Hydrophobicity, Solubility, and Emulsifying Properties of Soy Protein Peptides Prepared by Papain Modification and Ultrafiltration

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ABSTRACT: Peptide size control is important for obtaining desirable functional properties so that these peptides can be better utilized. Proteolytic enzymatic modification of soy protein isolates (SPI), followed by ultrafiltration, is an effective way to fractionate these proteins into peptides with controlled molecular size. SPI was predenatured by mild alkali at pH 10 and heated at 50°C for 1 h prior to partial hydrolysis by papain at pH 7.0 and 38°C for 10, 30, and 60 min (PMSPI10, PMSPI30, and PMSPI60). The hydrolysate PMSPI60 was further fractionated by ultrafiltration with a stirred cell and disc membranes (100-, 50-, and 20-kDa molecular weight cut-off) into one retentate (R100) and three permeates (P100, P50, and P20). Molecular weight distribution, surface hydrophobicity (S_0), protein solubility (PS), emulsifying activity index (EAI), and emulsion stability index (ESI) of the control SPI (without added papain), hydrolysates, and ultrafiltrates were investigated. Significant increases ($P < 0.001$) in S_0, PS, EAI, and ESI were observed in the hydrolysates. Peptides in the permeates had higher PS and EAI but lower S_0 than the peptides in the retentate and hydrolysate. Soy protein peptides that were prepared from SPI by papain modification and ultrafiltration had lower molecular weight, higher solubility, and higher emulsifying properties. They could find use in products that require these properties, especially in the cosmetic and health food industries.

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KEY WORDS: Emulsifying properties, enzymatic modification, hydrophobicity, papain, solubility, soy protein isolate, ultrafiltration.

Proteolytic enzyme modification of proteins is an effective way to improve their functionality. The peptides produced by partial proteolysis of proteins have smaller molecular size and less secondary structure than the original proteins. They contribute to increased solubility, decreased viscosity, and significant changes in the foaming, gelling, and emulsifying properties when compared with those of the original proteins (1). To obtain desirable functional properties in soy protein hydrolysates, hydrolysis must be carried out under strictly controlled conditions to a specified (generally low) degree of hydrolysis (2). A limited degree of hydrolysis usually improves the emulsifying and foaming capacities, whereas excessive hydrolysis often causes loss of some of these functionalities (3). Therefore, peptide size control is essential if optimal and reproducible changes in functional properties are to be achieved (1).

Ultrafiltration has been successfully used to separate proteins (peptides) from salts and sugar and to fractionate proteins (peptides) from each other (4). Partial hydrolysis of soy protein isolates (SPI) (5–9) generates soy protein peptides with different molecular weight distributions that can be separated with ultrafiltration membranes of different pore sizes.

To understand the basis of peptide functionality, it is essential to know the fundamental properties of the peptide, including protein surface hydrophobicity, solubility, and emulsifying properties. This study investigated these fundamental properties of soy protein peptides that were prepared by papain hydrolysis and subsequent membrane ultrafiltration.

EXPERIMENTAL PROCEDURES

Materials. Soybeans (*Glycine max* var. Hutcheson) were obtained from the Variety Laboratory, Department of Agronomy, University of Arkansas. Papain (I.U.B. 3.4.22.2, 30,000 PU/mg) was purchased from Solvay Enzymes (Elkhart, IN). The stirred ultrafiltration cell (Model 8400, volume size 400 mL) was purchased from Amicon (Danvers, MA). Disc membranes (20-, 50-, and 100-kDa molecular weight cutoff) were purchased from Spectrum Medical Industries, Inc. (Houston, TX). Laboratory chemicals were of analytical grade, obtained from standard commercial sources, and used as received.

Preparation of SPI. The soybeans were finely ground, defatted with hexane and dried under the hood at ambient temperature. SPI was prepared according to the method of Wolf and Cowan (10) by alkaline (pH 9) extraction of the defatted soy flour, followed by acid (pH 4.5) precipitation, washing, and freeze-drying.

