydrolysis (DH 7% to 17%) of soy protein isolates (Hettiarachchy and Kalapathy 1997) and a more extensive hydrolysis of casein (Mahmoud and others 1992) decreased emulsifying properties with increasing DH. Our protein for hydrolysis differed from soy protein isolate and casein respectively in protein conformation and amino acid sequence, hence inducing different properties. If we compare EAIs of soy protein isolate, considered a good emulsifier, with our fractions; only FI's EAI was not significantly different. Probably it is because FI is the fraction with less hydrolysis, and it could be used for emulsified products where a very high solubility at low pH is not a major requisite.

On the other hand, although the overall effect of the synthesis was to decrease EAI there was not a correlation between EAI values and molecular size of the fractions, neither with S_o values. Even more so, there were no differences ($p > 0.05$) in EAI values of FI-E and FIII-E, and the fraction with the higher EAI (FII-E)

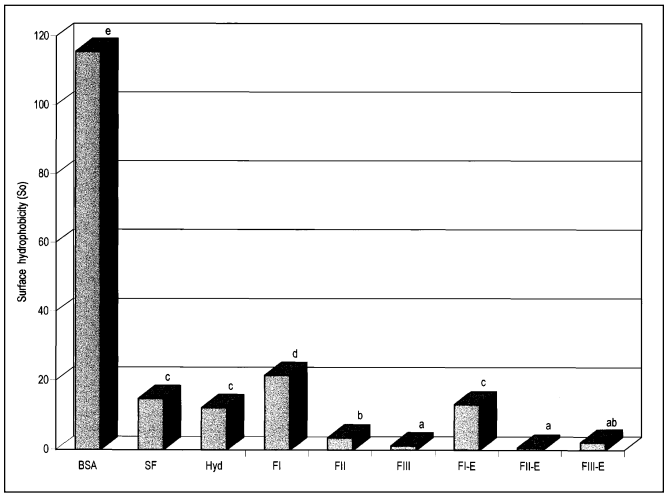

Fig. 2—Surface hydrophobicity (S_o) of bovine serum albumin, soy flour, hydrolysate and UF fractions. S_o values which do not bear the same letter are significantly different ($p<0.05$).

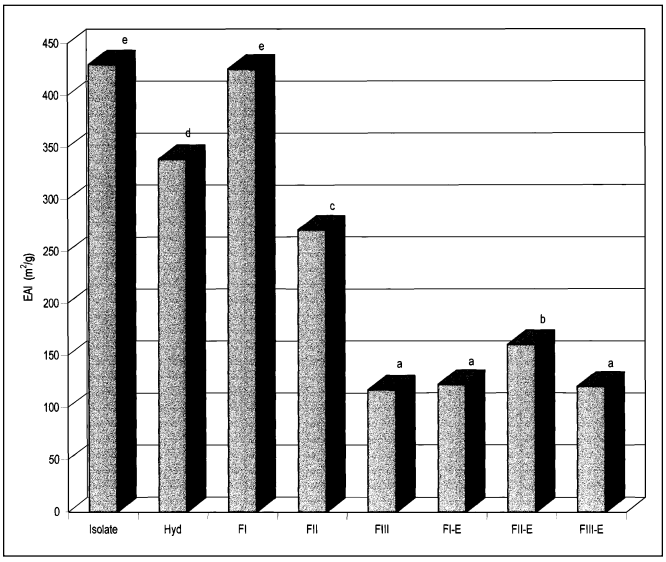

Fig. 3—Emulsifying activity index (EAI) of comercial soy protein isolate, hydrolysate and UF fractions. EAI values which do not bear the same letter are significantly different ($p<0.05$).

Table 5—Foaming properties of soy isolate, hydrolysate and UF fractions (mL/100 mL of solution)[1].

pH	Time	Isolate	Hyd	FI	FII	FIII	FI-E	FII-E	FIII-E
4.0	0 min	33.75^{bc}	293.00^{mn}	311.00^{mn}	377.67^{o}	164.50^{fg}	150.00^{fg}	573.00^{q}	493.50^{p}
4.0	30 min	24.00^{ab}	140.25^{f}	178.00^{gh}	200.67^{hij}	0.00^{a}	18.00^{ab}	165.00^{fg}	105.00^{e}
7.0	0 min	223.00^{ij}	299.75^{mn}	255.25^{kl}	279.75^{lm}	198.00^{hi}	145.00^{fg}	332.00^{no}	291.00^{mn}
7.0	30 min	176.75^{gh}	151.25^{fg}	156.75^{fg}	15.00^{ab}	6.75^{ab}	94.00^{de}	0.00^{a}	13.50^{ab}

[1]Data followed by different superscripts are significantly ($p < 0.05$) different.

presented the lowest S_o value. Although percentage of bound methionine correlated with EAI for each fraction, S_o values were not affected in the same way.

