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Effects of High Hydrostatic Pressure on Some Functional and Nutritional Properties of Soy Protein Isolate for Infant Formula

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ABSTRACT: The effects and mechanism of high hydrostatic pressure (HHP) on some functional and nutritional properties of soy protein isolate (SPI) for infant formula were investigated. Results indicated that solubility, water holding capacity, emulsification activity index, and foaming capacity were improved at lower pressure and time levels, whereas these properties declined at higher levels. However, the emulsification stability index dropped when the pressure increased and the foaming stability decreased with pressure and time levels rising. HHP-treated SPI gave better swallowing properties and in vitro digestibility than control. The hardness, adhesive force, and springiness of SPI gels increased with increaded pressure and elongated time, being lower than those of the control. Near UV circular dichroism spectra confirmed the alteration of tertiary and/or quaternary conformations caused by HHP. Sodiumdoecyl sulfate polyacrylamide gel electrophoresis results indicated that β-conglycinin was more pressure labile than glycinin, and high molecular weight subunits formed via disulfide linkage at higher treatment levels.

KEYWORDS: High hydrostatic pressure, functional properties, in vitro digestibility, SPI

INTRODUCTION

High hydrostatic pressure (HHP) is a promising nonthermal technology for the processing of foods. There is increasing worldwide attention in the utilization of HHP attributed to the advantages of this technology over other methods of processing and preservation. HHP offers homogeneity of treatment at every point in the product because the applied pressure is instantaneously and uniformly distributed within the HHP chamber.[1] Other obvious benefits in using this technology are significant or total inactivation of microorganisms and better functional and nutritional retention of ingredients in the processed products, with improved food quality parameters.[2−5] Additionally, there are considerable energy savings in comparison to traditional thermal stabilization techniques, because once the desired pressure is reached, it can be maintained without the requirement for additional energy input.[6]

Soy protein is the predominant vegetable protein in the diet of Oriental and Western countries and may be the most inexpensive source of protein for nutritional or technological properties.[7] There is a myriad of literature describing the applications of HHP to modify the functional properties of soy protein isolate (SPI) such as texture, emulsifying, solubility, and rheological properties.[7−15] The influence of various process parameters (e.g., pressure, time, temperature, protein concentration, pH, and presence of salts) on functional and structural properties of SPI has been investigated.[7−15] Different food systems require special types of SPI. Therefore, SPI has been subdivided according to its use. SPI is widely used as an ingredient in meat products, baby foods, beverages, and wheat flour products.[14] To our knowledge, there are very limited data available on the effects of HHP on SPI especially for infant formula. Moreover, few studies have been conducted about the influences of HHP on in vitro digestibility, thixotropy, foaming properties, and near UV circular dichroism (CD) spectra, which play crucial roles in the appraisal of the quality of SPI for infant formula.

The objective of this study is to investigate the influences of HHP treatment on some functional and nutritional properties of SPI for infant formula, including solubility, water holding capacity (WHC), emulsifying activity index (EAI), emulsifying stability index (ESI), foaming capacity (FC), foaming stability (FS), thixotropy, gelling property, and in vitro digestibility of protein. In addition, the mechanism of HHP influence on SPI will be analyzed by measurement of the ζ-potential, near UV CD spectra, and subunit molecular weight distribution. This study will provide practical information for HHP application in the modification of SPI for infant formula.

MATERIALS AND METHODS

Materials. SPI (model: special for infant formula) was purchased from Henan Anyang Mantianxue Food Co. Ltd. (China). According to the Chinese National Food Safety Standard-infant formula, SPI for infant formula must be qualified with the following requirements: protein content (on dry basis) ≥ 90%, and nitrogen soluble index (NSI) ≥ 80%, made from nongenetically modified soybean cultivars, negative urease activity. Its protein content on a dry basis and NSI were, respectively, 91.61 and 82.8%, mainly made from nongenetically modified soybean cultivars Zhonghuang35 and Beidou10. Its urease activity was negative.

Bovine serum albumin (BSA) was purchased from Sinopharm Chemical Reagent Co., Ltd. Low molecular weight protein markers and β-mercaptoethanol (2-ME) were purchased from Shanghai

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Shengzheng Biotech. Co., Ltd. (China). All other chemicals used were of analytical grade.

HHP Treatment. High pressure equipment (model UHPF-750 MPa-3 L; maximum pressure, 750 MPa; KEFA Hitech Food Machine Co., Ltd., Baotou, China) with a hydraulic type cell with an inner capacity of 3 L (1000 mm in diameter and 2000 mm in height) and a water jacket for temperature control was used in this study. Prior to pressure processing, SPI solutions with a suitable volume were vacuum-conditioned in a polyethylene bag. The temperature during treatment was controlled to avoid overheating. Water was used as the pressure transmitting medium in the machine, and its temperature was kept at 20 °C during the pressure processing. Distilled water was used as the solvent. SPI solutions at 1% (w/v) and pH 6.8 were subjected to HHP treatment at 200, 300, 400, and 500 MPa for 15 min, and SPI solutions were subjected to HHP treatment at a constant pressure of 300 MPa for 5, 10, 15, and 20 min. The target pressure was reached at a rate of about 250 MPa/min and released at a rate of about 300 MPa/min. After HHP treatment, the SPI solutions were freeze-dried. The unpressurized SPI solutions were also freezed-dried and used as experimental controls.