Preparation of protein hydrolysates by papain. Three 10-g portions of SPI were each dispersed in 200 mL deionized water, adjusted to pH 10 with 1.0 N NaOH, and incubated at 50°C for 1 h with constant 120-rpm shaking (Orbital Lab-Line Shaker, Model 1288, Lab-Line Instruments, Inc., Mel-

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rose Park, IL). Each portion was adjusted to pH 7.0 and hydrolyzed with 0.01 g of papain at 38°C. One dispersion was hydrolyzed for 10 min (PMSPI10), the second dispersion was hydrolyzed for 30 min (PMSPI30), and the third dispersion was hydrolyzed for 60 min (PMSPI60) with constant shaking at 120 rpm. The resulting hydrolysates were each adjusted to pH 7.0 and heated at 95°C for 5 min to inactivate the enzyme. Each hydrolysate was then freeze-dried, ground, sealed in glass bottles, and stored at 4°C until analyzed. As a control, SPI was also treated similarly without added enzyme.

Peptide fractionation by ultrafiltration. The hydrolysate PMSPI60 was further fractionated by ultrafiltration with a stirred cell and disc membrane system. A 4-g batch of the hydrolysate was dissolved in 400 mL deionized water, adjusted to pH 7.0, and first separated by a 20-kDa molecular weight cutoff (MWCO) membrane in the cell at 70 psi and 4°C. The separation generated two streams—permeate (designated P20) and retentate 1. The P20 was dialyzed against deionized water at 4°C for 24 h to remove salt through cellulose membrane with a MWCO of 2,000 Da (Spectrum). Retentate 1 was further separated by a 50-kDa MWCO membrane and generated two other streams—permeate (designated P50) and retentate 2. Finally, retentate 2 was separated by a 100 kDa-MWCO membrane and generated a permeate (designated P100) and a final retentate (designated R100). For satisfactory separation, each retentate was passed through the same membrane twice before being separated with the next membrane. Each separation was performed until the volume of retentate was reduced to about 30 mL. Then the retentate was diluted to 400 mL with deionized water and passed through the same membrane. The resulting four fractions (R100, P100, P50, and P20) were each freeze-dried, finely ground in a grinder, sealed in glass bottles, and stored at 4°C until analyzed.

Molecular weight distribution. Molecular weight distributions of SPI, hydrolysates (PMSPI10, PMSPI30, and PMSPI60), and ultrafiltrates (R100, P100, P50, and P20) were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Samples (1 μg/μL) were prepared in a buffer that contained 62.5 mM Tris (pH 6.8), 8 M urea, 2% SDS, and 5% 2-mercaptoethanol. SDS–PAGE was carried out on a slab gel with an SDS–Tris–glycine discontinuous buffer system with 4% stacking gel and 7–15% gradient gel to obtain good resolution for lower-molecular-weight bands. The gels were stained with 0.25% Coomassie blue, R-250, and destained until the background stain was removed (11).

Surface hydrophobicity. Protein surface hydrophobicities of the SPI, hydrolysates, and ultrafiltrates were determined with 1-anilino-8-naphthalene sulfonate (ANS) as a hydrophobic probe (12). The protein was serially diluted with 0.01 M phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.00056 to 0.015%. Twenty microliters of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) were added to 4 mL of the diluted protein solution. The fluorescence intensity (FI) of the protein was measured with a spectrofluorometer (Model SFM23/B; Kontron Ltd., Zurich, Switzerland). Excitation and emission wavelengths were 390 and 470 nm, respectively. The FI reading was standardized by adjusting the spectrofluorometer reading for 10 μL of ANS in 5 mL methanol to 80% of full scale. The slope of the plots of FI vs. percentage of protein concentration was calculated by least-squares linear regression and used as the surface hydrophobicity (S_0).

Protein solubility. To determine protein solubility, 20 mg of protein samples was dispersed in 20 mL deionized water, adjusted to pH 3, 5, 7, 9, or 11 with 0.1 N HCl or 0.1 N NaOH, magnetically stirred at ambient temperature for 30 min, and centrifuged (Model J2-21; Beckman Instruments, Fullerton, CA) at 12,100 × *g* for 10 min. Protein contents were determined by the Bradford method (13) with SPI as the standard, for which the protein content was determined by the Kjeldahl method. Protein solubility was calculated as:

$$\text{solubility (\%)} = \frac{(\text{protein content in supernatant})}{(\text{total protein content in sample})} \times 100 \qquad [1]$$