Differential Scanning Calorimetry (DSC). UF fractions showed 2 thermal transition peaks in their thermograms. The 1st one, centered at around 85 °C corresponds to 7S protein (Sorgentini and others 1995), and it was shown only for 4 of the 6 fractions. These ΔH values were negligible as compared to those of the 2nd transition observed for all of the fractions and can be explained as one result of total protein denaturation. Therefore, only the analysis for the 2nd peak is presented (Table 6). No differences were found ($p > 0.05$) in T_{max} among the fractions. This transition is similar to that reported for 11S protein (Wagner and Añón 1990; Sorgentini and others 1995).

There were no differences among the ΔH of the fractions under 10 kDa, while ΔHs of the fractions above 10 kDa were higher with FI's ΔH significantly ($p > 0.05$) higher than that of FI-E. ΔH values were higher in the high molecular weight and hydrolysis fractions than those for low molecular weight and synthesis fractions, as it corresponds to the degree of denaturation. According to Henn and Netto (1998), there is a decreasing in the proportion of the basic peptide of 11S from soy protein isolates with an increasing of degree of denaturation. Additionally, the areas of endotherms correlated directly to the surface hydrophobicity values. Indeed, the S_o value of FI was higher than that of FI-E and both of them had higher S_o values than those of the fractions under 10 kDa with no differences among them.

Finally, although no studies on flavor were made, some preliminary assumptions can be made. Firstly, fraction FI (above 10 kDa) so resembles soy protein isolate in amino acid composition and functional properties, that both of them may elicit similar flavor response. Commonly bitterness is associated with small molecular weight peptides under 2400 kDa or less (Arai and others 1970). On the other hand, regarding fractions under 10 kDa, Deeslie and Cheryan (1988) produced ultrafiltrated (under 10 kDa) enzymatic hydrolysates from soy protein and found that hydrolysates were 'less than half as bitter as the reference solution, which itself was a barely detectable level of bitterness'. Hydrolysates obtained by the former authors presented very similar functional properties than those obtained by us. So it seems reasonable to anticipate that a very similar flavor would be expected. With respect to methionine enriched hydrolysates, we have no information on flavor to draw any assumption.

Table 6—Peak 2's transition temperatures and calorimetric entalphies of UF fractions[1,2].

Fraction	T_{tr}(°C)	T_{max} (°C)	ΔH (J/g)
FI	92.97 ± 0.97^{bc}	95.11 ± 0.83^{d}	2.956 ± 0.39^{e}
FI-E	92.01 ± 2.27^{bc}	93.23 ± 2.21^{cd}	1.017 ± 0.16^{d}
FII	92.80 ± 1.35^{bc}	93.91 ± 1.22^{cd}	0.585 ± 0.11^{c}
FII-E	94.71 ± 1.62^{c}	95.84 ± 1.84^{d}	0.562 ± 0.08^{c}
FIII	91.87 ± 2.80^{bc}	92.87 ± 2.64^{cd}	0.482 ± 0.04^{bc}
FIII-E	94.69 ±1.06^{c}	95.54 ± 1.00^{d}	0.315 ± 0.08^{abc}

[1]Means ± SD of four replicates.
[2]Values in the same column with different superscripts are significantly ($p < 0.05$) different.

In conclusion, all the fractions under 10 kDa (FIII, FIII-E, FII, and FII-E) presented 100% solubility at all pHs. The less hydrolyzed fractions (FI and FI-E) showed lower solubilities especially at pHs 4 to 6. The majority of the analyzed functional properties were predictable from solubility of the fractions obtained from hydrolysis but after methionine binding the molecular weight and content of bound Met were interacting together to modify and determine the functional properties.

