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ARTICLE

Structural and Emulsifying Properties of Soy Protein Isolate Subjected to Acid and Alkaline pH-Shifting Processes

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Structural unfolding of soy protein isolate (SPI) as induced by holding (0, 0.5, 1, 2, and 4 h) in acidic (pH 1.5−3.5) and alkaline (pH 10.0−12.0) pH solutions, followed by refolding (1 h) at pH 7.0, was analyzed. Changes in emulsifying properties of treated SPI were then examined. The pH-shifting treatments resulted in a substantial increase in protein surface hydrophobicity, intrinsic tryptophan fluorescence intensity, and disulfide-mediated aggregation, along with the exposure of tyrosine. After the pH-shifting processes, soy protein adopted a molten globule-like conformation that largely maintained the original secondary structure and overall compactness but lost some tertiary structure. These structural modifications, consequently, led to markedly improved emulsifying activity of SPI as well as the emulsion stability.

KEYWORDS: Soy protein isolate; pH shifting; emulsifying properties; molten globule

INTRODUCTION

Soy protein as an inexpensive functional and health-promoting ingredient is widely utilized in the formulation of food, beverage, and neutraceutical products. For example, soy protein isolate (SPI) has been included in a wide variety of formulated foods to impart stability against phase separation in food systems such as milk products, ice cream, mayonnaise, and comminuted and restructured meats (*1*). However, aside from their structure-forming properties, native soy proteins in general are of limited functionality, including emulsifying and other surface properties. Therefore, continuing efforts are required to modify native soy protein structures to a more surface-active state for a broadened functionality, which may be accomplished through physical, chemical, or enzymatic means or the combination thereof.

As with many other food protein ingredients, native SPI has the potential to decrease the interfacial tension in an O/W emulsion and prevent coalescence of lipid droplets through the formation of a physical barrier at the interface. Yet, SPI as an emulsifying agent is generally recognized to be less efficient when compared with many other food proteins or modified proteins, such as casein and whey proteins (*2*, *3*). Because surface and emulsifying properties of proteins are strongly correlated with their structure (*4*), SPI could have an improved emulsifying activity if the proteins are partially unfolded prior to emulsification. Several attempts have been made to alter the native structure of soy proteins to maximize their emulsifying capacity, for example, high-pressure or combined temperature/high-pressure treatments (*5*−*7*), exposing the proteins to extreme pH conditions (*8*), and enzymatic treatments (*9*). In particular, altering the pH of a protein solution is one of the oldest techniques employed to unfold proteins. As the pH of the medium increases or decreases from a protein's isoelectric point, an increased charge repulsion drives the protein molecules to a partially unfolded state (*10*). Results from several studies revealed that globular proteins may be partly unfolded under extreme pH conditions (usually low pH); globulins have the tendency to lose many side-chain interactions to become more flexible but yet retain a relatively intact conformation. This dynamic structure is referred to as the "molten globule" structure (*11*, *12*).

Significant functionality improvements caused by structural changes have been reported for muscle proteins and egg albumen following acid or alkali treatments (*10*, *13*, *14*). However, the impact of extreme acid and alkaline unfolding processes on soy protein conformation and functionality is not well documented. A few available studies noted that exposures of soy glycinin (*15*, *16*) and SPI (*17*) to extreme acid pH conditions resulted in protein hydrophobicity and molecule size changes and improved the foaming and emulsifying properties. However, the possible disruptions of secondary and tertiary structures of soy proteins under both extreme pH (acid, alkaline) conditions have not been fully investigated. For example, it remains unclear how the exposure of SPI to extreme alkali pH for various times followed by adjustment of the pH to neutrality (i.e., pH shifting) would affect its protein conformation and surface properties.

In the present study, we investigated the effect of pH-shifting treatments on the conformation of soy proteins and determined how the conformational changes related to emulsifying properties. In particular, SPI was treated with a series of acid pH values (1.5, 2.5, 3.5) and alkaline pH values (10.0, 11.0, 12.0) to induce a molten globular structure, followed by refolding upon adjustment of the pH to 7.0. Samples subjected to these pH-shifting treatments were analyzed for protein solubility, surface properties (hydrophobicity and exposures of sulfhydryl, tryptophan, and

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tyrosine residues), secondary structures (circular dichroism), and disulfide bond aggregation. Emulsifying properties of treated protein samples were subsequently measured, and their relationship with altered protein structures was described.

MATERIALS AND METHODS

SPI Preparation. SPI was prepared from Taiwan 292 soybeans (harvested in 2007). Soybeans were first milled. After removal of the hull, the flour was treated with *n*-hexane/ethanol (10:1, v/v) to extract oil, and the extraction was repeated twice. The defatted meal was dispersed in distilled water (1:10, w/v) and adjusted to pH 8.0. The dispersion was stirred for 2 h to extract protein and then centrifuged at 13500*g* for 30 min at 4 °C. The supernatant was adjusted to pH 4.5 with 2 M HCl and centrifuged at 3300*g* for 20 min. The pellet was washed twice with distilled water, each done by suspension in 5-fold (w/w) distilled water followed by centrifugation at 8000*g* for 10 min. Thereafter, the protein pellet was resuspended in 5-fold (w/w) distilled water and neutralized to pH 7.0 with 2 M NaOH. The samples were freeze-dried and stored in a 2 °C cooler. Protein content in the prepared SPI powder was 95% (w/w) as determined with the biuret method (*18*) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