Emulsifying properties. Emulsifying properties were measured by the method of Pearce and Kinsella (14). Pure corn oil (2 mL) and 6 mL of 0.1% protein solution (pH 7.0) were homogenized in a mechanical homogenizer (Virtishear Tempest, The Vir Tis Co., Gardiner, NY) at the highest setting 6 for 1 min. Fifty-microliter portions of the emulsions were pipetted from the bottom of the container at 0 and 10 min after homogenization. Each portion was diluted with 5 mL of 0.1% SDS solution. Absorbances of these diluted emulsions were measured at 500 nm (Varian series 634 double-beam spectrophotometer; Springvale, Australia). The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI):

$$\text{EAI (m}^2\text{/g)} = 2T\,(A_0 \times \text{dilution factor}/C \times \Phi \times 10{,}000) \qquad [2]$$

where T = 2.303; A_0 = absorbance measured immediately after emulsion formation; dilution factor = 100, C = weight of protein/unit volume (g/mL) of aqueous phase before emulsion formation; and Φ = oil volume fraction of the emulsion; and

$$\text{ESI (min)} = A_0 \times \Delta t/\Delta A \qquad [3]$$

where Δt = 10 min and $\Delta A = A_0 - A_{10}$.

Statistical analysis. Experiments were performed and the data were analyzed with the general linear model procedure of SAS Institute, Inc. (Cary, NC) to determine differences between treatment means. The significance of differences between means was determined by the least significant difference test procedure at $P < 0.05$ (15).

RESULTS AND DISCUSSION

Molecular weight distribution. The SDS–PAGE patterns of SPI contained five major bands; three of them (80, 76, and 50

kDa) were identified as 7S globulin, and two (35 and 25 kDa) as 11S globulin (Fig. 1). The minor bands of 38 and 33 kDa were identified as subunits of 11S globulin, and the 94-kDa band as lipoxygenase(16). The subunits of 11S globulin were more susceptible to papain than those of 7S globulin, as evidenced by the diffused SDS–PAGE pattern. After hydrolysis, the band of lipoxygenase disappeared from all hydrolysates.

The SDS–PAGE pattern of the ultrafiltrates showed that the disc membranes separated the hydrolysate into four peptide fractions (Fig. 1). The pattern of the retentate (R100) was similar to that of the original hydrolysate (PMSPI60). This observation indicated that some small peptides of PMSPI60 could not be separated from large peptides in deionized water at pH 7.0. In the system, these small peptides could still be bound together with large peptides through hydrophobic, hydrogen, and electrostatic bonds. These small peptides were well separated in the SDS–PAGE buffer system, which contained urea, SDS, and 2-mercaptoethanol. However, in contrast to PMSPI60, the band of 28 kDa in R100 was substantially reduced and permeated to the P100 fraction. Three major bands (32, 25, and 15 kDa) were observed with the SDS–PAGE pattern of the peptides in the P100 fraction. The distribution of P50 showed two major bands (25 and 14 kDa), while that of P20 showed one major band of 14 kDa. Two weak bands (45 and 35 kDa) were also observed in all permeates. The peptide fractions of the soy protein hydrolysate that separated through the stirred cell and disc membrane system were comparable with those obtained by other types of ultrafiltration, such as continuous ultrafiltration (6,8) and the vortex flow filtration membrane (9). The yields were 48, 12, 20, and 20% for R100, P100, P50, and P20, respectively. The high yield of R100 could be due to the fact that R100 contained small peptides that resulted from protein–protein interactions during separation.

Surface hydrophobicity. Papain modification greatly improved ($P < 0.001$) the surface hydrophobicity of SPI from 12.2 for control SPI to 28.7, 30.5, and 26.2 for PMSPI10, PMSPI30, and PMSPI60, respectively (Fig. 2). No significiant difference was observed between hydrolyzed samples ($P < 0.05$). The slight decrease in the surface hydrophobicity of PMSPI60 could be due to production of more small peptides in PMSPI60 as compared to PMSPI10 and PMSPI30. These small peptides could have fewer hydrophobic binding sites than larger peptides. This observation indicated that the surface hydrophobicity of protein could be decreased by excessive enzyme hydrolysis. The effect of protein hydrolysis on surface hydrophobicity is well known (17). In a native protein molecule, hydrophobic groups are buried inside the core of the folded structure. After partial hydrolysis, some of these groups are exposed, resulting in increased surface hydrophobicity. No significant difference ($P > 0.05$) was found between the surface hydrophobicities of the peptides in PMSPI60 and in R100. The surface hydrophobicities of the peptides in PMSPI60 (26.2) and in R100 (24.9) were significantly ($P < 0.001$) higher than that of SPI (12.2). The surface hydrophobicities of the peptides in permeates were 1.35, 1.40, and 1.42 for P100, P50, and P20, respectively, which were significantly ($P < 0.0001$) lower than that of SPI (Fig. 2). The