Because of its perfect amino acid composition for infants and a hypothetical no allergenic responses (due its lower molecular weight as established by other authors), FIII-E could be used in hypoallergenic formulas as the only source of protein. FII-E meets the amino acids requirement for children and adults, and it could be used in fortified soluble formulas and as the only protein source in special medical diets. FI-E, because of its high methionine content (4.2%) and foam stability and emulsifying activity, presents a good source of protein for baby food. FI resembles commercial soy protein isolate in its functional properties, and it could be an alternative for different products, using soy isolate to amortize production. In the same way, FII and FIII because of their solubility and clarity at low pH, could be used in sparkling or carbonated fortified beverages.

Currently, additional nutritional and sensorial evaluations are in progress and studies using modified hydrolysates in formulation should be done since there is an increasing demand for dietetic products for therapeutic use.

Materials and Methods

Materials

Defatted soybean flour was purchased from Gamesa, S.A. (Cd. Obregón, Sonora, México). Soy protein isolate SUPRO® 500E was from Protein Technologies International (St. Louis, MO). After grinding, the flour was assayed for total protein content, ash, moisture, particle size, and amino acid composition according to methods described below. High performance liquid chromatography (HPLC) grade chemicals were from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). The rest of the reagents were of analytical grade from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) or Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.).

Preparation of pancreatic enzyme concentrate

Extraction and fractionation. Fresh porcine pancreas were gathered from a processing plant (Alpro, S.A. Hermosillo, Sonora, México) and transported on ice to the laboratory. Extraction steps with 0.125 M H_2SO_4 and 30% ammonium sulfate precipitation were done according to Mullally and others (1995). Precipitate was resuspended in 0.1 M NaCl and ultrafiltrated in a CH2 system (Amicon, Beverly, Mass., U.S.A.) through a 10 kDa molecular weight cut-off (MWCO) spiral membrane. Ultradiafiltration was achieved with 0.1 M NaCl until permeate was ammonium sulfate-free using the qualitative $BaCl_2$ test.

Activation of zymogens. After ultradiafiltration, samples were buffered with 0.1 M Tris-HCl, pH 7.0, containing 10 mM

$CaCl_2$, in a 1:1 ratio (v/v). Activation was done according to Mullally and others (1995) with 2% exogenous trypsin type II-S (Sigma Chemical Co. St. Louis, Mo., U.S.A.) solution (1 mg/mL). Aliquots of 5 mL were taken at different times (2, 4, 6, 8, 10, and 12 h), at 37 °C prior to freeze-drying. The enzymatic activities of trypsin and chymotrypsin were determined according to Erickson and others (1983) with synthetic substrates α-N-Benzoyl-D-L-Arginine-p-Nitroanilide (BAPNA) and N-Glutaryl-L-Phenylalanine-p-Nitroanilide (GNPA) using commercial trypsin (type II-S) and chymotrypsin (type II) (Sigma Chemical, Co. St. Louis, Mo., U.S.A.) as controls.

Partial characterization by SDS-PAGE. The enzyme concentrate and commercial trypsin and chymotrypsin were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 17 % gel according to Laemmli (1970).

Enzymatic hydrolysis

Temperature optimization. The effect of temperature (40, 45, 50, and 55 °C) on the enzymatic concentrate activity was determined using bovine serum albumin as substrate, according to Erickson and others (1983). Briefly, 5 mL of a 1% (w/v) enzymatic concentrate solution were added to a buffered (pH 8) 1% albumin solution (15 mL) previously equilibrated to 40, 45, 50, or 55 °C, and incubated for 4 h. Aliquots of 2 mL were taken each hour, precipitated with 3.5 mL of 5% TCA, and soluble protein was measured in the supernatant by absorbance at 280 nm.

Time and degree of hydrolysis. Hydrolysates were produced from defatted soy flour by a batch process. A preliminary assay of enzymatic hydrolysis was done in order to establish the optimum hydrolysis time. A 4% (w/v) suspension of soy flour (250 mL) was hydrolyzed with the pancreatic enzyme preparation at 50 °C, pH 8.0 for 24 h. Enzyme: substrate ratios were 0.02 and 0.04. The pH was maintained by addition of 0.5 M NaOH. Aliquots were taken at 0, 6, 12, and 24 h, and the degree of hydrolysis (DH, %) calculated as the percentage of peptide bonds cleaved, using volume and molarity values of the NaOH used to maintain the pH constant (Adler-Nissen, 1986). Following hydrolysis, proteolytic activity was inactivated by heating at 85 °C for 30 min. Hydrolysates were centrifuged at 403 × *g*, 15 min at 4°C (IEC Centra GP8R). At the optimum time (as determined after ultrafiltration), a 15-L suspension of soy flour was hydrolyzed using a 4% (w/v) suspension of soy flour, an enzyme: soy protein ratio of 0.04, 50 °C, pH 8, and 12 h. Proteolytic activity was inactivated as described above, and the hydrolysate was freeze-dried.