pH-Shifting Process. Lyophilized SPI (9 g) was suspended in 300 mL of distilled water and stirred with a magnet bar for 30 min to a uniform dispersion. The dispersion was centrifuged at 12000*g* for 15 min at 4 °C. The supernatant was diluted to the concentration of 20 mg/mL. The protein solution was adjusted to either low pH values (pH 1.5, 2.5, or 3.5) with 2 M HCl or high pH values (pH 10.0, 11.0, or 12.0) with 2 M NaOH and then held at the specific pH for 0, 0.5, 1, 2, and 4 h at 20 ± 2 °C to induce unfolding (*14*). Finally, the protein dispersions were neutralized to pH 7.0 and held for 1 h to induce refolding. The ionic strengths, expressed as the concentration of NaCl of the acid and alkaline pH-shifting-treated SPI solutions, determined using an S30 SevenEasy conductivity meter (Mettler Toledo GmbH Analytical, Sonnenbergstrausse, Switzerland), were 0.01−0.02 and 0.03−0.04 M, respectively.

Protein Solubility. Treated and nontreated (control) SPI dispersions (20 mg/mL) refolded at pH 7.0 were centrifuged at 12000*g* for 15 min at 4 °C. Soluble protein content in the supernatant was determined with the biuret method. Total protein was also determined with the Biuret method. Protein solubility was calculated as the percent distribution of protein in the supernatant over the total protein content in the dispersion.

Surface Free Sulfhydryl Group. The sulfhydryl group of protein was determined according to the method of Beveridge (*19*). Control and pH-shifting-treated SPI solutions (20 mg/mL) were diluted with distilled water to 4 mg/mL. After reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co.) for 15 min, absorbance at 412 nm was recorded against a blank. A molar extinction coefficient of 13600 M^{-1} cm^{-1} was used for the conversion of absorbance to sulfhydryl concentration. Reagent blank and sample blank were prepared to correct for the color from reagents and protein solution.

Surface Hydrophobicity. Surface hydrophobicity was measured using the 1-anilino-8-naphthalenesulfonate (ANS) (Sigma Chemical Co.) fluorescence probe (*20*). Because the fluorescence intensity (FI) is directly proportional to soybean protein concentration in the range from 0.005 to 0.5 mg/mL, as determined from our preliminary trial, control and each pH-shifting-treated SPI sample were diluted with 0.01 M phosphate buffer (pH 7.0) to yield final concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL before reaction with ANS. FI was measured with an emission wavelength of 484 nm and an excitation wavelength of 365 nm (both with a slit width 5 nm) on a Hitachi 650-60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). The initial slope of the FI versus protein concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity.

Intrinsic Tryptophan Fluorescence. The protein concentration of control and pH-shifting-treated SPI samples was diluted to 1 mg/mL in a 10 mM phosphate buffer at pH 6.2. Tryptophan fluorescence was measured with a Hitachi 650-60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) at a 295 nm excitation wavelength (slit width 5 nm) and a 300−400 nm emission wavelength (slit width = 5 nm) at a 10 nm/s scanning speed. The phosphate buffer used to dissolve soy protein was used as blank solution for all samples.

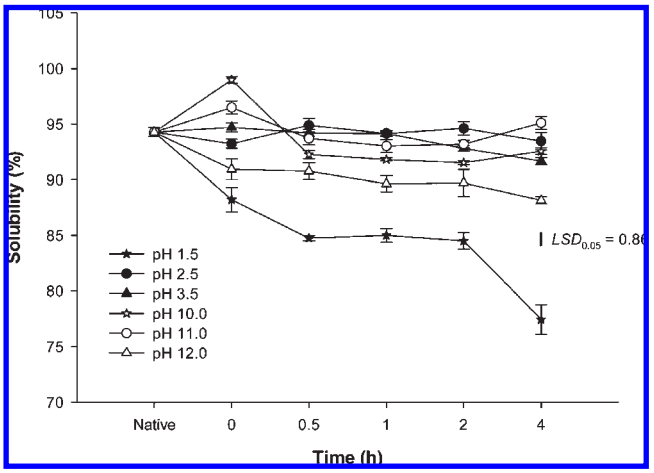

Figure 1. Solubility of soy protein isolate (SPI) after low pH (1.5, 2.5, 3.5) or high pH (10.0, 11.0, 12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

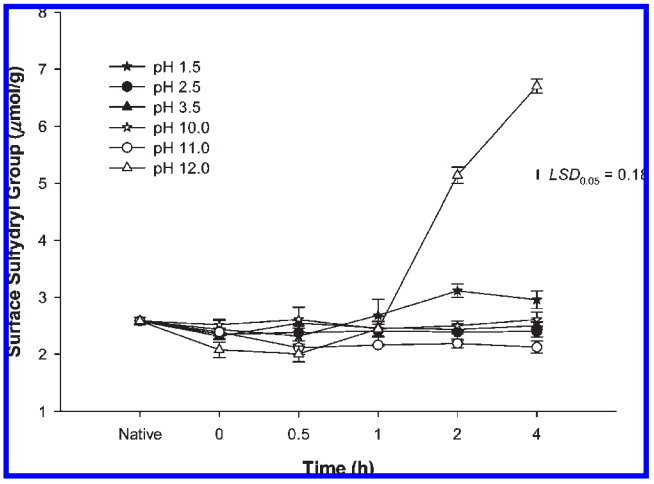

Figure 2. Surface free sulfhydryl group content of soy protein isolate (SPI) after low pH (1.5, 2.5, 3.5) and high pH (10.0, 11.0, 12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