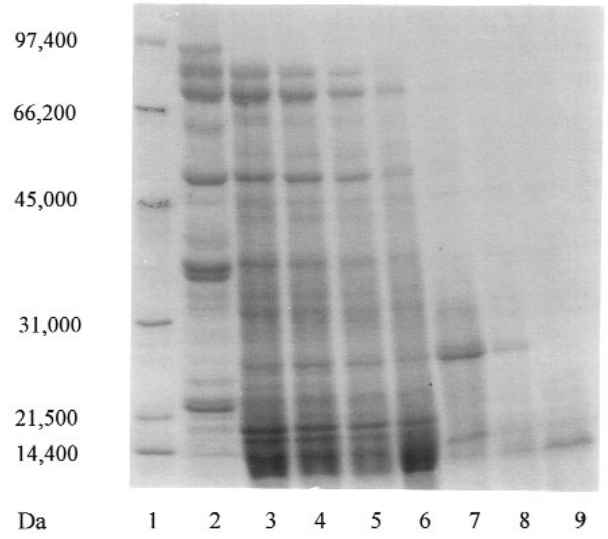

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) pattern of soy protein isolate (SPI), papain-modified soy protein isolates (PMSPI10, PMSPI30, and PMSPI60), and the retentate (R100) and permeates (P100, P50, and P20) from PMSPI60. Lane 1: molecular weight standard; lane 2: SPI; lane 3: papain-modified SPI for 10 min (PMSPI10); lane 4: papain-modified SPI for 30 min (PMSPI30); lane 5: papain-modified SPI for 60 min (PMSPI60); lane 6: retentate by 100-kDa-MWCO membrane (R100); lane 7: permeate from 100-kDa-MWCO membrane (P100); lane 8: permeate from 50kDa-MWCO membrane (P50); lane 9: permeate from 20-kDa-MWCO membrane (P20). MWCO, molecular weight cutoff.

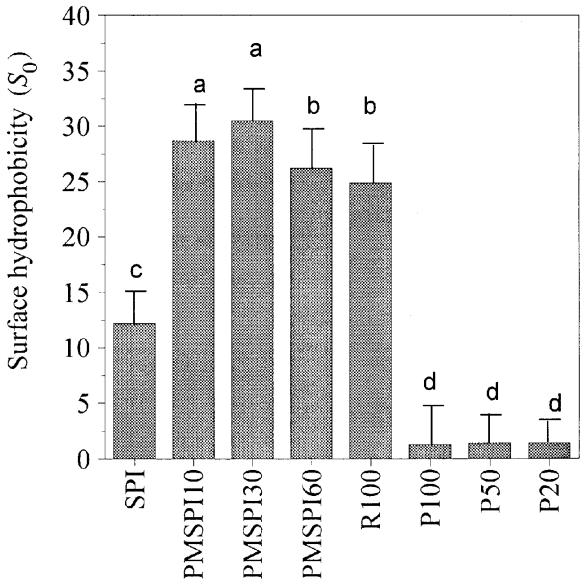

FIG. 2. Protein surface hydrophobicity (S_0) of SPI, papain-modified SPI (PMSPI10, PMSPI30, and PMSPI60), retentate (R100), and permeates (P100, P50, and P20). See Figure 1 for abbreviations. Means with different letters on the top of the bars were significantly different ($P < 0.001$).

low surface hydrophobicity suggests that the small peptides in the permeates had fewer hydrophobic binding sites for the ANS or that the binding sites for the probe in the peptides were modified during hydrolysis.

