JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

DOI:10.1021/jf101045b

pH Shifting Alters Solubility Characteristics and Thermal Stability of Soy Protein Isolate and Its Globulin Fractions in Different pH, Salt Concentration, and Temperature Conditions

JIANG JIANG,[†,‡] YOULING L. XIONG,*[,‡] AND JIE CHEN[†]

[†]State Key Laboratory of Food Science and Technology and School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China, and [‡]Department of Animal and Food Sciences, University of Kentucky, Lexington, Kentucky 40546

Soy protein isolate (SPI), β-conglycinin (7S), and glycinin (11S) were subjected to pH-shifting treatments, that is, unfolding at pH 1.5 or 12.0 followed by refolding at pH 7.0, to induce molten globule structures. Treated samples were analyzed for protein solubility, thermal stability, and aggregation in 0, 0.1, and 0.6 M NaCl solutions at pH 2.0−8.0. The pH_{12} shifting resulted in drastic increases (up to 2.5-fold) in SPI solubility in the pH 6.0−7.0 range, especially at 0 M NaCl. The $pH_{1.5}$ shifting had a generally lesser effect on solubility. 11S exhibited a solubility pattern similar to that of SPI, but the solubility of 7S was unaffected by pH shifting except at 0.6 M NaCl. The pH shifting, notably at pH 12.0, produced soluble, disulfide-linked polymers from 11S and reduced ($P < 0.05$) its enthalpy but not its temperature of denaturation. Soy proteins structurally altered by pH shifting had a reduced sensitivity to thermal aggregation.

KEYWORDS: Soy protein isolate; β-conglycinin; glycinin; pH shifting; solubility

INTRODUCTION

Many globular proteins when exposed to extreme pH conditions undergo significant conformational changes. These proteins, referred to as molten globule (MG) state, maintain a relatively compact structure (i.e., retention of most secondary structure) but tend to lose some of the tertiary structure (*1*). It has been demonstrated that incubation of myosin (*2*), egg albumin (*3*), and hemoglobin (*4*) at extreme acidic or alkaline pH followed by readjustment of the pH back to neutrality, a process known as pH shifting, induces the MG state of the respective proteins. In the MG state, proteins show improved functional properties, notably emulsifying and foaming activities.

A few available studies have described the influence of extreme pH values on the structure and surface properties of soy glycinin (*5*, *6*) and soy protein isolate (SPI) (*7*). In our previous study (*8*), we demonstrated that extreme pH-shifting treatments could induce MG structure in soy proteins, leading to improved emulsifying properties. Because native SPI is a complex structure, it is of interest to understand how different subunits, particularly β-conglycinin (7S) and glycinin (11S), would respond to pH-shifting treatments. For example, the influence of pH shifting on the solubility and thermal properties of SPI and its main components, 7S and 11S globulins, in different pH, ionic strength, and temperature environments warrants investigation.

A notable feature of soy proteins is the strong pH and ionic strength dependence of the molecular conformation and the associated functional properties (*9*−*12*). Denaturation temperatures depend strongly on pH and ionic strength (*13*). The onset denaturation temperature in deionized water is around 80−90 °C for soy 11S globulin and 60−70 °C for 7S globulin; the presence of salt increases the denaturation temperatures (*14*). On the other hand, a good solubility generally correlates with "optimum" gelling, emulsifying, and foaming properties.

It has been reported that soy 7S globulin is sensitive to heating at higher ionic strengths, forming aggregates directly, whereas 11S globulin is sensitive at lower ionic strengths, dissociating to subunits that form aggregates partly (*15*). Because pH-shifting-treated soy proteins have undergone considerable changes in tertiary structures (*6*, *8*), it is conceivable that both 7S and 11S globulins in SPI after exposures to extreme pH values become more (or less) susceptible to salt and temperature. In screening soy proteins for potential applications, their solubility is often used as a marker. Thus, a clear understanding of the solubility profiles of pH-shifting-treated soy proteins over a broad range of pH and ionic strengths as well as their sensitivity to temperature is important for the food and beverage industry.

The substantial changes in protein structure and exposures of hydrophobic groups of SPI after extreme acid (pH 1.5) and alkaline (pH 12.0) pH-shifting processes, as observed in our previous investigation (*8*), led us to hypothesize that SPI components would have solubility profiles and thermal properties different from their native (untreated) controls. The overall objective of the present study was to prepare structurally modified SPI for an improved solubility in certain ionic strength and pH conditions of food products. Accordingly, native SPI was subjected to pH shifting and then suspended in aqueous solutions at different ionic strengths (0, 0.1, and 0.6 M NaCl) and pH values (2.0−8.0). Protein solubility and thermal aggregation were determined. To establish the relative contributions of SPI

*Corresponding author [telephone (859) 257-3822; fax (859) 257-5318; e-mail ylxiong@uky.edu].

© 2010 American Chemical Society
Published on Web 06/04/2010

subunits, the solubility of isolated 7S and 11S globulins was also evaluated.

MATERIALS AND METHODS

SPI, 7S, and 11S Preparation. SPI was prepared from defatted soy flour as described previously (*8*). Briefly, dehulled, milled soybean flour was treated with *n*-hexane/ethanol (10:1, v/v) to extract oil, and the extraction was repeated twice. SPI was obtained by alkaline treatment (pH 8.0) of the defatted flour and subsequent precipitation at the isoelectric point (pH 4.5). The 7S and 11S fractions were prepared according to the method of Ruíz-Henestrosa (*16*). Protein samples were freeze-dried and stored at 2 °C. Protein contents in SPI (92%), 7S (97%), and 11S (95%) powders were determined according to the biuret method using a Kjeldahl protein concentration calibrated SPI as standard.

pH-Shifting Process. Lyophilized SPI, 7S, and 11S were suspended in nanopure water to obtain a 30 mg/mL protein concentration and stirred at room temperature (21 ± 1 °C) with a magnet bar for 30 min to uniform dispersions. The dispersions were centrifuged at 12000*g* for 15 min to remove any particulates. The supernatant was diluted to a protein concentration of 20 mg/mL and then titrated to either low pH (1.5) with 2 M HCl or high pH (12.0) with 2 M NaOH. The solutions at these extreme pH values were held for 1 h at room temperature to induce unfolding, subsequently neutralized to pH 7.0, and held for 1 h to induce refolding (*8*). The ionic strengths (expressed as molar concentration of NaCl) in the final acid and alkaline pH-shifting-treated protein solutions, as determined using an S30 SevenEasy conductivity meter (Mettler Toledo GmbH Analytical, Sonnenbergstrausse, Switzerland), were found to be minimal (< 0.03). The pH-shifting-processed samples were freeze-dried and kept at 4 °C before use.