Solubility. The protein solubility of SPI was determined according to the method of Wang et al.,[11] with minor modification. Aliquots (200 mg) of SPI samples were dispersed and stirred at 200 rpm in 20 mL of distilled water (pH 6.8) or distilled water (pH 3, which was adjusted with 0.5 M HCl) at room temperature for 1 h. Then, the dispersions were centrifuged at 10000*g* for 30 min at 20 °C. The protein content in the supernatant was measured by micro-Kjeldahl method (N × 6.25). Measurements were performed in duplicate.

WHCs. The WHC was determined according to the method of Tomotake et al.,[16] with slight modification. Briefly, 1.5 g of sample was weighed into 10 mL centrifuge tubes preweighed. For each sample, distilled water (pH 6.8) was added in small increments to a series of tubes under continuous stirring with a glass rod. After the mixture was thoroughly wetted, samples were centrifuged (2000*g*, 10 min). After the centrifugation, the supernatant liquid was discarded. The WHC was calculated as WHC = the weight of water held by sample/the weight of dry sample. Measurements were performed in triplicate.

Emulsifying Activity. The EAI and ESI of SPI were determined according to the method of Pearce et al.,[17] with minor modifications made by Tang et al.[18] For emulsion formation, 6 mL of 0.2% SPI dispersion in 0.05 M Tris-HCl buffer (pH 7.5) and 2 mL of soybean oil were homogenized in FA25 model, High-Speed Homogenizer (Fluko Co., Germany) for 1 min at the maximum velocity (about 15000 rpm). An aliquot (50 μL) of emulsion was taken from the bottom of the homogenized emulsion, immediately (0 min) or 10 min after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution. After the emulsion was vortex mixed, the absorbance of diluted emulsions was read at 500 nm in the spectrophotometer.

EAI and ESI values were calculated by the following equations:[17,18]

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{c \times \varphi \times (1-\theta)}$$

$$\text{ESI } (\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10$$

where DF is the dilution factor (100), c is the initial concentration of protein (0.1 g/100 mL), φ is the optical path (0.01 m), θ is the fraction of oil used to form the emulsion (0.25), and A_0 and A_{10} are the absorbances of the diluted emulsions at 0 and 10 min, respectively. Measurements were performed in triplicate.

Foaming Activity. The foam capacity and stability were determined according to the method of Venktesh et al.,[19] with slight modification. Two grams of SPI was dispersed in 100 mL of distilled water (pH 6.8). Then, it was whipped in FA25 model, High-Speed Homogenizer (Fluko Co.) for 1 min at 10000 rpm and was poured into a 250 mL graduated cylinder. The volume of the foam after 30 s was calculated, and the volume increase is expressed as percent foam capacity. The foam stability was determined by measuring the decrease in foam volume as a function of time up to a period of 30 min: fFoam capacity (%) = volume after whipping (mL) − volume before whipping (mL)} × 100/volume before whipping (mL). Measurements were performed in triplicate.

Thixotropic Property Determination. The thixotropy was measured according to the method of Zhang et al.,[20] with some modification. The thixotropy was determined at 25 °C with a controlled stress rheometer, model Rheometer AR-G2 (TA Instruments, England). The rheometer was equipped with a stainless steel plane having a diameter of 40 mm. For the investigation of the thixotropic property of aqueous SPI solutions, the flow curves were measured by increasing the shear rate from a minimum of 2.0 s^{-1} to a maximum of 200 s^{-1} and then decreasing the shear rate in the same equal steps. The duration of shear at each step was about 3 min. If the system investigated exhibits the thixotropy, a hysteresis loop could be obtained from these "upward" and "downward" curves developed during round-trip shear stress−shear rate paths, and the corresponding enclosed area could be used to evaluate the magnitude of the thixotropy. Measurements were performed in triplicate.

Textural Measurements of SPI Gels. Gels were prepared by heating 16% (w/v) soy protein distilled water dispersions in cylindrical glass molds with an inner diameter of 20 mm and a height of 40 mm. The molds were filled three-quarters full to enable air bubbles to escape, and the air bubbles were removed using a vacuum pump and then placed vertically in a water bath. The sample was heated at 90 °C for 30 min and then cooled to room temperature by immersion in an ice bath, and then, the sample was kept at 4 °C for 24 h.[21]

Before analysis, the gels were removed from the container and transferred to Texture Analyzer (TMS-PRO, Food Technology Corp., VA). A cylinder probe with a diameter of 10 mm was chosen. The probe test speed, starting trigger force, deformation, back-off distance, and holding time between two cycles were set as follows: 120 mm/min, 0.05 N, 40%, 40 mm, and 0 s. The tests were performed at 25 °C, and measurements were performed in triplicate. Four simple parameters were utilized to determine the manner in which a food is handled and behaves within the mouth: hardness, peak force in the first compression cycle; adhesive force, maximum negative force generated during upstroke of probe; cohesiveness, the ratio of positive area during the second to that of the first compression cycle (downward strokes only); and springiness, height that the food recovers during the time elapsed between the end of the first bite and the start of the second.[22] Four parameters were analyzed by Texture Lab Pro Application, Version 1.13.2.0 (VA).