UV Absorption Spectra. Both zero-order and second-derivative ($dA^2/d\lambda^2$) UV spectra (275−310 nm) of control and pH-shifting-treated soy protein samples, after dilution to 1 mg/mL in phosphate buffer (10 mM, pH 6.2), were obtained with a Varian Cary 50 UV−vis spectrophotometer (Varian Inc., Palo Alto, CA) at room temperature. Phosphate buffer used to dissolve protein was used as blank solution for all samples.

Circular Dichroism (CD). Conformational changes in the secondary structure of pH-shifting-treated protein samples were analyzed using a model Mos-450 CD spectropolarimeter (Biologic, Claix, France) at 25 °C. Secondary structure determination was performed by scanning diluted protein samples (50 μg/mL) between 200 and 260 nm. Deionized water was used as blank solution for all samples. The values of scan rate, response, bandwidth, and step resolution were 100 nm/min, 0.25 s, 1.0 nm, and 0.2 nm, respectively. Five scans were averaged to obtain one spectrum. The molar ellipticities of protein samples were calculated as $[\theta]$ (deg cm^2 $dmol^{-1}$) = $(100 \times X \times M)/(L \times C)$, where X is the signal (millidegrees) obtained by the CD spectrometer, M is the average molecule weight of amino acid residues in the protein (assumed to be 115), C is the protein concentration (mg/mL) of the sample, and L is the cell path length (cm).

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the procedure of Liu and Xiong (*21*). The stacking gel and the resolving gel contained 3 and

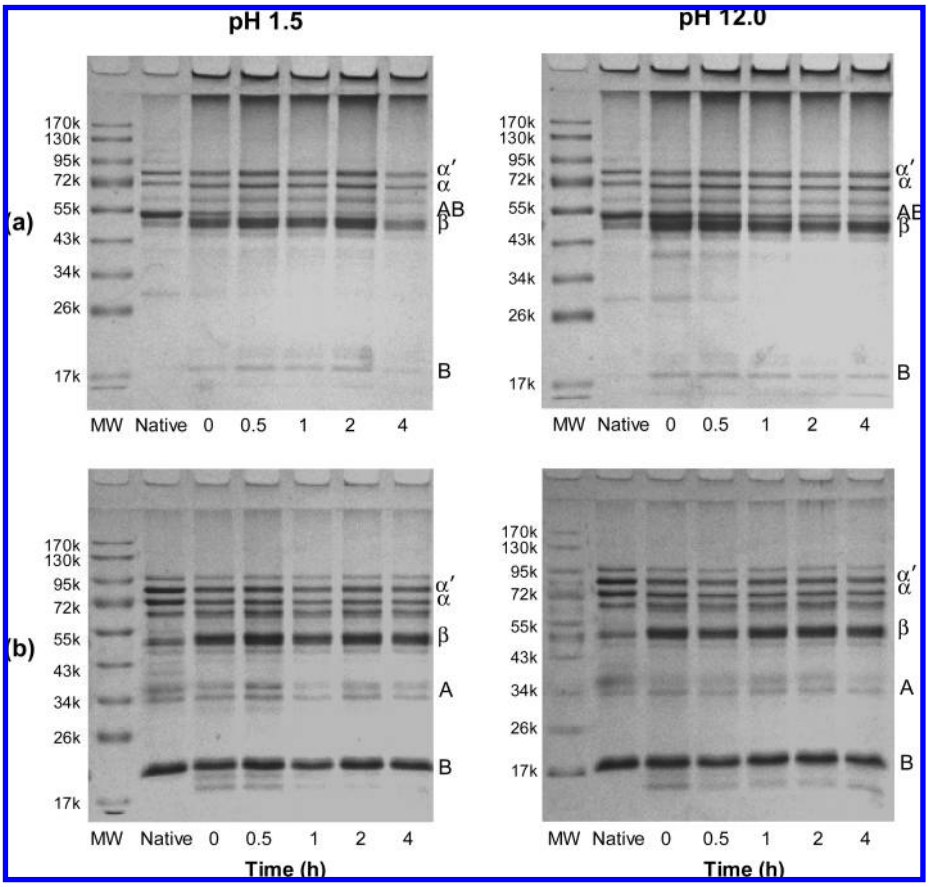

Figure 3. SDS-PAGE pattern of soy protein isolate (SPI) after low pH (1.5) and high pH (12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment. The SDS-PAGE samples were without β-mercaptoethanol (row a) or treated with 5% β-mercaptoethanol (row b). MW: molecular weight marker (Da). SPI constituents: α' (86 kDa), α (66 kDa), and β (51 kDa) for conglycinin; A, the acidic subunit (34−43 kDa); B, the basic subunit (17−26 kDa) for glycinin.

12% acrylamide, respectively. SDS-PAGE samples were prepared with and without 5% β-mercaptoethanol. For samples without β-mercaptoethanol, 1 mM *N*-ethylmaleimide was added to prevent formation of disulfide artifacts during sample preparation.