Solubility. The solubility of pH-shifting-treated samples (SPI, 7S, 11S) in comparison with their respective controls was investigated at three ionic strengths (0−0.6 M NaCl) and nine pH levels (2.0−8.0). Specifically, sample proteins were suspended (20 mg/mL) in a 10 mM sodium phosphate buffer (pH 7.0). Aliquots of the protein suspensions were then diluted to a final concentration of 5 mg/mL with 50 mM sodium citrate buffers (pH 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5) or 50 mM sodium phosphate buffers (pH 7.0 and 8.0), each containing 0, 0.1, or 0.6 M NaCl. The final pH of these protein solutions was readjusted, if necessary, to the specific target values. The solutions were centrifuged at 5000*g* for 15 min at 21 °C. Protein concentration in the supernatants and in the whole suspensions was determined according to the biuret method as described above. Solubility was calculated as the percent distribution of protein in the supernatant over the total protein content in the dispersion.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Liu and Xiong (*17*) under nonreducing conditions. The stacking gel and the resolving gel contained 5 and 12% acrylamide, respectively. SDS-PAGE samples were prepared with 1 mM *N*-ethylmaleimide to prevent formation of disulfide artifacts during sample preparation. The relative amounts of SPI subunits on SDS-PAGE gels were estimated densitometrically using a model Ultroscan XL laser scanner (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Differential Scanning Calorimetry (DSC). A relatively high concentration (10% w/v, or 100 mg/mL protein) of SPI, 7S, and 11S dispersions was used for DSC analysis to ensure adequate signal output. Although the residual amount of NaCl in pH-shifting-treated protein samples was minimal, as indicated above, its ionic effect may become accentuated at the 10% protein concentration. Hence, all native and pH-shifting-treated protein samples subjected to DSC were first dialyzed (MWCO = 6000−8000, Spectra/Por tubings, Spectrum, LA) at 20 °C for 24 h against nanopure water with three water changes to remove salts and then lyophilized. Dried protein powders were subsequently dispersed in 25 mM sodium phosphate buffer (pH 7.0) containing 0, 0.1, or 0.6 M NaCl to obtain 10% (w/v) protein slurries. Approximately 15 mg of each slurry was transferred into a polymer-coated aluminum pan and hermetically sealed. A sealed, empty pan was used as the reference. Samples were scanned at 10 °C/min through a range of 25−100 °C using a model 2920 differential scanning calorimeter (TA Instruments, Inc., New Castle, DE). The maximum temperature (T_{max}) and enthalpy (ΔH) associated with every thermal transition were determined using Universal Analysis ver. 1.2N software (TA Instruments) as described in the DSC user's manual.

Turbidity. A turbidity experiment was carried out to determine the susceptibility of pH-shifting-treated proteins to thermal insolubilization and aggregation. Aliquots of 5 mL each of dilute protein solutions (2 mg/mL in 25 mM sodium phosphate buffer, pH 7.0) at different ionic strengths (0, 0.1, 0.6 M NaCl) were placed in test tubes. The tubes were closed with screw caps to prevent evaporation of water during heating. Samples were heated at 1 °C/min in a programmable water bath (Haake L D3 heating circulator, Fisher Scientific, Waltham, MA). When a target temperature was reached (30, 50, 60, 70, 80, 90, 97 °C), two tubes (duplicate) were removed and immediately chilled in an ice slurry. The turbidity of the heated solutions was measured at 600 nm. Moreover, heated solutions were centrifuged at 5000*g* for 15 min; the concentration of proteins that remained in the supernatant (soluble) was determined according to the biuret method.

Statistical Analysis. Up to three preliminary trials were conducted to establish the experimental conditions and optimize the testing procedures. The initial trials showed high consistency and repeatability of results; therefore, two subsequent independent experiments (replicates), each with duplicate or triplicate sample analysis from a new batch of pH-shifting -processed SPI, 7S, and 11S samples, were conducted to obtain the means reported herein. Data were processed using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). Significant differences ($P < 0.05$) between means were identified using the least significance difference (LSD) procedure.

RESULTS AND DISCUSSION

Protein Solubility. The pH−solubility profile of native and pH-shifting-treated SPI (**Figure 1**) and those of 7S (**Figure 2**) and 11S (**Figure 3**) globulins all exhibited a U-shaped curve typical of most globular proteins. However, the pH dependence of the protein solubility was significantly affected by sample pH-shifting treatments prior to the solubility test and influenced by salt concentrations. For example, at both 0 and 0.1 M NaCl, the solubility of pH_{12}-shifting-treated SPI had an improved solubility at pH 2−3 and 6−8 ($P < 0.05$) when compared with native SPI, and at pH 4−5, the isoelectric point region of β-conglycinin and glycinin, the solubility reached a minimum (~6%) (**Figure 1**). However, in 0.6 M NaCl solution, in which the solubility of all samples was significantly raised in the isoelectric pH range (4−5), the solubility of native SPI was greater at pH 3−5 but less at pH 6−8 than that of both $pH_{1.5}$- and pH_{12}-shifting-treated samples, which were identical in their solubility behavior.

Analysis of isolated SPI fractions showed that pH-shifting treatments had much less effect on the solubility of 7S (**Figure 2**) when compared to the SPI at all pH levels. A notable exception was that, for 7S suspended in 0.6 M NaCl, the solubility of pH_{12}-shifting-treated sample exhibited a sharp decline to a minimum at pH 4.5, which was not seen for control and $pH_{1.5}$-shifting-treated samples that maintained a constant high solubility (80−90%) at all pH values evaluated. An insensitivity of 7S to pH at high ionic strengths, including the pH at the isoelectric point, was also observed by Anderson et al. (*18*). The results can be explained because electrostatic interactions, which led to protein insolubilzation, between polypeptides in native or $pH_{1.5}$-shifting-treated β-conglycinin were weakened by the presence of 0.6 M NaCl. However, the same high concentrations of Na^+ and Cl^- neutralized protein charges in pH_{12}-shifting-treated β-conglycinin around the isoelectric pH, thereby promoting hydrophobic aggregation and precipitation.

On the other hand, 11S globulin showed a remarkable similarity to SPI in pH solubility profile responding to the pH-shifting treatments. These included a substantially greater solubility for pH_{12}-shifting-treated sample than control (native) and $pH_{1.5}$-shifting-treated samples suspended in pH 6−7 solutions at 0 and 0.1 M NaCl (**Figure 3**). As a result of the alkaline pH treatment, the pH range causing insolubility of 11S proteins also became