Sequential in Vitro Digestibility of Protein Procedure. The in vitro digestibility of the proteins was evaluated by the method of Tang,[23] with some modification. Pepsin (Sigma, P7000, 1:10000, 600−1000 units/mg) and trypsin (Genview, DH355-1, 1:250) were utilized for in vitro digestion investigation. Briefly, 5 mL of 1% (w/v) SPI solutions was subjected to HHP treatment at 300 MPa, pH 6.8, and 20 °C. Then, the HHP-treated solutions and control sample were adjusted to pH 1.5 with 1 M HCl and were preincubated in a water bath at 37 °C for 3−5 min. Then, an amount of pepsin (20 mg of pepsin per mL of 0.1 M KH_2PO_4, pH 2, buffer) was added, and the ratio of enzyme to protein substrate was 1:100 (u/w). The mixtures of pepsin and protein solution were mixed well and incubated at 37 °C at 0, 1, 5, 10, 20, 30, 60, and 120 min periods of time, aliquots of the mixtures were taken, and the pH was adjusted to 7.0 with 1.0 M NaOH to stop the enzymatic reactions. Additionally, the final pepsin-digested hydrolysates (pH 7.0) were further digested by the addition of trypsin at an enzyme: substrate ratio of 1:20 (u/w) (20 mg of trypsin per mL of 0.1 M Tris-HCl buffer, pH 7.0) at 37 °C for 0, 1, 5, 10, 20, 30, 60, and 120 min, respectively.

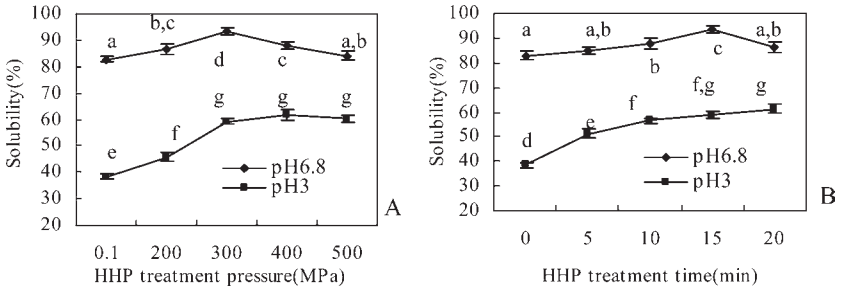

Figure 1. Effects of HHP treatment pressure and time on the solubility of SPI (A, HHP treatment time is fixed at 15 min; B, HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

Nitrogen Release during Digestion. The % nitrogen release during digestion process was determined by the method of Iwami et al.,[24] with some modifications. Ten milliliters of 10% (w/v) trichloroacetic acid (TCA) was added into 10 mL of the digested mixtures, and the final concentration of TCA reached 5% (w/v). The mixtures were then centrifuged (8000*g*, 30 min) to obtain the precipitates. After they were washed with 10 mL of TCA (10%, w/v), the precipitates were obtained again by centrifugation at the same parameters. The N content of the samples was determined by Kjeldahl method (N × 6.25). The % N release during the digestion was calculated as % N release = $(N_0 - N_t) \times 100/N_{tot}$, where t is the digestion time (min), N_t (mg) is the TCA-insoluble N after digestion for t (min), N_0 (mg) is the TCA-insoluble N in the protein sample, and N_{tot} (mg) is the total N of protein sample. Measurements were performed in triplicate.

ζ-Potential Determination. The ζ-potential was measured according to the method of Tang et al.[25] The ζ-potential profiles of various SPIs were measured using a Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, Worcestershire, United Kingdom). Freshly prepared protein dispersions were diluted to 2 mg/mL with 10 mM PBS (pH 7.0) and filtered through a 0.45 μm HA Millipore membrane prior to analysis. Measurements were performed in triplicate.

Near-UV CD Spectroscopy. Near-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The Near-UV CD spectroscopic measurements were performed according to the method of Tang et al.[25] The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette with a protein concentration around 1.0 mg/mL. The sample was scanned over a wavelength range from 250 to 320 nm. For both measurements, the spectra were an average of eight scans. Used were the following parameters: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 0.5 nm; and sensitivity, 100 mdeg. The cell was thermostatted with a Peltier element at 2 °C unless specified otherwise. The concentration of the proteins was determined by the Bradford method, using BSA as the standard.[26] Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. A mean value of 112 for the amino acid residue was assumed in all calculations, and CD measurements were expressed as mean residue ellipticity (q) in deg cm^2 $dmol^{-1}$. Data were the means of duplicate measurements.