Emulsifying Properties. Control and pH-shifting-treated SPI solutions at pH 7.0 were diluted with distilled water to a concentration of 10 mg/mL. Emulsions were prepared by homogenization of 20 mL of the mixture of soybean oil and the protein solution (1:3, v/v) at 13500 rpm for 2 min using an Ultra-Turrax homogenizer (Ika T18 Basic, Staufen, Germany). The emulsions were immediately transferred into 25 mL capacity beakers (diameter = 3.8 cm) and allowed to stand at 20 ± 2 °C for exactly 1 min (for emulsifying activity testing) or 30 min (for emulsion stability testing) before sampling.

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined according to the turbidometric method of Pearce and Kinsella (*22*). For EAI measurement, aliquots of freshly prepared emulsions (20 μL) were taken from the bottom of the beakers exactly 1 min after emulsion preparation and diluted with 5 mL of 0.1% SDS (a 251 dilution factor). Absorbance (optical density) at 500 nm was read. EAI was calculated as

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{4.606 \times A_{500}}{C \times (1-\phi) \times 10^4} \times \text{dilution factor}$$

where C is the protein concentration (g/mL) before emulsification and ϕ is the oil volume fraction (v/v) of the emulsion.

For ESI measurement, the emulsions prepared as described above were kept undisturbed at 20 ± 2 °C. Aliquots were taken from the bottom of the beakers exactly 30 min after emulsion preparation, diluted, and measured for absorbance at 500 nm as described above. ESI was defined as: ESI (m^2/g)=(A_t/A_0)×100, where A_t and A_0 represent the absorbances at 30 min and at time zero, respectively.

Statistical Analysis. Unless specified otherwise, all experiments were repeated three times each with a new batch of SPI preparation. In each repeated trial, duplicate or triplicate samples were analyzed. Data were processed using a one-way SPSS program (SPSS Statistical Software, Inc., Chicago, IL). Significant differences ($P < 0.05$) between means were identified using the least significance difference (LSD) procedure.

RESULTS AND DISCUSSION

Protein Solubility. The solubility of native and pH-shifting-treated SPI samples is shown in **Figure 1**. The solubility of native SPI was 94%. When the protein solution was titrated to pH 1.5 and immediately adjusted back to pH 7.0 (pH 1.5 → 7.0), the solubility dropped to 87.5% ($P < 0.05$). Holding the SPI at the extremely low pH further reduced the protein solubility, to 77% after 4 h. A similar trend in protein solubility reduction was observed for samples treated with extremely high pH (12.0), although the magnitude of reduction was less ($P < 0.05$) than that for the pH 1.5 shifting treatment. Samples treated at other pH values (2.5, 3.5, 10.0 and 11.0) overall did not exhibit appreciable solubility changes. Adjustment of the pH of the SPI solution to 10.0 slightly improved protein solubility ($P < 0.05$), apparently due to a stronger hydrogen bond formed between charged proteins and water, but the solubility gain diminished with holding time. The solubility decrease by extremely low or high pH-shifting treatments indicated that soy proteins were susceptible to denaturation (unfolding → hydrophobic aggregation) under these conditions. Moreover, as described later, disulfide cross-linking also contributed to the loss in protein solubility for samples subjected to the extremely low or high pH-shifting treatments.

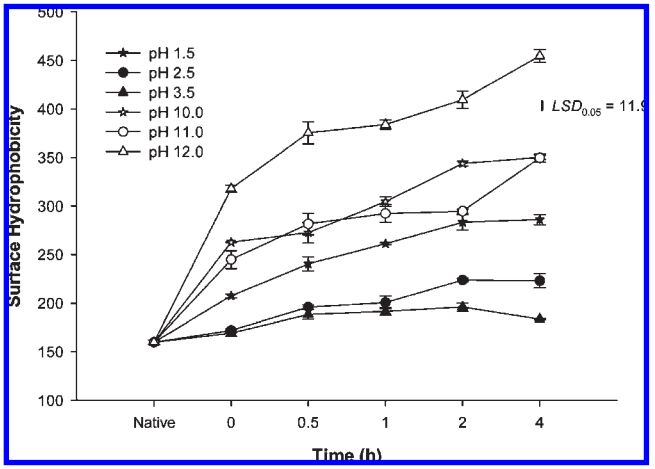

Figure 4. Surface hydrophobicity of soy protein isolate (SPI) after low pH (1.5, 2.5, 3.5) and high pH (10.0, 11.0, 12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

Surface Free Sulfhydryls (SH). The content of exposed SH groups in control SPI samples was 2.5 μmol/g, which was not affected by the pH-shifting treatments at pH 2.5, 3.5, 10.0, and 11.0. On the other hand, adjustment of the protein solution to pH 12.0 slightly lowered the amount of detectable SH (**Figure 2**), probably due to the formation of disulfide bonds via SH oxidation and SH/SS interchange reactions. Thiol groups tend to be more reactive via deprotonation to form mercaptide ion species (S^-) as the alkali pH rises, and SH oxidation also accelerates in an alkaline pH environment (*23*, *24*). However, incubation of SPI at pH 12.0 for more than 1 h before adjustment of the pH back to 7.0 resulted in substantial increases ($P < 0.05$) in the free SH content, up to nearly 3-fold when compared with the control (**Figure 2**). Intramolecular disulfide bonds might be broken after holding at the extreme alkali pH (*25*), and glycinin, which contains 48 mol of half-cysteine mol^{-1} of proteins, was likely involved in the SH change. A slightly increased amount of SH was also detected in samples held at pH 1.5 for more than 1 h followed by adjustment to pH 7.0. Acid-induced cleavage of disulfide bonds may be responsible for such an increase (*26*).