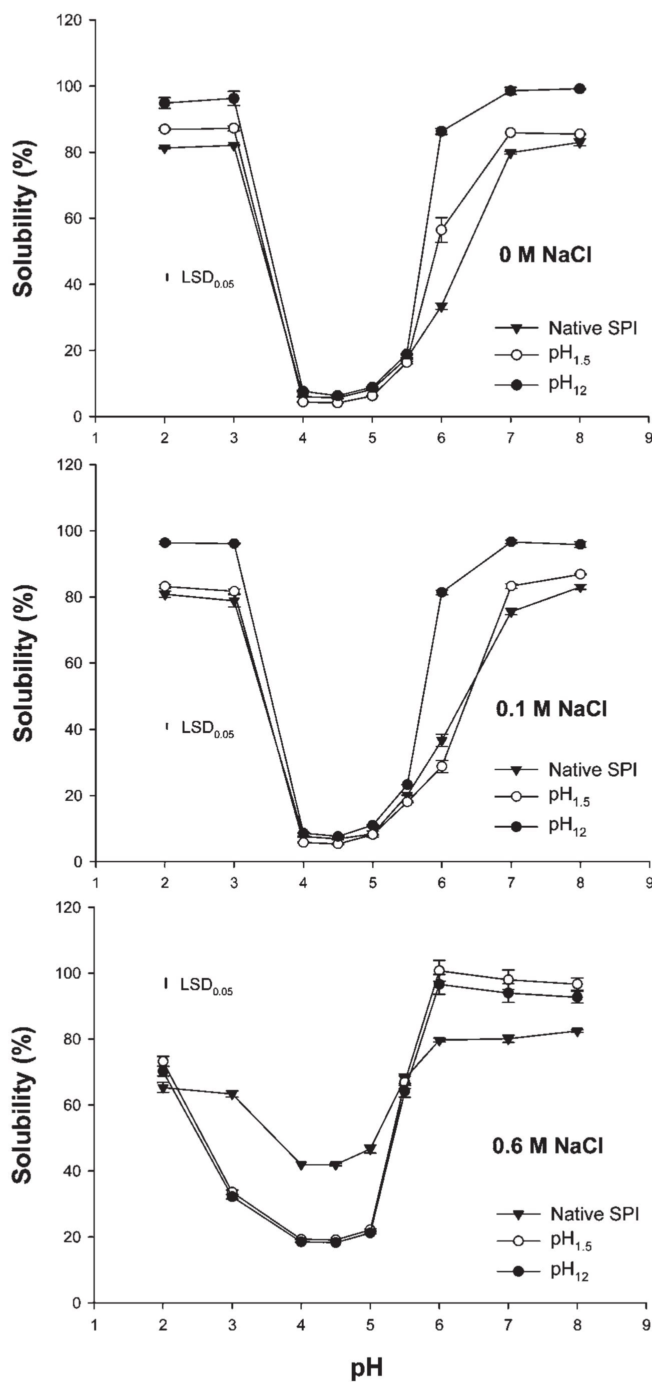

Figure 1. Effect of pH-shifting treatments (pH 1.5 or 12) on the solubility of soy protein isolate (SPI) in different salt solutions (0, 0.1, and 0.6 M NaCl) under various pH conditions.

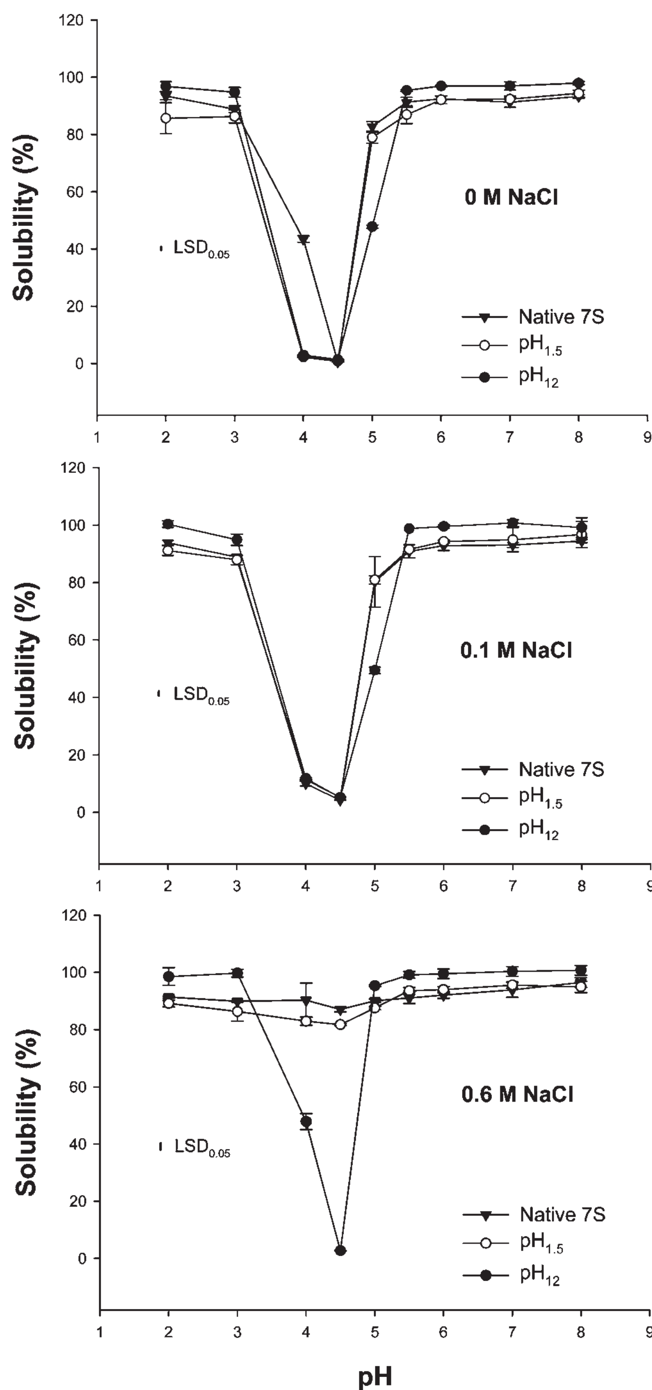

Figure 2. Effect of pH-shifting treatments (pH 1.5 or 12) on the solubility of soy β-conglycinin (7S) in different salt solutions (0, 0.1, and 0.6 M NaCl) under various pH conditions.

narrower (pH 4−5.5 instead of 4−6 for native 11S). Because hydrophobic and van der Waals interactions are among the main forces stabilizing the individual globulin units in native glycinin (11S) (*19*), the pH_{12} alkaline treatment must have permanently disrupted some of the forces and, hence, reduced the range of isoelectric pH. Nonetheless, adjustment of the ionic strength to 0.6 M NaCl lowered the solubility of pH_{12}-shifting-treated samples at pH 2−4 but barely affected the native and $pH_{1.5}$-shifting-treated 11S when compared with SPI samples.

The marked solubility enhancements (up to 2.5-fold) for pH_{12}-shifting-treated SPI in pH 2−3 and 6−8 solutions at low ionic strengths (0 and 0.1 M NaCl) are noteworthy. Our previous investigation demonstrated extensive protein structural unfolding of SPI when subjected to treatment at pH 12, which was accompanied by the cleavage of some disulfide bonds between subunits in glycinin (*17*). These physicochemical changes ostensibly contributed to the solubility improvements of pH_{12}-shifting-treated samples.

SDS-PAGE and Densitometry. SDS-PAGE was conducted to identify the specific SPI components extracted, which may help explain the solubility changes. As displayed in **Figure 4**, at both 0 and 0.1 M NaCl, the A and B subunits of glycinin were more prominent and the AB complex band was less intense in the pH 3 or 6 extracts (soluble fractions) of pH_{12}-shifting-treated SPI than in control SPI. Even in native SPI, both whole and supernatant samples in 0 and 0.1 M NaCl showed some dissociated A and B