Protein solubility. Papain modification significantly improved protein solubility. After hydrolysis, protein solubility at pH 7.0 increased from 56% for control SPI to 89, 93, and 94% for PMSPI10, PMSPI30, and PMSPI60, respectively. The increased protein solubility could be due to smaller molecular peptides being produced by papain hydrolysis. In addition, papain hydrolysis could lead to unfolding of protein molecules. Not only nonpolar amino acid groups but also some polar amino acid groups, buried inside protein molecules, could be exposed on the surface of protein molecules after unfolding. These exposed polar amino acids may interact with water molecules through hydrogen bonds and electrostatic interactions, resulting in increased protein solubility. Improvement in protein solubility due to enzyme hydrolysis is well documented (1,18).

The protein solubility of unmodified SPI, PMSPI60, and the ultrafiltrates, as a function of pH, was also studied (Fig. 3). Hydrolysis increased the solubility of the soy protein at all pH ranges in this study. At pH 3, 5, 7, 9, and 11, the protein solubility increased from 36, 3, 56, 89, and 94% for unmodified SPI to 42,18, 94, 98, and 100% for PMSPI60, respectively. Slightly increased protein solubilities at pH 3, 9, and 11 were observed with PMSPI60 in comparison to those of unmodified SPI. At these pH values, both unmodified SPI and PMSPI60 carried negative (pH 9 or 11) and positive (pH 3) electric charges, thus contributing to solubility. The effects of smaller peptides and of exposure of hydrophilic groups on protein solubility were minor compared to those of electric charges at pH 3, 9, and 11. However, at pH 5 and 7, electrostatic repulsions of protein/peptide molecules were less than those at pH 3, 9, and 11, so protein solubility at those pH values could depend on molecular size and the presence of hydrophilic groups. Papain hydrolysis could result in smaller peptides and exposure of more hydrophilic groups. Greatly increased protein solubilities of PMSPI60 at pH 5 and 7 were observed in comparison with unmodified SPI. The solubility of peptides in the retentate R100 was similar to that in PMSPI60, and the peptides in permeates (P100, P50, and P20) were almost completely soluble (100%) at all pH ranges studied except at pH 5, where the solubility of P100 was 89%. High solubility of the permeates was expected because almost all hydrophilic groups in the small molecules were exposed to the water environment.

Emulsifying properties. Papain modification significantly ($P < 0.001$) improved the emulsifying activity index of SPI from 102 to 198, 228, and 207 m^2/g for PMSPI10, PMSPI30, and PMSPI60, respectively (Fig. 4). The lower EAI of PMSPI60, compared to that of PMSPI30, could be due to the lower surface hydrophobicity of PMSPI60. After partial hydrolysis, the protein structure and function were altered. The hydrolysate with a higher solubility and smaller molecular size might facilitate the diffusion and spread at oil–water interfaces. The exposed hydrophobic groups enhanced the interaction between proteins and lipids. High emulsifying activities for the hydrolysates were obtained. EAI of peptides in permeates (287, 309, and 269 m^2/g for P100, P50, and P20, respectively) were significantly higher than those in R100 (137 m^2/g) and PMSPI60 (207 m^2/g), and even higher than that of bovine serum albumin (243 m^2/g). High correlation between surface hy-

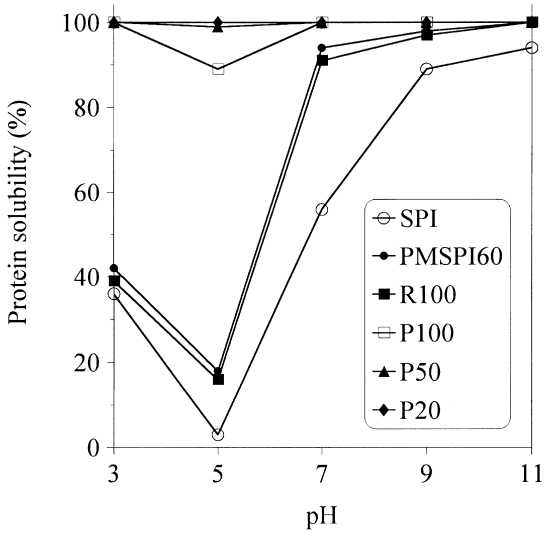

FIG. 3. Protein solubility of SPI, PMSPI60, retentate R100 and permeates P100, P50, and P20. For abbreviations see Figure 1.