SDS-PAGE. Electrophoresis of SPI samples treated with or without β-mercaptoethanol (a disulfide bond breaking agent) was performed to determine the role of S−S linkages in the formation of protein aggregates in samples incubated under acid and alkaline pH conditions. As shown in **Figure 3** (row a), under nonreducing conditions (no β-mercaptoethanol), extremely large proteins that accumulated on the top of the stacking gel as well as the resolving gel appeared in both low (pH 1.5) and high (pH 12.0) pH-shifting-treated samples. Samples treated with intermediate pH shifting exhibited similar but less intense protein aggregation (data not shown). These high molecular weight (MW) bands, which were almost absent in control (native) samples, were covalent polymers and aggregates from soy globulins. Furthermore, the AB complex of glycinin gradually diminished during incubation, suggesting that glycinin was the precursor of those high MW polymers. The disappearance of the AB complex was rapid in the pH 1.5 samples and gradual in the pH 12.0 samples. On the other hand, the β-conglycinin subunits (α', α, and β) showed but unremarkable changes during incubation. Petruccelli and Añón (*27*) reported that at pH 9−10, the β subunit from 7S globulins and the B subunit from 11S globulins formed aggregates.

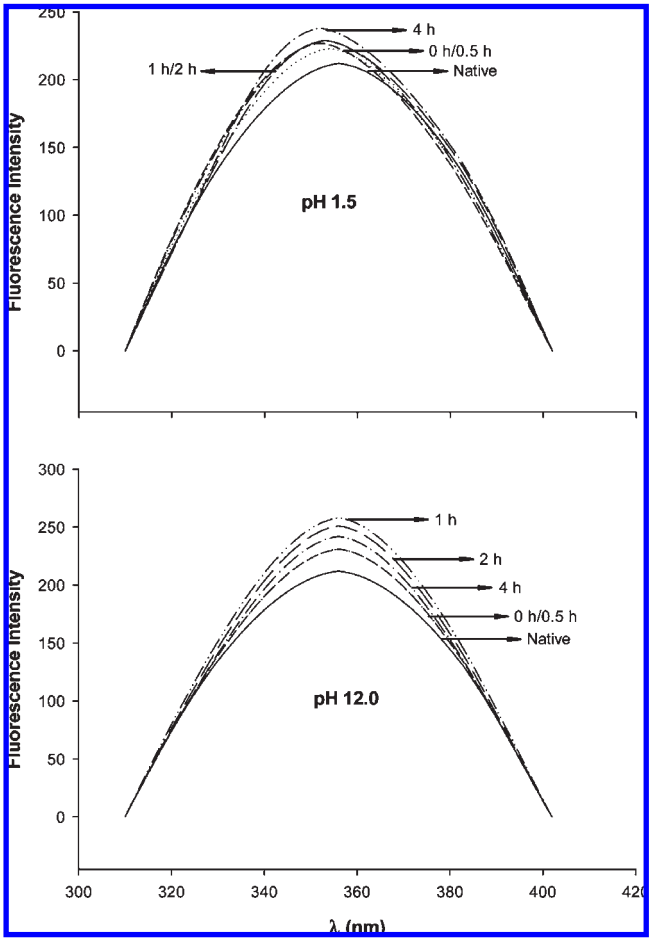

Figure 5. Tryptophan fluorescence of soy protein isolate (SPI) after low pH (1.5) and high pH (12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

When β-mercaptoethanol was applied to the pH-shifting-treated samples, the large MW polymers and aggregates were completely dissociated into the constituting A and B subunits of glycinin (**Figure 3**, row b). The recovered basic subunit of glycinin (B) appeared as a rather intense band. All samples, irrespective of the specific treatment pH, showed an identical electrophoretic pattern. These results demonstrated that disulfide cross-linking of glycinin and, to a lesser extent, β-conglycinin, was responsible for the production of protein polymers and aggregates in the pH-shifting and holding process, which also explained the decline in protein solubility. Because both intramolecular and intermolecular SH/SS interchanges are dynamic and are expected in the observed SPI polymer formation, whether such reactions would result in less or more SH groups being detected would depend on many factors, such as the holding time, the pH, and the transitional state of the protein structure.