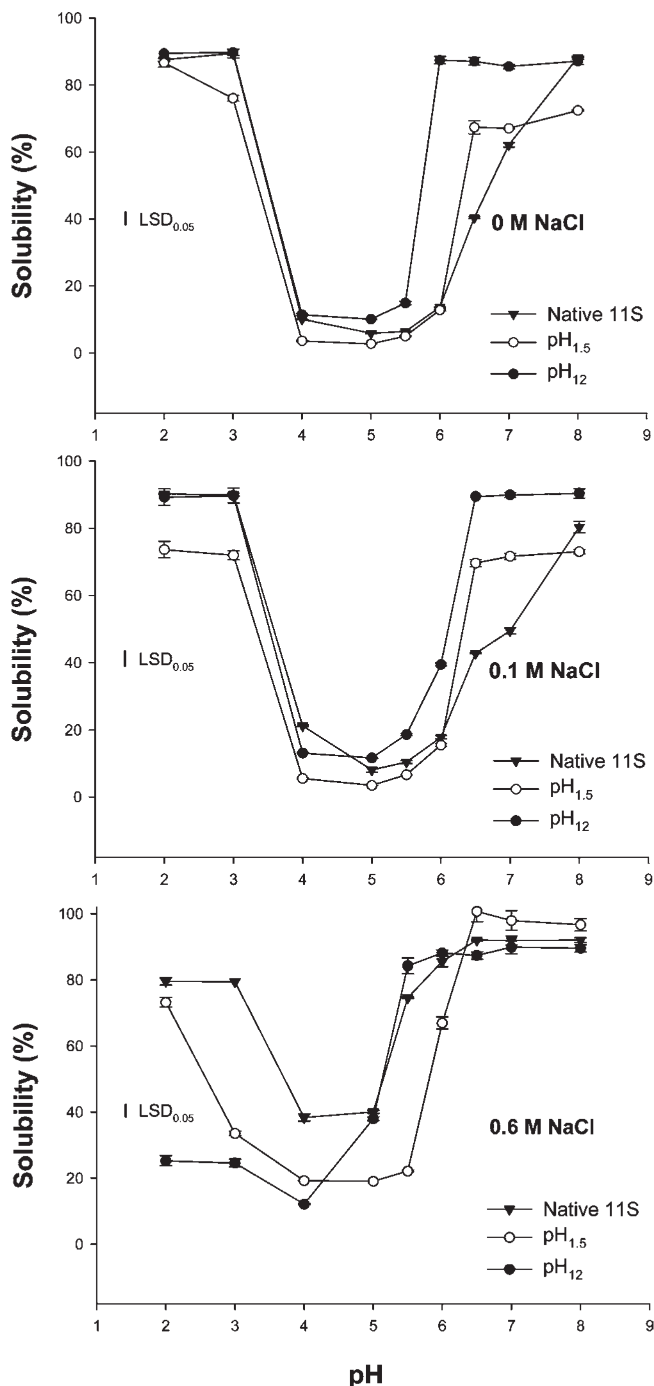

Figure 3. Effect of pH-shifting treatments (pH 1.5 or 12) on the solubility of soy glycinin (11S) in different salt solutions (0, 0.1, and 0.6 M NaCl) under various pH conditions.

subunits, indicating that these acidic and basic polypeptides were partially present along with disulfide-linked AB subunit. A similar finding was reported by Renkama et al. (*10*). In comparison, changes in the main subunits of β-conglycinin (α', α, β) were less obvious or more variable.

Densitometric scanning of the gels was run to determine the composition and relative distribution of individual polypeptides in the soluble fractions. The data confirmed losses of AB subunit and concomitant increases in dissociated A and B subunits of glycinin in the extracts of pH_{12}-shifting-treated SPI samples dissolved in the low ionic strength solutions at pH 3 and 6 (**Table 1**). In contrast, the acidic pH-shifting did not induce appreciable changes in protein extraction except that it produced more protein polymers or aggregates that were soluble at pH 3 (band 1). Furthermore, although not obvious in the SDS-PAGE, the scanned data revealed remarkable percentage reductions of the β-subunit of β-conglycinin following both acidic and alkaline pH-shifting treatments at any pH and ionic strength when compared with native SPI. The disappearance of the β-subunit in the pH_{12}-shifting-treated samples was notably more progressive than that in $pH_{1.5}$-shifting-treated samples. Thus, protein aggregates that were induced by pH-shifting processes originated mostly from the AB complex in 11S and the β-subunit of 7S.

These results demonstrated that 11S globulins were more sensitive than 7S globulins to pH-shifting treatments and that the alkaline pH shifting (pH_{12}) promoted cleavages of native disulfide bonds, whereas the acidic pH shifting ($pH_{1.5}$) favored cross-linking of glycinin subunits. The results were consistent with the observation that the solubility profiles of 11S were similar to those of SPI, whereas those of 7S deviated. Therefore, it can be concluded that the response of disulfide-rich 11S subunits to extreme pH-shifting treatments was a determinant factor for solubility increases of treated SPI dissolved in low ionic strength solutions (0−0.1 M NaCl) under low-pH (< 3) and high-pH (> 5.5) conditions.

Comparisons of the pH−solubility curves of SPI, 7S, or 11S at the three NaCl solutions revealed a general trend of decreasing sensitivity of protein solubility to pH as the salt concentration was raised. For example, the solubility gaps between the minimum and maximum of native SPI at 0, 0.1, and 0.6 M NaCl were, respectively, 77.3, 76.1, and 40.5%; for the pH_{12}-shifting-treated SPI, the gap also reduced from 92.8% (0 M NaCl) to 89.0 (0.1 M NaCl) and 78.4% (0.6 M NaCl) (**Figure 1**). This trend held true also for 7S (**Figure 2**, except for the pH_{12}-shifting-treated sample in 0.6 M NaCl) and 11S globulins (**Figure 3**). The decrease in solubility of 11S at ionic strength of 0.5 M was previously reported by Lakemond et al. (*20*).

The effect of NaCl can be explained by its ionic effects. In the isoelectric pH range (4−5), the presence of sodium (Na^+) and chloride (Cl^-) ions that interact with charged groups on proteins would allow the formation of an electric double layer at the crystal−solution interface, thereby increasing the apparent solubility of soy proteins, a "salt-in" effect. The result supported the findings by Yuan et al. (*19*) on soy 7S and 11S proteins. However, at either side of the isoelectric pH, high-concentration ions (i.e., 0.6 M NaCl) would neutralize respective opposite charges on proteins and, therefore, diminish the gain in net charges brought about by adjusting the pH of the solution away from a protein's isoelectric point. The electrostatic interaction would also explain why the substantial augmentation in the solubility of SPI and its subunits via extreme pH-shifting treatments was diminished at increasing NaCl concentrations. Our results were in good agreement with those published in the literature (*20*, *21*).

Thermal Stability. As solubility is important for proteins used in beverages, cold emulsions, and other nonheated food products, the resistance of functional protein ingredients to thermal aggregation and insolubilization can be equally essential in the processing of many heated foods. To determine the effect of pH-shifting on protein stability, treated SPI samples dispersed in different ionic strength solutions at pH 7.0 were subjected to DSC analysis. As depicted in **Figure 5**, native SPI samples displayed two distinct endothermic transitions attributed to 7S and 11S (*22*). Increasing the salt concentration caused the denaturation temperature (T_{max}) of both constituents of SPI to shift to higher ($P < 0.05$) values (**Table 2**). The total enthalpy of denaturation (ΔH) also rose ($P < 0.05$) with increasing NaCl concentrations, suggesting a stabilizing effect of salt. The $pH_{1.5}$-shifting treatment induced no significant changes in T_{max} of either 7S or 11S, but it