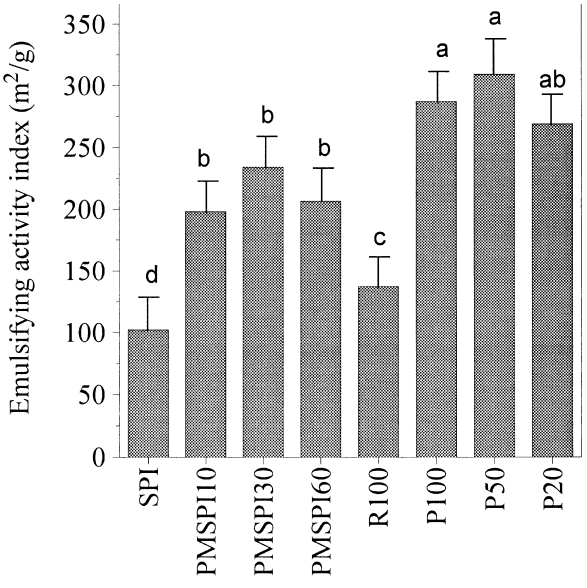

FIG. 4. Emulsifying activity index of SPI, PMSPI10, PMSPI30 and PMSPI60, retentate R100, and permeates P100, P50, and P20. Means with different letters on the top of the bars were significantly different ($P < 0.001$). For abbreviations see Figure 1.

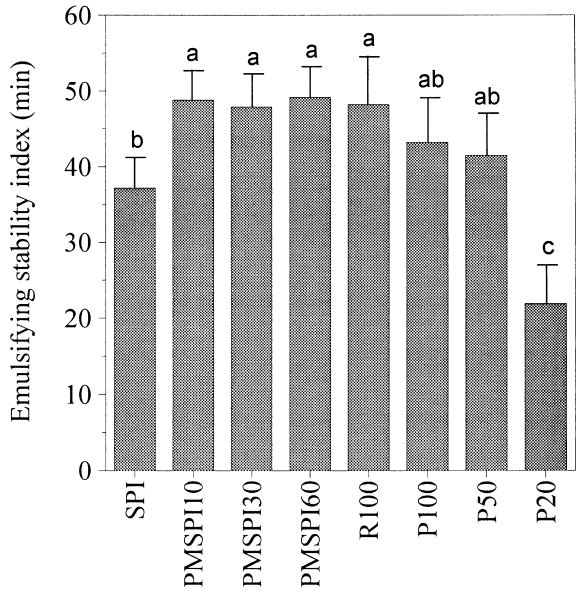

FIG. 5. Emulsion stability index of SPI, PMSPI10, PMSPI30, PMSPI60, retentate R100, and permeates P100, P50, and P20. Means with different letters on the top of the bars were significantly different ($P < 0.001$). For abbreviations see Figure 1.

drophobicity and emulsifying activity has been reported (19–24). However, Shimizu *et al.* (25) reported that there was no correlation between them. The correlation between surface hydrophobicity and EAI for the unmodified SPI, hydrolysates, and retentate was high ($r = 0.816$, $P < 0.001$), but for the unmodified SPI, hydrolysates, retentate, plus permeates it was low ($r = 0.439$, $P > 0.005$). The low surface hydrophobicity of the permeates cannot be credited for their high emulsifying activity. Hence, solubility and molecular size, rather than surface hydrophobicity, might be the major factors for the high emulsifying activity of the small peptides.

Papain modification also significantly ($P < 0.001$) improved the ESI of SPI from 37.2 to 48.0, 47.2, and 48.9 min for PMSPI10, PMSPI30, and PMSPI60, respectively (Fig. 5). The ESI of R100 was the same as that of the hydrolysates. No significant difference in ESI ($P > 0.05$) between the permeates (P100 and P50) and the retentate (R100) was found. However, the ESI of the small peptide fraction of P20 was significantly lower ($P < 0.001$) than other peptide fractions.

Papain is one of the least expensive FDA-approved vegetable-derived enzymes used in food formulation and processing. Soy protein peptides prepared by papain modification and ultrafiltration had high emulsifying properties and protein solubility and could find use in products that require these properties. Currently, we are conducting a study to investigate the use of these specific soy peptides, prepared by enzymatic modification followed by ultrafiltration, in formulating cosmetic and personal care products.

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