SDS−Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli,[27] using 12% separating gel and 4% stacking gel. The protein samples were solubilized in 0.125 M Tris−HCl buffer (pH 6.8), containing 1% (w/v) SDS, 2% (v/v) 2-ME, 5% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, and heated for 5 min in boiling water. Then, samples were centrifuged (10000*g*, 10 min) before electrophoresis. Samples were loaded at 10 μL/channel and run at 25 mA constant current. After the electrophoresis, the gel was dyed in 0.25% Coomassie blue (R-250) in the 50% trichloroacetic acid and then destained in 7% acetic acid [methanol:acetic acid:water, 227:37:236 (v/v/v)]. The samples for nonreducing SDS-PAGE experiments were conducted as the same mentioned-above procedure, just without 2-ME.

Statistical Analysis. Data reported are mean values and standard deviations. Data were analyzed using SPSS for Windows (version 15.0, SPSS Inc. Chicago, IL) following an analysis of variance (ANOVA) one-way linear model. Mean comparisons were performed using the Duncan test, and the significance level was established for $P < 0.05$.

RESULTS AND DISCUSSION

Solubility and WHC. Influences of HHP treatment on solubility of SPI are shown in Figure 1. SPI presented higher solubility at pH 6.8 than at pH 3. This phenomenon can be attributed to protein aggregation near the isoelectric point (pI). For SPI treated at pH 3 and pH 6.8, solubility significantly ($P < 0.05$) increased in the range of 200−300 MPa and 5−15 min, while no differences of solubility at pH 3 were observed, and significant ($P < 0.05$) decreases of solubility were seen at pH 6.8 with pressure increasing and time elongating. At lower pressure and time levels, unfolding of SPI structure can enhance the protein−solvent interactions, thereby enhancing the solubility. On the other hand, at higher pressure and time levels, the declines of solubility can be related to the formation of some insoluble high molecular weight aggregates due to exposure of hydrophobic residues and/or SS formation. It is noteworthy that the HHP-treated SPI showed the significant increase in surface hydrophobicity (data not shown). Moreover, the following SDS-PAGE result (without 2-ME) may provide some explanations for the decrease of solubility.

Similar results were observed by Puppo et al.,[12] which showed that HHP treatment at 200−400 MPa increased the solubility of SPI at pH 3.0 and 8.0. Additionally, our results are consistent with some findings obtained by Molina et al.,[7] which indicated that 7S and SPI with 10% (w/v) concentration treated at 200 MPa and pH 6.5 gave the highest increase of solubility, while the solubility decreased at 400−600 MPa. However, solubility of 7S globulin at pH 7.5 and 11S glycinin at pH 6.5 did not change significantly. Wang et al.[11] reported that HHP treatment at 200−600 MPa resulted in a slight but gradual decline in solubility of SPI. The discrepancy between our results and those reported by the above authors could be related to different cultivars and SPI preparation methods. The solubility was closely associated with the relative content of the acidic and basic subunits in the 11S glycinin.[7] Reconstituted freeze-dried solutions for HHP treatment were utilized in the current study, whereas in Wang et al.'s study, SPI solutions were freshly prepared from defatted soybean meal and were not freeze-dried prior to HHP treatment.[11]

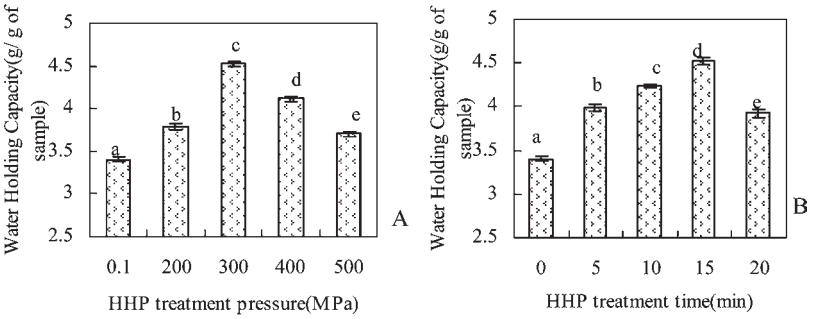

Figure 2. Effects of HHP treatment pressure and time on the WHC of SPI (A, HHP treatment time is fixed at 15 min; B, HHP treatment pressure is fixed at 300 MPa). Means with different letters are significantly different at the 5% level.

The concentration of SPI solutions is crucial for eliciting the influence of HHP treatment on functional properties, since protein−protein and protein−solvent interactions during HHP-induced aggregation would be greatly dependent upon protein concentration.[11] In the current study, the following determinations of functional properties adopted different concentrations. To alleviate the effects of concentration, the protein concentration was fixed at 1% (w/v) level. Therefore, reconstituted freeze-dried solutions for HHP treatment were utilized.

WHC and solubility belong to protein functionality related to hydration. The influences of HHP pressure and time on WHC (Figure 2) indicated the similar alteration tendency to solubility. At lower pressure and time levels, WHC of SPI significantly ($P < 0.05$) increased as compared to the native sample. It is likely that the partial unfolding of the protein will allow interactions between the subunits and help form a flexible network in which water is entrapped. However, at higher treatment levels, aggregations and precipitation of proteins are enhanced, leading to a significant ($P < 0.05$) drop of WHC. This changing trend of WHC agrees with the results obtained by Molina et al.,[22] which indicated that 11S and SPI with 20% (w/v) concentration treated at 500 MPa gave the highest of WHC, while WHC decreased at 600−700 MPa.