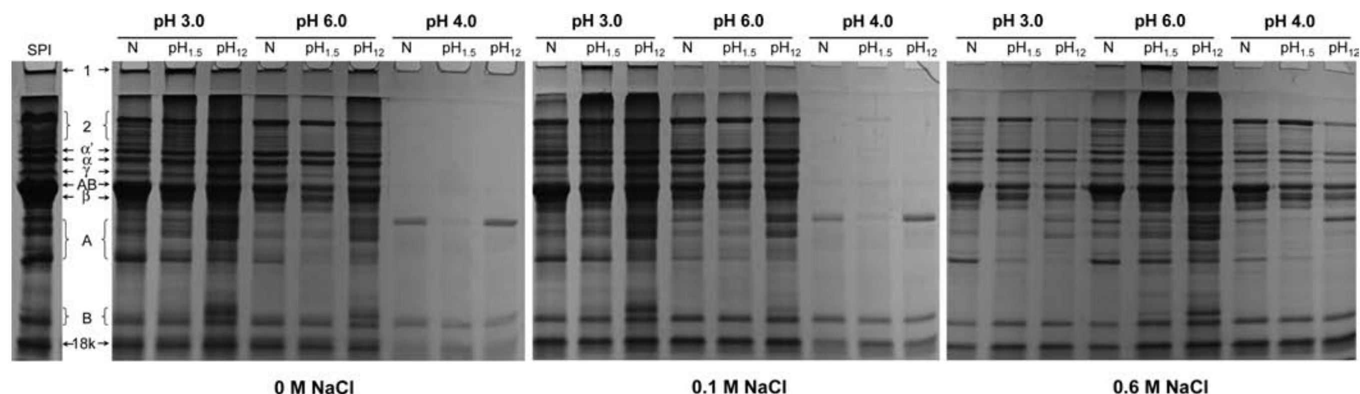

Figure 4. SDS-PAGE patterns of soluble fractions of SPI (in 0, 0.1, and 0.6 M NaCl at different pH values) after $pH_{1.5}$- or pH_{12}-shifting treatments. Lanes: SPI, whole SPI; N, native SPI as control. SPI constituents: α′ (86 kDa), α (66 kDa), and *β* (51 kDa) for conglycinin, and A, the acidic subunit (34−43 kDa), and B, the basic subunit (17−26 kDa), for glycinin.

Table 1. Percentage of Soy Protein Subunits in Native and pH-Shifting-Treated SPI Samples That Remained Soluble in Different NaCl Solutions at Three pH Levels (3.0, 4.0, 6.0)

		pH 3.0			pH 4.0			pH 6.0		
SPI subunit	SPI (pH 7.0)	native	$pH_{1.5}$	pH_{12}	native	$pH_{1.5}$	pH_{12}	native	$pH_{1.5}$	pH_{12}
0 M NaCl										
α′	7.3	6.2	6.2	6.9	0.0	0.0	0.0	9.9	13.2	7.3
α	9.4	7.9	7.9	7.7	0.0	0.0	0.0	10.8	13.8	9.7
β	19.3	19.8	16.4	12.3	0.0	0.0	0.0	19.4	14.5	10.9
AB	8.5	8.4	4.0	4.9	0.0	0.0	0.0	5.2	6.7	5.1
A	19.4	17.0	13.3	27.7	39.2	16.1	52.2	14.4	13.0	17.0
B	7.6	6.4	7.4	11.3	60.8	83.9	47.8	9.1	11.7	10.4
polymers	28.5	34.3	44.7	29.2	0.0	0.0	0.0	31.3	27.0	39.6
0.1 M NaCl										
α′	7.3	7.6	5.8	7.2	0.0	0.0	0.0	11.7	13.3	9.9
α	9.4	7.6	7.4	9.8	0.0	0.0	0.0	12.2	13.3	11.5
β	19.3	27.3	14.8	13.8	0.0	0.0	0.0	18.6	18.6	14.3
AB	8.5	9.7	6.7	8.5	0.0	0.0	0.0	5.1	6.3	4.0
A	19.4	15.0	12.8	16.1	50.2	0.0	52.5	16.0	13.4	21.4
B	7.6	7.0	6.4	8.2	49.8	100.0	47.5	7.3	8.9	8.4
polymers	28.5	25.8	46.2	36.2	0.0	0.0	0.0	29.1	26.3	30.5
0.6 M NaCl										
α′	7.3	8.1	11.5	8.1	8.3	9.8	12.0	6.9	6.7	7.3
α	9.4	7.0	10.3	8.6	7.1	9.3	6.5	7.4	7.6	8.7
β	19.3	33.5	16.7	14.1	27.1	15.9	15.4	31.7	14.0	12.0
AB	8.5	12.8	15.5	12.3	14.7	15.9	16.1	8.6	7.7	8.4
A	19.4	16.2	11.3	18.9	8.9	8.5	18.4	9.7	7.1	13.9
B	7.6	6.4	9.9	10.5	9.5	13.0	11.8	4.6	3.9	8.4
polymers	28.5	16.1	24.7	27.5	24.4	27.6	19.8	31.2	53.0	41.2

substantially reduced ΔH, when compared with native SPI. On the other hand, the pH_{12}-shifting destabilized 7S and 11S in SPI at all ionic strengths, which was evidenced by the almost complete elimination of thermal transitions irrespective of the salt concentration. The major reductions in ΔH following pH shifting can be ascribed to the disruption of the tertiary structure of SPI globulins rather than their secondary structure due to electrostatic repulsions (*8*).

The effect of salts on the thermal denaturation of soy proteins has been extensively studied. For example, Bikbov et al. (*23*) found that the 11S globulin at pH 7.6 had an increased stability when the salt concentration was elevated from 0 to 0.7 M. As enthalpy of denaturation is a measure of the total energy required to disrupt intramolecular forces/bonds (*24*), raising the salt concentration, which weakened charge interactions, seemed to drive SPI into a state in which the contacts of hydrophobic groups and hydrogen bonds became a more dominant stabilizing factor.

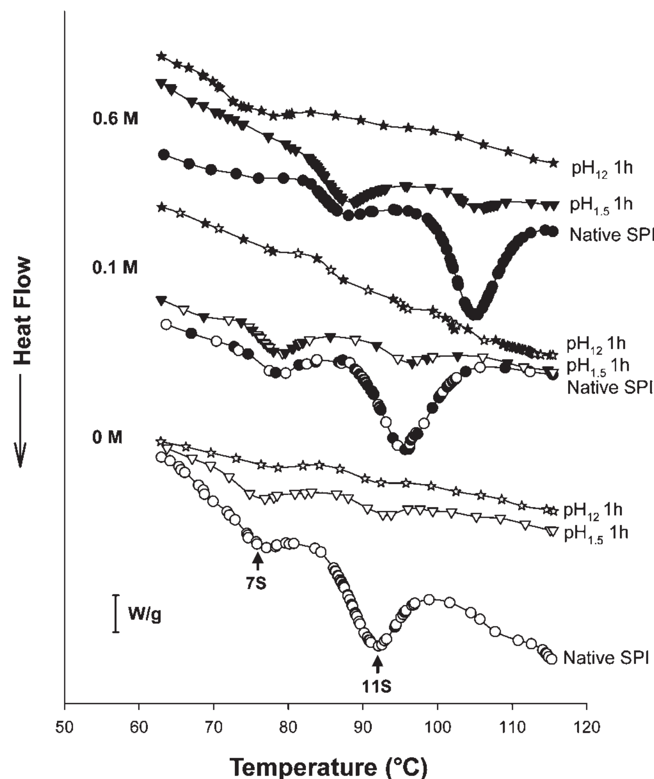

Figure 5. DSC thermograms of SPI in different ionic strength solutions (0, 0.1, and 0.6 M NaCl, pH 7.0) after $pH_{1.5}$- or pH_{12}-shifting treatments.