Enzymatic synthesis

Reaction conditions. The powder hydrolysate of soy protein was reconstituted to 20 g/100 mL. Enzymatic synthesis was done at 37 °C using chymotrypsin as catalyzer (1:100, enzyme:hydrolysate ratio), methionine methyl ester (Met-ME), and 3 M glycerol. Firstly, the effects of pH (6 and 7) and reaction time (3, 6, and 9 h) were optimized, and then methionine was added (0.2275 to 0.0569 g Met/g hydrolysate). The pH was maintained constant by addition of 2 N NaOH or 2 N HCl. Aliquots were extracted from the reaction mixture at 3, 6 and 9 h in order to quantify the synthesis product. Chymotrypsin was inactivated by heating to 85 °C for 15 min.

Optimization. Aliquots were exhaustively dialyzed against 1% NaCl. The synthesis products were analyzed for amino acid composition after precipitation with 20% trichloroacetic acid (TCA) and centrifugation at 5500 × *g* for 10 min. Both supernatant and precipitate and dialysis solution were analyzed in order to determine the best reaction time and pH conditions to obtain 4% methionine bound to the hydrolysate. Afterward, using the chosen time and pH, methionine addition was optimized by the same procedure.

Ultrafiltration

Both unmodified and modified hydrolysates were fractionated in a UF system (Amicon, Beverly, Mass., U.S.A.) through a series of spiral membranes of decreasing pore size. Hydrolysates were first passed through a 10 kDa molecular weight cut-off (MWCO) membrane. It was diafiltered with 2 volumes of water. Permeates were then pumped through a 3 kDa MWCO membrane and a 1 kDa MWCO membrane, diafiltering with 2 volumes of water each time. Fractions obtained from no-modified hydrolysate were designated as FI> 10 kDa, 10>FII> 3 kDa and 3>FIII> 1 kDa, and fractions from methionine enriched hydrolysate were called: FI-E> 10 kDa, 10>FII-E> 3 kDa and 3>FIII-E> 1 kDa. All the fractions will be referred to as UF fractions in subsequent sections.

Analytical methods

Molecular weight distribution. The molecular weight of each UF fraction was evaluated by gel filtration chromatography using Sephadex G-50 as recommended by Pharmacia (1991). The eluant was 0.02 M phosphate saline buffer, pH 7.2. The column void volume (DI=1cm and L=90 cm) was determined with Dextran blue (1 mg/mL), and the peaks were registered using an Econo BioRad system (Hercules, Calif., U.S.A.) by UV-detection at 280 nm . The flow rate was 18 mL/h. The column was calibrated with solutions of ovoalbumin, trypsin, chymotrypsin, lysozyme, aprotinin, and vitamin B12 (1 mg/mL), and a calibration plot was calculated by linear regression ($r = -0.99$). Fraction samples (0.5 to 1 mL) were injected into the column, and the elution volume was determined. Then, the molecular weight was calculated from the calibration plot.

Protein content. Protein in dried samples (in triplicate of 2 different samples) was quantified as total nitrogen by the micro-Kjeldahl method 960.52 of AOAC (1990). The factor used to obtain protein percentage was 6.25. Protein in solution was determined by the Lowry and others (1951) technique.

Moisture and ash. Moisture was determined by oven-drying at 130 ± 3 °C until constant weight was obtained (AOAC 1990, method 925.10). Ash was analyzed according to AOAC (1990) method 923.03.

Amino acid analysis. The amino acid content of supernatants, precipitates, and dialyzed solutions for optimization of synthesis conditions, the UF fractions, and soy-based products was determined according to Vázquez and others (1995), using a Varian 9010 High Performance Liquid Chromatography (HPLC) equipped with a Fluorochrom II detector. Samples were hydrolyzed under vacuum at 150 to 155 °C in 6 N HCl using a Reacti-Therm 18870 digestor (Pierce, Rockford, Ill., U.S.A.).