Emulsifying and Foaming Properties of SPI. Figure 3 indicated that HHP treatment resulted in gradual and significant ($P < 0.05$) increases of EAI in the ranges of 200−300 MPa and 5−15 min, when compared to control. However, EAI significantly ($P < 0.05$) dropped with a further increase of pressure and time, being higher than that of the native sample. The results suggest that unfolding of proteins and subsequent exposure of hydrophobic groups by HHP treatment improved the emulsifying property of SPI. Similarly, Molina et al.[7] reported that 0.25 and 0.50% SPI solutions showed the same patterns of EAI alteration after HHP treatment. Our result is also consistent with the report of Wang et al.,[11] who concluded that HHP treatment remarkably increased the EAI values of SPI at 200 MPa, while a further increase in pressure (400 and 600 MPa) did not result in a significant change in the EAI. Puppo et al.[13] also showed that SPI (1%) treated at 200−400 MPa had a significantly lower oil droplet size in emulsions and flocculation index when compared to the control. Nevertheless, the highest value of EAI was obtained at 200 or 400 MPa, which is different from the level (300 MPa) in the present study. The discrepancy may be attributed to the soybean cultivar difference. Different soybean cultivars will lead to the different ratio of 7S/11S in the SPI. The 7S, a trimer without any disulfide bonds, is more pressure labile than the 11S whose subunits are linked by many disulfide bonds.[7]

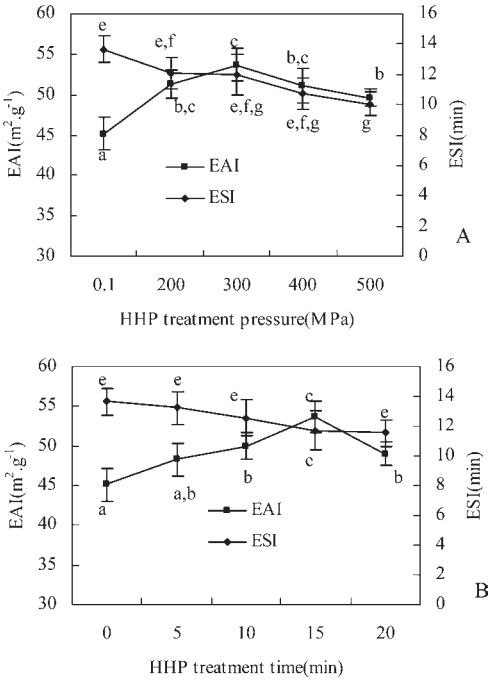

Figure 3. Effects of HHP treatment pressure and time on the EAI and ESI of SPI (A, HHP treatment time is fixed at 15 min; B, HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

HHP processing led to a gradual and significant ($P < 0.05$) decrease in ESI (Figure 3) with the increase of pressure, while no differences were observed with the elongation of time. This may result from a decline in molecular flexibility of the proteins because of aggregation, since molecular flexibility is an important factor influencing emulsion stability.[28] Moreover, Molina et al.[7] found the same ESI alteration patterns of 7S at pH 6.5 and 11S at pH 6.5 and 7.5 with pressure increasing, who presumed that this phenomenon was caused by the higher adsorption of the protein at the interface produced by high pressure. Tang et al.[25] proposed the mechanism for the emulsification process. The emulsification process could be considered to consist of at least three steps: the adsorption of protein on the O/W interface, followed by changes of quaternary and tertiary conformations, and last, formation of a stable viscoelastic film (which stabilizes the oil particles in the water medium). The first step is clearly affected by the hydrophobicity/hydrophilicity balance (or protein solubility) and highly dependent on the flexibility in quaternary conformation.[25]

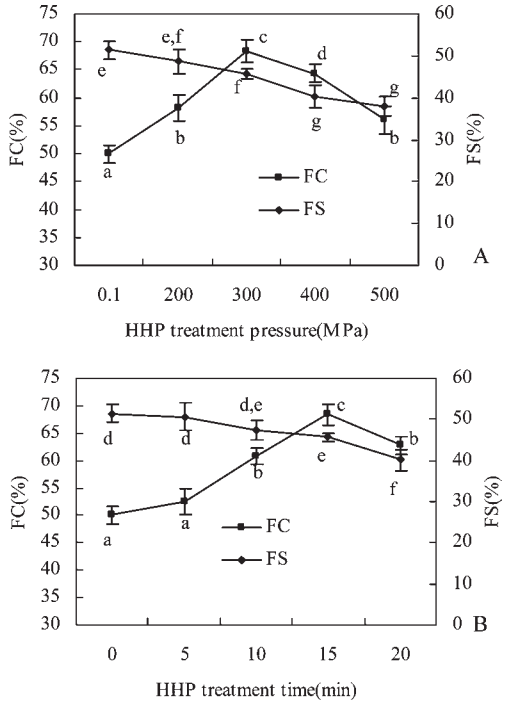

Figure 4. Effects of HHP treatment pressure and time on the FC and FS of SPI (A, HHP treatment time is fixed at 15 min; B, HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