Surface Hydrophobicity and Tryptophan Fluorescence. Determined using the ANS fluorescent probe, surface hydrophobicity of treated SPI samples, except those at pH 2.5 and 3.5, was found to be significantly higher ($P < 0.05$) than that of the control (**Figure 4**). The hydrophobicity further increased with holding time at the acid pH values of 1.5 and 2.5 and at all of the alkaline pH values (10.0, 11.0 and 12.0). However, the increase was less for acid pH when compared with the alkaline pH treatments. Although not specifically measured, SPI typically contains 1.75−2% phytic acid (*28*). It has been shown that treatment of

Table 1. Effect of pH-Shifting Treatments on the Intrinsic Fluoresence of SPI[a]

			acidic pH						alkaline pH					
	native		pH 1.5		pH 2.5		pH 3.5		pH 10.0		pH 11.0		pH 12.0	
holding time (h)	FI [b]	λ_{max} (nm)	FI	λ_{max} (nm)	FI	λ_{max} (nm)	FI	λ_{max} (nm)	FI	λ_{max} (nm)	FI	λ_{max} (nm)	FI	λ_{max} (nm)
0	212	356	223	354	221	355	218	356	221	354	221	356	231	356
0.5	212	356	227	352	223	355	217	356	220	355	225	356	231	356
1	212	356	229	353	223	354	219	355	220	355	225	356	258	356
2	212	356	229	353	223	354	219	355	220	355	225	354	251	356
4	212	356	238	352	227	354	219	356	220	355	230	355	242	356

[a] pH shifting was performed by holding the SPI solutions (20 mg/mL) at the designated pH for the specified time periods followed by adjustment to pH 7.0. [b] Fluoresence intensity.

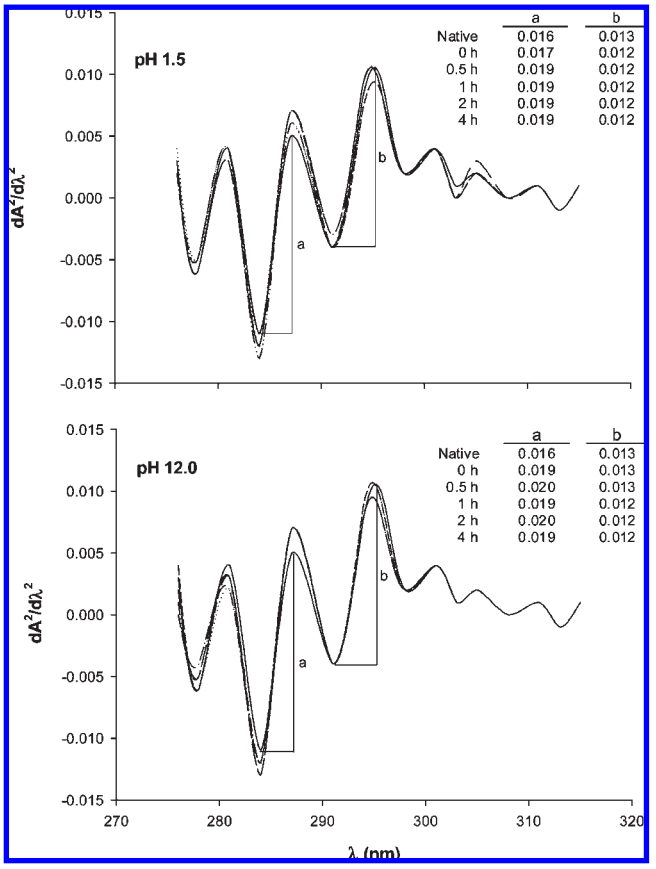

Figure 6. Second-derivative UV spectra of soy protein isolate (SPI) after low pH (1.5) and high pH (12.0) unfolding treatments followed by refolding at pH 7.0. The insets show changes in the "a" and "b" values as a function of holding time at the specific pH. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

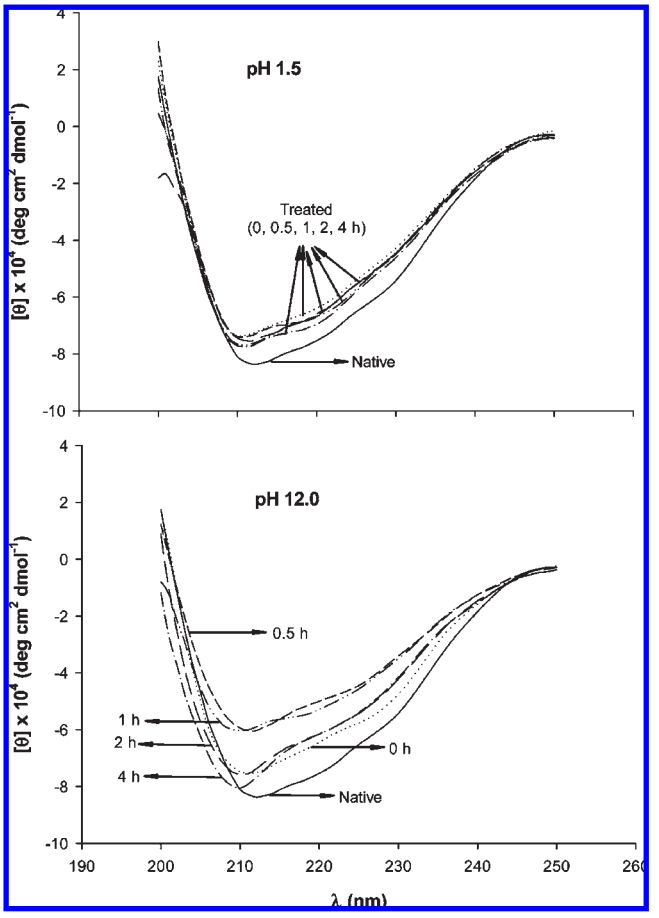

Figure 7. Circular dichroism spectra of soy protein isolate (SPI) after low pH (1.5) and high pH (12.0) unfolding treatments with various holding times followed by refolding at pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