Table 2. Denaturation Temperature (T_{max}) and Enthalpy (ΔH) of Native and pH-Shifting-Treated Soy Protein Isolate (SPI), As Affected by Salt Concentrations

		denaturation parameter[a]		
sample	NaCl (M)	T_{max} 7S (°C)	T_{max} 11S (°C)	total ΔH (J/g)
native SPI	0	75.6 d	91.7 c	0.60 b
	0.1	78.6 b	95.8 b	0.77 a
	0.6	87.9 a	104.6 a	0.78 a
$pH_{1.5}$ shifting	0	77.0 bcd	92.3 c	0.17 de
	0.1	79.0 b	96.4 b	0.18 d
	0.6	87.6 a	104.5 a	0.26 c
pH_{12} shifting	0	76.4 cd	92.2 c	0.09 fg
	0.1	78.2 bc	87.1 d	0.05 g
	0.6	76.0 d	92.5 c	0.11 ef

[a] Data represent the means of two replications each with duplicate or triplicate sample analyses. Means within the same column that lack a common letter differ significantly ($P < 0.05$).

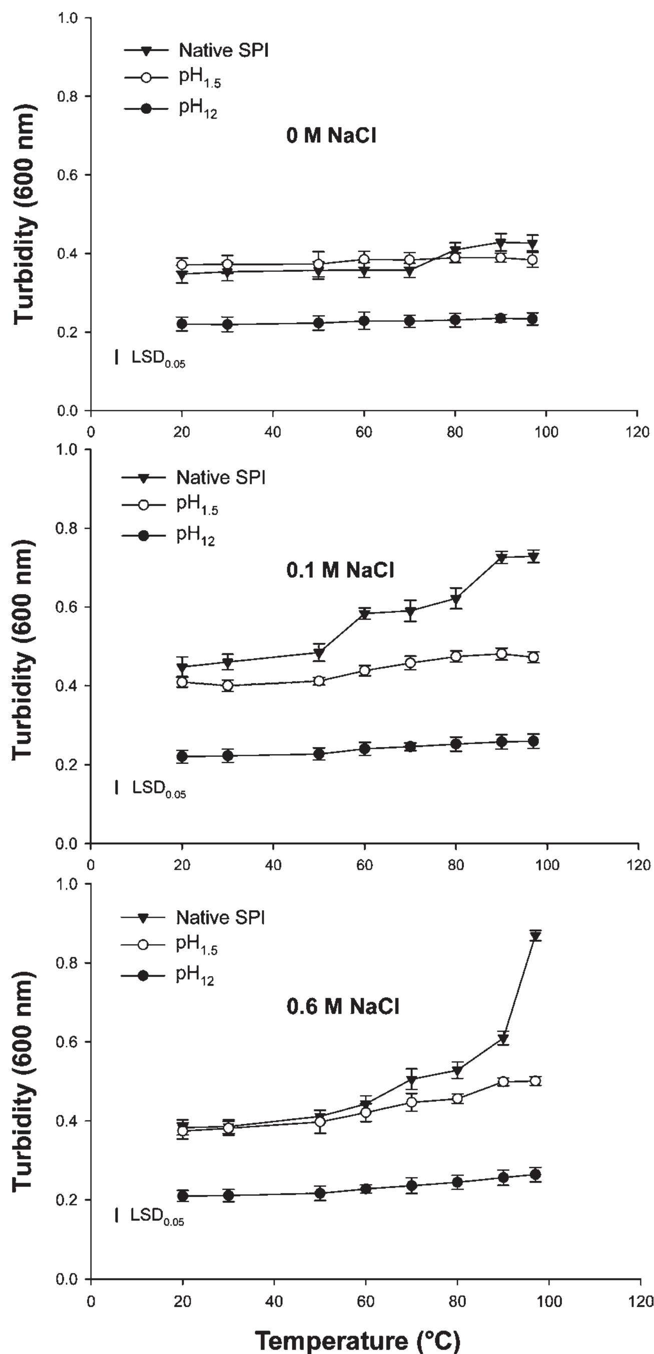

Figure 6. Turbidity of soy protein isolate (SPI) after $pH_{1.5}$- or pH_{12}-shifting treatments. Turbidity of heated samples at different ionic strengths (pH 7.0) was measured as optical density at 600 nm. Native SPI, control SPI solutions at pH 7.0 without pH-shifting treatment.

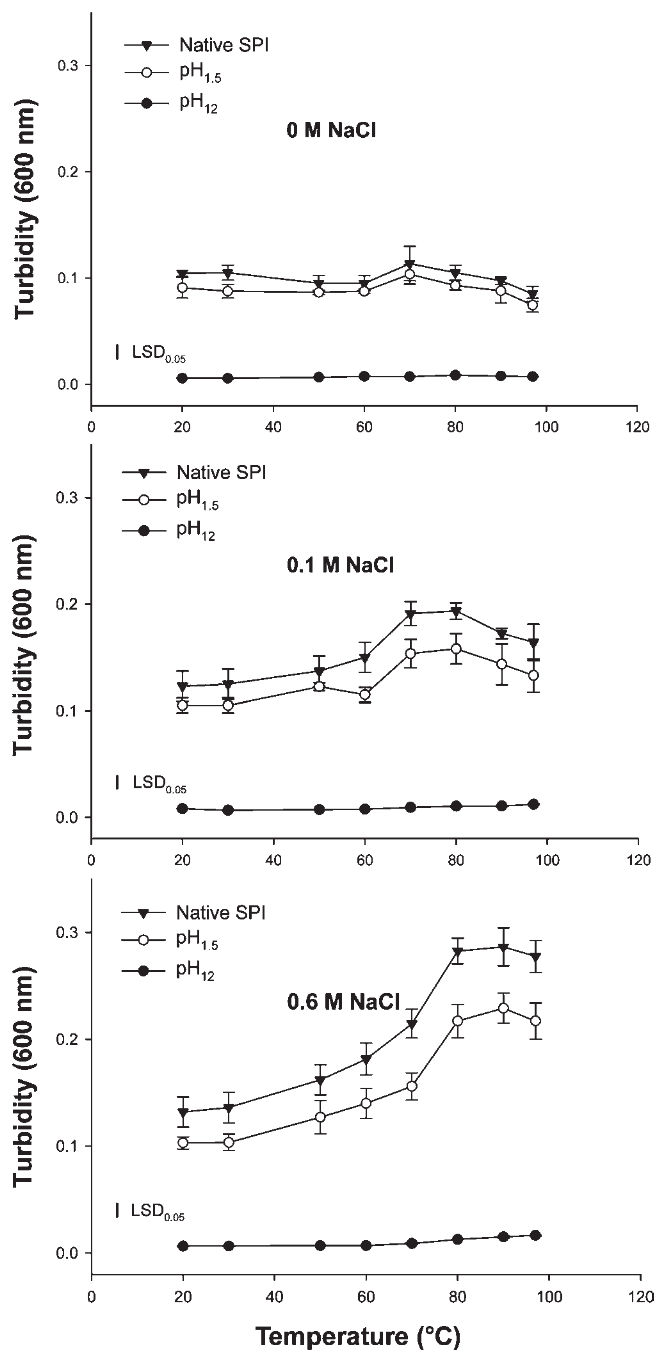

Figure 7. Turbidity of soy β-conglycinin (7S) after $pH_{1.5}$- or pH_{12}-shifting treatments. Turbidity of heated samples at different ionic strengths (pH 7.0) was measured as optical density at 600 nm. Native 7S, control 7S solutions at pH 7.0 without pH-shifting treatment.