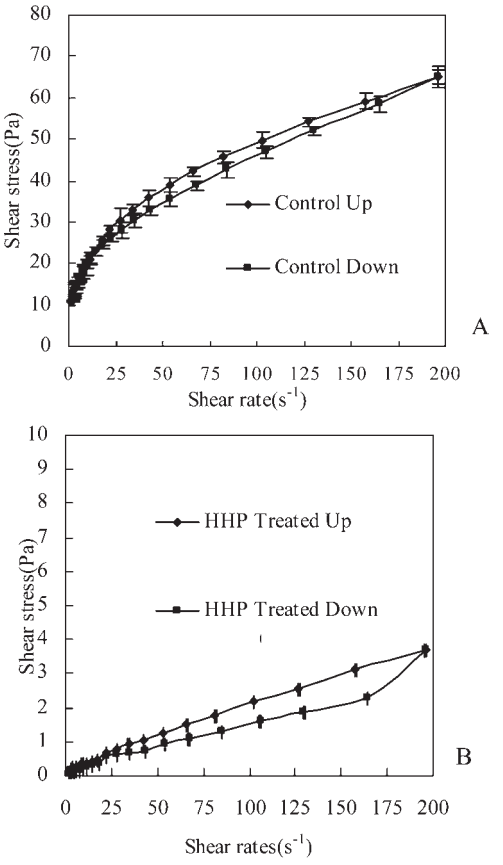

Figure 5. Thixotropic response of aqueous SPI solutions (A, control SPI; B, HHP treated at 300 MPa for 15 min).

Unfolding of the tertiary conformation caused by HHP is of vital importance for the emulsion stability. Therefore, the tertiary conformation was determined in the following steps.

Emulsifying and foaming activities belong to the functional properties related to the protein surface. Figure 4 indicated that FC was significantly ($P < 0.05$) enhanced in the range of 200−300 MPa and 5−15 min, and then, at higher treatment levels, FC significantly ($P < 0.05$) decreased. In contrast, HHP processing led to a gradual and significant ($P < 0.05$) decrease in FS. However, Krešić et al.[29] observed that the increases in both hydrostatic pressure and treatment time significantly improved the foaming ability and stability of whey protein isolate. The discrepancies may be caused by the type of food protein and the protein concentration.

HHP improved the solubility in the aqueous phase and partially unfolded the structures of the SPI in the range of 200−300 MPa and 5−15 min, thus promoting the adsorption during bubbling and reduction of the air−water interface tension. However, if the structures of proteins are excessively unfolded, the intermolecular interactions are not prone to form. On the contrary, the partial unfolding of structures tends to form networks via nonconvalent interactions with high viscoelastic and mechanical properties. If the protein owns good FC and stability, its structure should keep a suitable balance between flexibility and rigidity.[30] Therefore, at higher pressure and time levels, the decrease of FC and stability maybe result from the unbalance of the conformational flexibility and rigidity. The following tertiary conformation measurements will provide some explanations for the foaming activity alteration.

Thixotropy of SPI and Textural Properties of SPI Gels. It is known that the thixotropy of a macromolecular solution quantified by the solution ability to regain its gel structure when the solution is allowed to rest for a longer period of time, which is usually attributed to the breakdown/alignment of the macromolecular chains or segments.[31] To characterize the thixotropic property of aqueous SPI solutions, the "upward" and "downward" curves developed during round-trip stress−shear rate paths are presented in Figure 5, and the areas of the corresponding hysteresis loops are determined in Table 1. HHP-treated and native SPI solution both followed the Power law model. As can be seen, the hysteresis area of HHP-treated SPI was remarkably smaller than that of the native SPI, and the viscosity of HHP-treated SPI was considerably lower than that of control. Igarashi et al.[32] demonstrated that the thixotropic property of foods can affect the aspect of oropharyngeal swallowing. Therefore, HHP-treated SPI indicated low difficulty or shear stress in swallowing for infants. To our knowledge, this thixotropic behavior has not been revealed for aqueous SPI solutions.

Table 1. Analysis of Rheology Results by Rheology Advantage Data Analysis Software[a]

	control	HHP treated
fluid model	power law	power law
b: viscosity (Pa s)	8.301	0.04630
c: rate index	0.381	0.7973
thixotropy (Pa s^{-1})	2384	191.4
standard error	12.74	13.03

[a] Power law model equation: stress = $b \times$ rate^c. The standard error was from the curve fitting.