Functional properties

Solubility. Solubility of the soy protein hydrolysate, UF fractions, and soy products was determined by a protein dispersibility index (PDI) method reported by Saeed and Cheryan (1988) and Parrado and others (1991). A 1% w/v aqueous solution was blended for 10 min, with either 2 N HCl or NaOH to adjust the pH. After 15-min resting period and readjustment of the pH if necessary, the sample was centrifuged at 1400 × *g* for 10 min. The protein content of the supernatant was deter-

mined. Solubility was expressed as the percent of total protein of the original sample in the supernatant.

Clarity. This was evaluated according to the method reported by Deeslie and Cheryan (1988). A 1% w/v aqueous solution of soy protein hydrolysate, UF fractions, and soy products was blended. The pH was adjusted with either 2 N HCl or NaOH. Optical clarity was assessed quantitatively by turbidity measurements, that is, as the optical density at $\lambda = 660$ nm. Double distilled water was used as the blank.

Foaming properties. These properties were evaluated according to Puski (1975). A 1% w/v aqueous solution of soy protein hydrolysate, UF fractions, and soy products was blended in a T25 UltraTurrax homogenizer (Janke & Kunkel, IKA, Labortechnik, Staufen, Germany) for 10 min at 13,500 min^{-1}. The blender contents were immediately transferred to a 50-mL graduated cylinder, and the foam volume was registered. 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.

Surface hydrophobicity. Protein surface hydrophobicity (S_o) was determined by the cis-parinaric acid (CPA) flourescent method of Kato and Nakai (1980). The soy protein hydrolysate, UF fractions, and bovine serum albumin (BSA) were dissolved (1 mg/mL) in 0.01 M phosphate buffer, pH 7.0. The solutions were stirred for 2 h at 20 °C and centrifuged at 8000 $\times$ *g* for 20 min. Protein concentration of the supernatants was determined according to the method of Lowry and others (1951). Each supernatant was serially diluted with the same buffer to obtain protein concentrations ranging from 0.0005 to 0.1 mg/mL. Then 20 μL of CPA (1 mg/mL dissolved in absolute ethanol) were added to 4 mL of protein solution. Flourescent intensity was recorded at 325 nm excitation and 420 nm emission, using a Turner 430 Spectrofluorometer (GK Turner Associates, Palo Alto, Calif., U.S.A.). The slope of the plot of fluorescence intensity in contrast to protein concentration were calculated by linear regression ($r=0.99$) and designated as S_o.

Emulsifying activity. Emulsifying activity index (EAI) was determined by a modification of the turbidimetric method of Pearce and Kinsella (1978), described by Li-Chan and others (1984). Emulsions were prepared with 6 mL of a 1% w/v protein solution (in a 10 mM pH 7.0 phosphate buffer) and 2 mL of corn oil, homogenized at 8000 min^{-1} for 1 min in a T25 UltraTurrax homogenizer. Aliquots of the emulsion were taken from the bottom of the vessel after 1 min, and diluted in 2 mL of 0.3% w/v sodium dodecyl sulfate (SDS) solution. Absorbance at 500 nm was measured in a Spectronic 21 Spectrophotometer (Milton Roy Co., Rochester, N.Y., U.S.A.), using 0.3 % SDS solution as the blank. The EAI (in m^2/g) was calculated as (Pedrosa and others 1997)

$$EAI = (2.303 \times 2 \times A_{500}) / (C \times \phi \times L)$$

where A_{500} is the absorbance at 500 nm, C is the weight of protein per unit volume of aqueous phase before emulsion formation (g/mL), ϕ is the oil volume fraction of the emulsion, and L is cell length (cm).

Differential Scanning Calorimetry (DSC). Protein samples were dissolved in double distilled water (0.2 g/mL) and hermetically sealed in stainless steel pans. A double distilled water double pan was used as reference. Heating thermograms were registered at 10 °C/min in a Perkin-Elmer Model DSC-4 calorimeter (Norwalk, Conn., U.S.A.) with a controller (Perkin-Elmer System 4 Thermal Analysis Microprocessor Controller) to determine transitional and maximal temperatures, and entalphies (ΔH in Joules/g of dry matter).

Statistical analysis

Three replicates were performed in a completely randomized design. General linear model procedure was used to test analysis of variance. The effects of hydrolysis, synthesis, and fractionation by ultrafiltration on functional properties were evaluated by Tukey-Kramer Multiple-Comparison test (NCSS 1996). Amino acid patterns were compared to the human requirement patterns (FAO/WHO 1991), by using Fisher's LSD Multiple-Comparison test (NCSS, 1996). Significance was defined at $p < 0.05$.

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