SPI with alkaline solutions causes the dissociation of phytic acid (*29*). Thus, it is tempting to suggest that the removal of the highly negatively charged acid probably contributed to the increased hydrophobicity of the samples treated with alkaline pH. It was also noticeable that the hydrophobicity increased with the decrease of acid pH or the increase of alkaline pH. Because the ANS fluorescence intensifies in a more hydrophobic environment, the pH-shifting-induced FI enhancements were strong evidence of protein unfolding. That is, it must have resulted from the exposure of hydrophobic side-chain groups that were originally occluded in the interior of the compact globular region of native soy proteins, an indication of change of tertiary structure. The results suggested that extreme alkaline pH-shifting treatments were more capable of inducing soy protein conformational changes than acid pH treatments.

Changes in soy protein structure could be reflected by the movement of side chains. After pH-shifting treatments, some previously buried side chains in native SPI globulins could be exposed to the polar surface. This will alter the intrinsic fluorescence of tryptophan residues as they are particularly sensitive to the polarity of microenvironments during the transition (*30*). As depicted in **Figure 5**, which included only pH 1.5 and 12.0 as examples, the pH-shifting treatments involving either the extremely low or high pH indeed changed the intensity of fluorescence attributed to tryptophan excitation and emission. The λ_{max} for emission maximum of the control SPI sample (356 nm) exhibited blue shifts after acid pH treatments (to as low as 352 nm) but barely any change upon alkaline pH treatments (**Table 1**). The FI increased from 212 (native SPI) to 217−238 for acid pH treatments and to 220−242 after alkaline pH treatments. These results

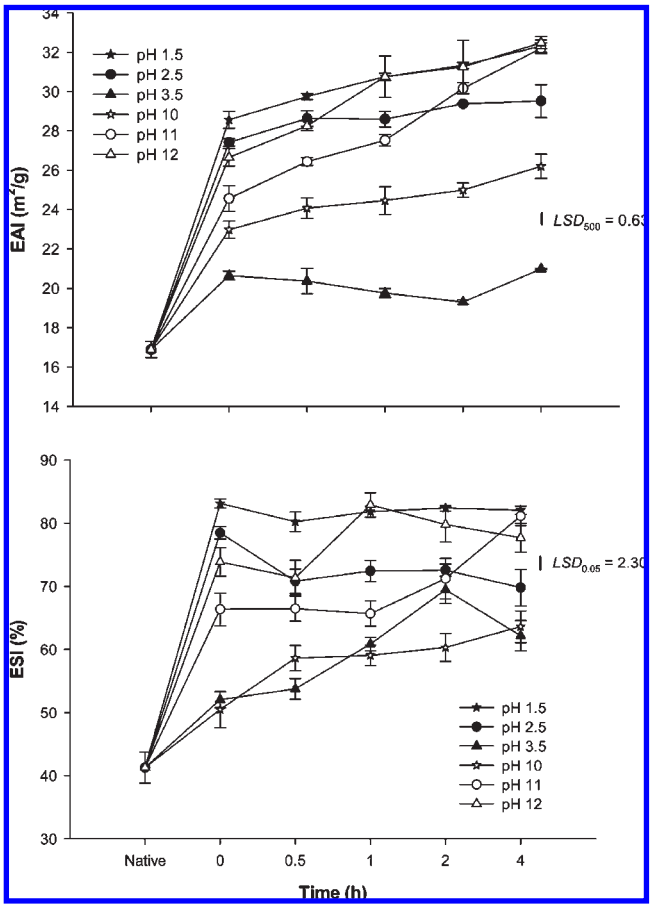

Figure 8. Emulsifying activity index (EAI) and emulsion stability index (ESI) of soy protein isolate (SPI) after low pH (1.5, 2.5, 3.5) and high pH (10.0, 11.0, 12.0) unfolding treatments with various holding times followed by refolding to pH 7.0. Native: control SPI solutions at pH 7.0 without pH-shifting treatment.

were indicative of conformational changes of soy globulins by the pH-shifting treatments.

The tryptophan fluorescence results were largely consistent with that of surface hydrophobicity measurement with the ANS fluorescence probe. For example, at acid pH, the FI change was less sensitive to incubation time when compared with that at alkaline pH. The difference may be explained by the alkaline pH values tested being farther away from the isoelectric points of most soy globulins (pH ~4.5). The protein structural changes seemed to be dynamic. Whereas tryptophan FI of SPI with unfolding pH 1.5, 2.5, 3.5, 10.0, and 11.0 all changed with incubation time, the FI of samples treated at pH 12.0 increased most remarkably after 1 h of holding and then declined afterward. Some soy globulins or their subunits might aggregate after long-time incubation at the extremely high pH. The result differed from that of the ANS fluorescence test, which showed a continuing rise in surface hydrophobicity of SPI upon pH 12.0 treatment (**Figure 4**). The difference can be attributed to the exposures of other hydrophobic residues when tryptophan residues were occluded.