The extent of denaturation of pH_{12}-shifting-treated samples was greater than that of $pH_{1.5}$-shifting-treated samples because the pH of the pH_{12} treatment medium was farther away from the proteins' isoelectric points (pH 4−5) (**Figures 2** and **3**), resulting in more extensive, irreversible disruptions of intramolecular ionic bonds. Such extensive structural unfolding due to the extreme alkaline pH treatment (pH 12) rendered the proteins less sensitive to ionic strength changes, because intramolecular bonds dictating thermal transition temperatures (T_{max}) were already severely weakened. In our previous study, we noted that soy proteins after pH-shifting treatments adopted a molten globule-like conformation that largely maintained the original secondary structure and overall compactness but lost some tertiary structure (*8*).

Thermal Aggregation. Heat-induced aggregation, analyzed using dynamic turbidity measurement (optical density at 600 nm), of SPI, 7S, and 11S samples at pH 7.0 is shown in **Figures 6**, **7**, and **8**, respectively. Native SPI in salt-free solution was resistant to aggregation, as was generally observed in similar studies (*25*). Increasing the NaCl concentration rendered SPI vulnerable to aggregation upon heating; in 0.6 M NaCl, the turbidity of SPI increased exponentially when heated from 80 to 97 °C (**Figure 6**), indicating protein aggregation. A similar trend was noted for native 7S (**Figure 7**) and 11S (**Figure 8**), although the former showed much less turbidity.

The gain in thermal stability at elevated ionic strength shown by the DSC analysis (**Figure 5**) cannot explain the rapid increase

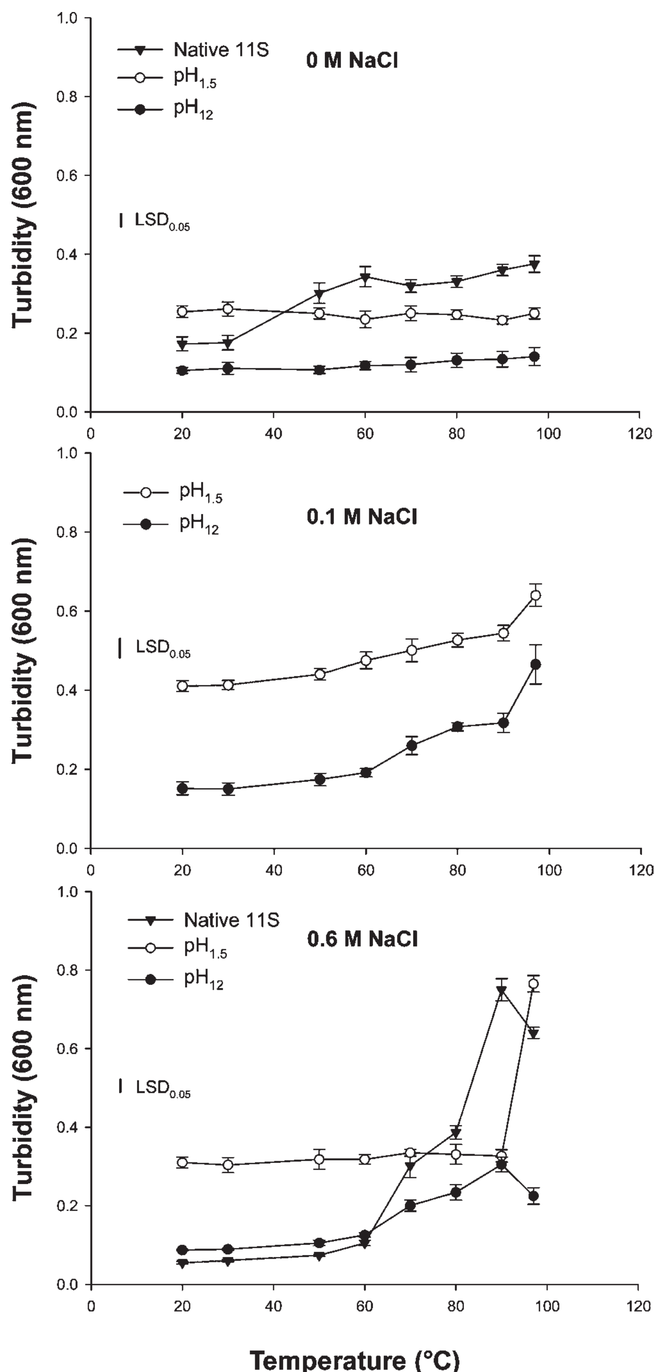

Figure 8. Turbidity of soy glycinin (11S) after $pH_{1.5}$- or pH_{12}-shifting treatments. Turbidity of heated samples at different ionic strengths (pH 7.0) was measured as optical density at 600 nm. Native 11S, control 11S solutions at pH 7.0 without pH-shifting treatment. Data for native 11S at 0.1 M NaCl were not collected due to protein precipitation.

in protein aggregation at high temperatures. For example, in 0.6 M NaCl, native 11S began to aggregate at temperature >50 °C (**Figure 8**), where unfolding of 11S did not commence (**Figure 5**). Even for the less stable 7S, it did not unfold until about 80 °C (**Figure 5**), a temperature that was far higher than the initial aggregation temperature of native SPI and 7S. Therefore, aggregation of SPI must be assigned to the ionic effects of NaCl, that is, diminishments of charge repulsions of exposed ionic groups on protein surface. Any electric double layer created around the protein surface would be removed at high temperatures.

In contrast, SPI samples after the extreme acidic ($pH_{1.5}$) or alkaline (pH_{12}) pH treatment were less labile to heat. The turbidity of the SPI and 7S after the pH_{12}-shifting treatment was the lowest and almost constant at any NaCl concentration with increasing temperatures compared with that of native and $pH_{1.5}$-shifting-treated proteins. Because pH-shifting-treated soy proteins were significantly unfolded (*8*), the present results further suggested that aggregation of SPI or their subunits was achieved mainly through the available surface active groups rather than those newly exposed during heating.

Because SPI is mainly composed of 7S and 11S, the turbidity characteristic of SPI will be dominantly influenced by these two globulin fractions. Despite the role of 7S in initiating SPI aggregation, the present results indicated that 11S globulins were a more important contributor to the overall aggregation of SPI because their turbidity values were much closer than those of SPI when compared with 7S. This effect can be attributed to the fact that more disulfide polymers were formed in heated 11S globulin (*26*). The effect of salt concentration on the overall turbidity increase of SPI and of 11S can be ranked in the order $0.1 > 0.6 \geq 0$ M NaCl. A precipitation was observed when native 11S was dissolved in 0.1 M NaCl solution at pH 7 (thus, no turbidity data were presented). However, this phenomenon was not seen in pH-shifting-treated samples. As Wolf (*27*) reported, 11S proteins were loosely associated at 0.1 ionic strength. Samples after extreme pH-shifting treatments were soluble at 0.1 M ionic strength because aggregated subunits were dissociated during the extreme high or low pH treatments.

It should be noted that the aggregation patterns of isolated 11S or 7S did not completely match those of SPI. This was because 7S globulin in SPI would interact and, thus, coaggregate with the basic polypeptides (B subunit) of 11S as the temperature increased (*28*, *29*). Moreover, the α and α′ subunits of 7S and the acidic polypeptide (A subunit) of 11S can associate through disulfide bonds to form soluble heteropolymers (*30*).