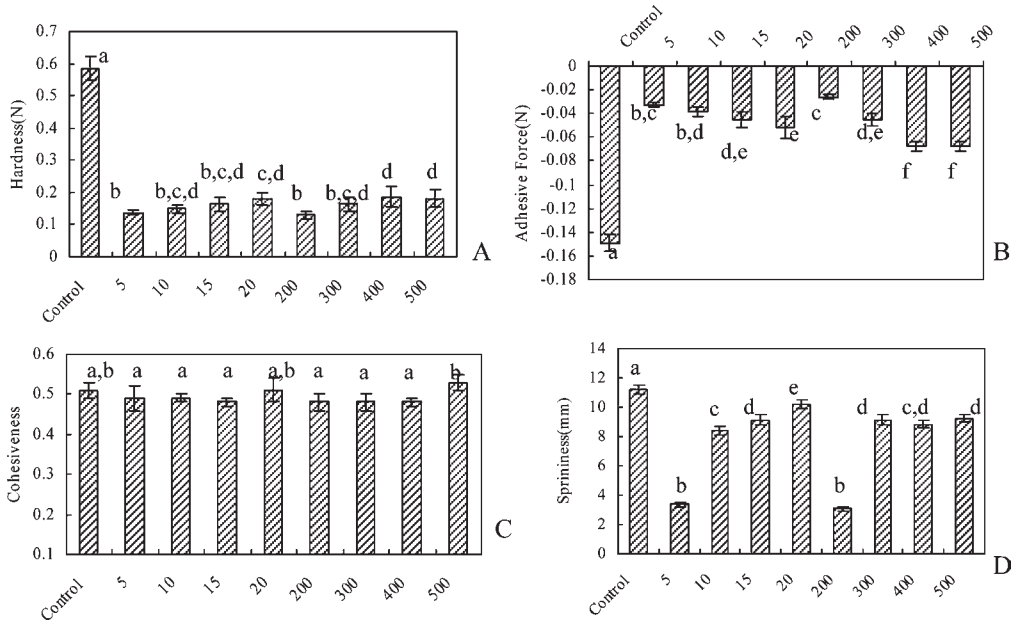

Figure 6. Effects of HHP treatment pressure and time on hardness, adhesive force, cohesiveness, and springiness of SPI gels (HHP treatment pressure is fixed at 300 MPa, and 5, 10, 15, and 20 separately indicate HHP treatment time; HHP treatment time is fixed at 15 min, and 200, 300, 400, and 500 separately indicate HHP treatment pressure). Means with different letters are significantly different at the 5% level.

Influences of HHP treatment on the textural properties are shown in Figure 6. The hardness, adhesive force, and springiness of HHP-treated SPI gels were significantly ($P < 0.05$) lower than those of control, while SPI yielded significantly ($P < 0.05$) increased hardness, adhesive force, and springiness with the elongation of time and increasing of pressure. The cohesiveness of HHP-treated SPI gels and control did not show significant differences. The alteration tendency of hardness, adhesive force, and cohesiveness was consistent with the findings obtained by Monila et al.[22] At 20% (w/v) protein concentration, SPI produced self-supporting gels at 300−700 MPa. HHP-induced gels gave significant lower adhesiveness and hardness and indicated slightly lower springiness and cohesiveness when compared to the heat-induced gels. Dumoulin et al.[33] found that the gels of soy protein produced at 300 MPa and above were softer than the heat-induced gels, but the hardness increased with increasing pressure. Okamoto et al.[34] demonstrated that the pressures (100−700 MPa) could affect the textural properties of some food proteins, including egg white and yolk, crude actomyosin, rabbit meat, and soy protein. In these HHP-induced gels, hardness increased and adhesiveness decreased with the increasing of pressures. However, in the present study, the hardness, adhesiveness, and springiness exhibited ascending trends as the pressure and time levels rose. These discrepancies may be attributed to the molecular weight of protein and the interaction forces involved in gels. Wang et al.[35] stated that the hardness of a globular protein gel at a given concentration was fundamentally related to the average molecular weight (M_W) of the protein. The greater the M_W, the greater would be the gelling power. The rise in the chain length of the polypeptide may enhance molecular entanglement in gel structure, which might restrict the relative thermal movements of the polypeptides.[35] The following SDS-PAGE results verified HMW subunits appearances. Therefore, the hardness increased at higher pressure and time levels. Furthermore, the proportion of hydrogen bonds, hydrophobic interactions, electrostatic interactions, and disulfide bonds are different in heat- and pressure-induced gels. More disulfide and other covalent bonds were found in heat-induced than in pressure-induced gels, explaining the higher hardness, adhesive force, and springiness of the former ones. Gels formed upon pressurization at lower temperature and pressure were characterized by a greater presence of ionic and hydrogen bonds, whereas gels obtained under higher pressure and temperature were characterized by a higher proportion of hydrophobic interactions.[36] Therefore, the following determination of ζ-potential, surface hydrophobicity, and SDS-PAGE will provide proof for the alteration tendency of textural properties of SPI gel.

In addition, the calculated parameter, chewiness, was the product of hardness × cohesiveness × springiness, which expressed energy required to chew a solid for swallowing. The chewiness values of control and sample treated at 300 MPa and 15 min were, respectively, 3.36 and 0.72 mJ, which was consistent with the thixotropic determinations.

In Vitro Digestibility of SPI. Figure 7 shows a typical profile of the nitrogen release of SPIs during sequential pepsin and trypsin digestion, as a function of digestion time. In addition, the % N release at the end of pepsin and trypsin digestion for SPI treated at 300 MPa and 15 min significantly ($P < 0.05$) increased by 6 and 7%, respectively, as compared with the native SPI.