UV Spectra. Second-derivative spectroscopy has been used to resolve the complex protein spectral transitions in the near-ultraviolet region and to delineate the contributions of the three aromatic amino acid residues (*31*−*33*). The second-derivative spectrum of SPI between 280 and 300 nm gave rise to two maxima at 287 and 296 nm and two minima at 284 and 291 nm (**Figure 6**). The peak at 296 nm can be assigned to tryptophan alone (*34*, *35*). Compared with the control samples, the second-derivative peak at 296 nm in pH-shifting-treated samples (pH 1.5 and 12.0) underwent a blue shift of 1−2 nm. Furthermore, whereas the "b" value of the transition peaks essentially remained unchanged, the "a" value became larger upon incubation; samples treated at the two extreme pH conditions showed no difference. The ratio of a/b increased substantially in all pH-shifting-treated SPI samples; for example, the value increased from 1.23 (control) to 1.58, 1.38, 1.31, 1.46, 1.50, and 1.58 after 4 h unfolding at pH 1.5, 2.5, 3.5, 10.0, 11.0, and 12.0, respectively. The ratio increase was attributed to the change in the three-dimensional positions and the movement of tyrosine residues from hydrophobic regions during the unfolding of the tertiary structure (*35*). The second-derivative UV results were in good agreement with those from the ANS and tryptophan fluorescence analyses, all indicative of structure unraveling when SPI was subjected to extreme pH-shifting processes.

CD Spectra. Circular dichroism scanning was done to determine the influence of pH shifting on the secondary structure of soy proteins, and the results from pH 1.5 and 12.0 are shown in **Figure 7**. The CD spectra displayed a negative band near 210 nm representing the α-helix conformation (*36*). The CD spectra of SPI from pH 1.5 → 7.0 shifting exhibited only slight changes; for samples treated at pH 12.0, the changes were variable and inconsistent. The small attenuation of the 210 nm negative peak and the blue shift of the peak wavelength suggested slight losses in the content of α-helical structure. The CD spectra for pH 1.5 samples did not change during incubation, indicating that holding time was not a main factor affecting secondary structure. Kristinsson and Hulin (*10*) also noted that unfolding time (up to 24 h) at low or high pH had a negligible effect on the CD spectra of cod myosin. The secondary α-helix conformation appeared to be undisrupted by pH-shifting treatments, and this has been noted for proteins in the molten globule state (*37*).

Emulsifying Properties. Both the acid and alkaline pH-shifting treatments markedly improved the emulsifying activity of SPI, and the EAI also increased ($P < 0.05$) with holding time except for samples treated at pH 3.5 (**Figure 8**). Thus, pH-shifting-treated soy proteins were more capable of forming an interfacial membrane, thereby facilitating the dispersion of oil droplets when compared with native proteins. The result was not surprising because an increased exposure of hydrophobic amino acid side chain groups due to partial structural unfolding in treated SPI samples, as described earlier, would render proteins with greater surface activity. The finding was consistent with that reported by Wagner and Guéguen (*15*), who noted that the foaming and emulsifying properties of soybean glycinin could be greatly improved by acid treatments. The EAI of the acid-pH-treated samples increased ($P < 0.05$) with decreasing pH and that of the alkaline-pH-treated samples increased ($P < 0.05$) with increasing pH (**Figure 8**). Holding time seemed to be quite beneficial for samples under the high pH (12.0) treatment and marginally advantageous for samples under low pH-shifting treatments.

The pH-shifting treatments also initiated substantial improvements of the stability of prepared emulsions (**Figure 8**). Similar to EAI, SPI samples treated with strongly acidic or alkaline unfolding pH, even without any holding time, were of high efficacy in stabilizing emulsion droplets. For samples treated at pH 1.5, 2.5, and 12.0, the substantial gain in ESI (up to 2-fold over the control sample) from the pH-shifting treatments was not further increased by holding. However, the stability of the emulsions prepared with pH 3.5, 10.0, and 11.0 treated SPI was enhanced ($P < 0.05$) when the holding time of the pH treatments was prolonged. The EAI of samples with pH 1.5 and 12.0 unfolding treatments were most improved because intramolecular repulsions

between like charges would lead to an unfolded state. The extreme pH induced structural changes could produce a relatively extended conformation and greater peptide chain flexibility, and the process seemed to be difficult to completely reverse upon subsequent refolding treatment at pH 7.0. Molecular flexibility and limited protein aggregation at the interface are important contributors to O/W emulsion stability (*20*). On the other hand, due to their smaller oil droplet size (indicated by the greater EAI values), emulsions stabilized by pH 1.5 and 12.0 treated proteins would have a reduced tendency to cream when compared with other emulsions. The observed improvements of emulsifying activity and emulsion stability of pH-shifting-treated SPI samples echoed the changes in surface activity of proteins as determined by surface hydrophobicity, intrinsic fluorescence, and UV absorption measurements. These findings agreed with previous papers describing the positive relationship between protein emulsifying activity and protein structural properties (*5*, *7*, *38*).

Overall, structural and surface property analyses and protein cross-linking measurement led to the general conclusion that treatment of SPI with extremely low pH (1.5) or high pH (12.0) induced significant protein unfolding. The structural changes appeared to primarily involve tertiary structure and could not be completely reversed with refolding treatment at pH 7.0, thus producing a "molten globule" structure. Whereas emulsifying properties were improved after treatments at all pH levels tested, unfolding pH values of 1.5 and 12.0 produced structures that were clearly more surface-active and more capable of stabilizing emulsion droplets than untreated SPI controls.

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