In conclusion, unfolding at extreme alkaline (pH 12) or acidic (pH 1.5) conditions followed by refolding at neutral pH significantly improved the solubility of SPI in low ionic strength (≤0.1 M NaCl) aqueous solutions at pH 2−3 and 6−8. This effect, which was largely attributed to the 11S globulins, also existed in high ionic strength (0.6 M NaCl) solutions at pH 6−8. The extreme alkali-treated SPI was remarkably stable against thermal aggregation due to reduced sensitivity of altered protein structure by the pH-shifting process. These results were of practical significance because the pH-shifting-treated SPI can be of great utility in beverage and other liquid-type food product applications.

LITERATURE CITED

(1) Goto, Y.; Calciano, L. J.; Fink, A. L. Acid-induced folding of proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 573–577.
(2) Kristinsson, H. G.; Hultin, H. O. Changes in conformation and subunit assembly of cod myosin at low and high pH and after subsequent refolding. *J. Agric. Food Chem.* **2003**, *51*, 7187–7196.
(3) Liang, Y.; Kristinsson, H. G. Structural and foaming properties of egg albumen subjected to different pH-treatments in the presence of calcium ions. *Food Res. Int.* **2007**, *40*, 668–678.
(4) Kristinsson, H. G.; Hultin, H. O. Changes in trout hemoglobin conformations and solubility after exposure to acid and alkali pH. *J. Agric. Food Chem.* **2004**, *52*, 3633–3643.
(5) Wagner, J. R.; Guéguen, J. Surface functional properties of native, acid-treated, and reduced soy glycinin. 2. Emulsifying properties. *J. Agric. Food Chem.* **1999**, *47*, 2181–2187.
(6) Wagner, J. R.; Guéguen, J. Surface functional properties of native, acid-treated, and reduced soy glycinin. 1. Foaming properties. *J. Agric. Food Chem.* **1999**, *47*, 2173–2180.

(7) Matsudomi, N.; Sasaki, T.; Kato, A.; Kobayashi, K. Conformational changes and functional properties of acid-modified soy protein. *Agric. Biol. Chem.* **1985**, *49*, 1251–1256.
(8) Jiang, J.; Chen, J.; Xiong, Y. L. Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. *J. Agric. Food Chem.* **2009**, *57*, 7576–7583.
(9) Molina Ortiz, S. E.; Añon, M. C. Hydrolysates of native and modified soy protein isolates: structural characteristics, solubility and foaming properties. *Food Res. Int.* **2002**, *35*, 511–518.
(10) Renkema, J. M. S.; Gruppen, H.; Vliet, T. V. Influence of pH and ionic strength on heat-induced formation and rheological properties of soy protein gels in relation to denaturation and their protein compositions. *J. Agric. Food Chem.* **2002**, *50*, 6064–6071.
(11) Rickert, D. A.; Johnson, L. A.; Murphy, P. A. Functional properties of improved glycinin and β-conglycinin fractions. *Food Chem. Toxicol.* **2004**, *69*, 304–311.
(12) Kinsella, J. E. Functional properties of soy proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242–258.
(13) Damodaran, S. Refolding of thermally unfolded soy proteins during the cooling regime of the gelation process: effect on gelation. *J. Agric. Food Chem.* **1988**, *36*, 262–269.
(14) Lakemond, M. C. C.; de Jongh, H. H. J.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Heat denaturation of soy glycinin: influence of pH and ionic strength on molecular structure. *J. Agric. Food Chem.* **2000**, *48*, 1991–1995.
(15) Hashizume, K.; Nakamura, N.; Watanabe, T. Influence of ionic strength on conformation changes of soybean proteins caused by heating, and relationship of its conformation changes to gel formation. *Agric. Biol. Chem.* **1975**, *39*, 1339–1347.
(16) Ruíz-Henestrosa, V. P.; Sánchez, C. C.; Escobar, M. M. Y.; Jiménez, J. J. P.; Rodríguez, F. M.; Patino, J. M. R. Interfacial and foaming characteristics of soy globulins as a function of pH and ionic strength. *Colloids Surf. A: Physicochem. Eng.* **2007**, *309*, 202–215.
(17) Liu, G.; Xiong, Y. L. Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidized myosin. *J. Agric. Food Chem.* **2000**, *48*, 624–630.
(18) Anderson, R. L.; Wolf, W. J.; Glover, D. Extraction of soybean meal proteins with salt solutions at pH 4.5. *J. Agric. Food Chem.* **1973**, *21*, 251–254.
(19) Yuan, Y. J.; Velev, O. D.; Chen, K.; Campbell, B. E. Effect of pH and Ca^{2+}-induced associations of soybean proteins. *J. Agric. Food Chem.* **2002**, *50*, 4953–4958.
(20) Lakemond, C. M. M.; de Jongh, H. H. J.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Soy glycinin: influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *J. Agric. Food Chem.* **2000**, *48*, 1985–1990.
(21) Shen, J. L. Solubility profile, intrinsic viscosity, and optical rotation studies of acid precipitated soy protein and of commercial soy isolate. *J. Agric. Food Chem.* **1976**, *24*, 784–788.
(22) Zhang, J.; Mungara, P.; Jane, J. Mechanical and thermal properties of extruded soy protein sheets. *Polymer* **2001**, *42*, 2569–2578.
(23) Bikbov, T. M.; Grinberg, V. Y.; Danilenko, A. N.; Chaika, T. S.; Vaintraub, I. A.; Tolstoguzov, V. B. Studies on gelation of soybean globulin solutions. *Colloid Polym. Sci.* **1983**, *261*, 346–358.
(24) Privalov, P. L.; Khechinashvili, N. N. A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *J. Mol. Biol.* **1974**, *86*, 665–684.
(25) Lee, K. H.; Ryu, H. S.; Rhe, K. C. Protein solubility characteristics of commercial soy protein products. *J. Am. Oil Chem. Soc.* **2003**, *80*, 85–90.
(26) Briggs, D. R.; Wolf, W. J. Studies on the cold-insoluble fraction of the water-extractable soybean proteins. I. Polymerization of the 11 S component through reactions of sulfhydryl groups to form disulfide bonds. *Arch. Biochem. Biophys.* **1957**, *72*, 127–144.
(27) Wolf, W. J. Chemical and physical properties of soybean proteins. *Baker's Dig.* **1969**, *43*, 30–37.
(28) Utsumi, S.; Damodaran, S.; Kinsella, J. E. Heat-induced interactions between soybean proteins: preferential association of 11S basic subunits and β subunits of 7S. *J. Agric. Food Chem.* **1984**, *32*, 1406–1412.
(29) Damodaran, S.; Kinsella, J. E. Effect of conglycinin on the thermal aggregation of glycinin. *J. Agric. Food Chem.* **1982**, *30*, 812–817.
(30) Yamagishi, T.; Miyakawa, A.; Noda, N.; Yamauchi, F. Isolation and electrophoretic analysis of heat-induced products of mixed soybean 7S and 11S globulins. *Agric. Biol. Chem.* **1983**, *47*, 1229–1237.

Received for review March 18, 2010. Revised manuscript received May 20, 2010. Accepted May 26, 2010. This research was supported, in part, by the Ministry of Science and Technology, China (Grant 2007AA10Z323), and the Ministry of Education, China (Grants NCET-07-0377 and IRT 0627). J.J. was an Exchange Student sponsored by the University of Kentucky. Approved for publication as journal article 10-07-037 by the Director of the Kentucky Agricultural Experiment Station.