Some proteins have been reported to exhibit an improved proteolytic digestibility when submitted to HHP.[37] This can be due to conformational changes in the protein that make it vulnerable to proteolysis, because, under high pressure conditions, protein unfolding can expose new cleavage sites to enzymatic hydrolysis. [38] In addition, enhancement of proteolysis under pressure has also been attributed to pressure effects on the enzyme and/or to effects on the substrate−enzyme interaction. [39] It is hypothesized that, when the enzyme treatments are conducted under pressure, unfolding of the hydrophobic core exposes new cleavage sites to enzymatic hydrolysis, so that proteolysis precedes protein aggregation by disulfide bonds that would otherwise diminish the accessibility to the hydrolytic enzymes. [40]

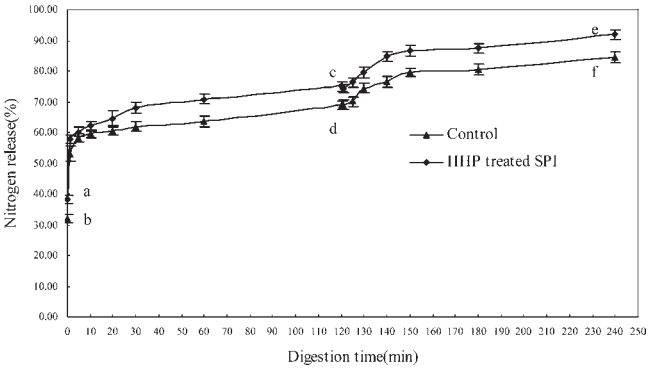

Figure 7. Typical profiles for the % nitrogen release during pepsin and trypsin digestion of native SPI and HHP treated SPI at 300 MPa and 15 min. Means with different letters at the same digestion time are significantly different at the 5% level.

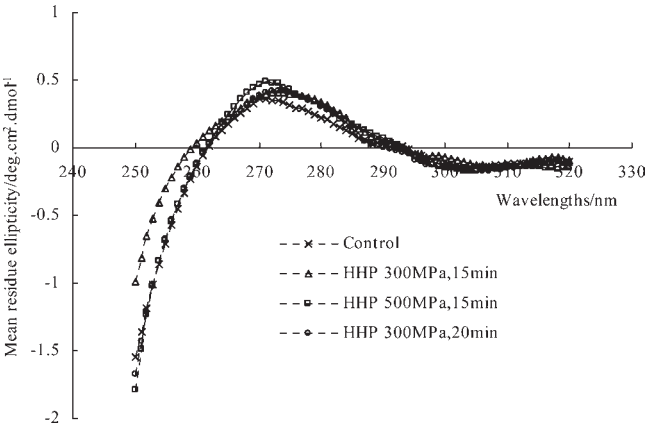

Figure 8. Typical near-UV CD [spectra of various SPIs solubilized in 10 mM phosphate buffer (pH 7.0)].

The enhanced in vitro digestibility of SPI for infant formula will benefit infants' growth. Overall, the novel processing technology, HHP, can significantly influence some functional and nutritional properties of SPI for infant formula.

ζ-Potential of SPI. It is well-known that the electrostatic force plays a key role in maintaining the structure of protein. Protein molecules behave as zwitterions carrying a net positive or negative charge depending on their isoelectric point and the pH of the environment. When charged groups are present in an ionic medium, a diffuse layer of ions is formed around the charge molecule to construct an electrical double layer.[15] Samples carried negative charges at pH 7.0, because the isoelectric point of SPI is 4.3. HHP treatment exhibited no evident influences on the ζ-potential of SPI (data not shown). The above double layer can result in the increase of volume of charged groups.[15] On the application of high pressure, the volume of a protein declines due to the compression of the internal cavities. In addition, the hydration of the proteins decreased, which counteracts this volume decline. As a result, high pressure leads to only a small decrease in volume in the case of several globular proteins.[41] SPI belongs to the above globular proteins. Therefore, HHP treatment cannot significantly influence the electrostatic interactions of SPI.

Near-UV CD Spectra. The tertiary and quaternary conformations of the SPIs were analyzed by near-UV CD spectroscopic technique. The CD spectra in the region 250−320 nm arise from the aromatic amino acids. Tryptophan (Trp) shows a peak close to 290 nm with fine structure between 290 and 305 nm; tyrosine (Tyr) exhibits a peak between 275 and 282 nm, with a shoulder at longer wavelengths; phenylalanine (Phe) indicates weaker but sharper bands with fine structure between 255 and 270 nm.[42] The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of aromatic amino acids present, their mobility, the nature of their environment (H-bonding, polar groups, and polarizability), as well as their spatial disposition in the protein.[42]

Figure 8 shows the typical near-UV CD spectra of the SPIs at pH 7.0. The near-UV CD spectra of these SPIs consisted of a prominent positive dichroic band at around 275 nm and a weak shoulder band at about 260 and 290 nm, clearly contributed by Tyr, Phe, and Trp residues, respectively. The Tyr band magnitude of HHP treated at 500 MPa and 15 min was much higher than that of control and the other HHP treated SPIs. The Phe band magnitude of HHP treated at 300 MPa and 15 min was much higher than that of control and the other HHP-treated SPIs. Because the Trp band magnitude in these SPIs was similar, the differences in the Tyr and Phe band magnitude could be mainly attributed to the differences in tertiary and/or quaternary conformations. Thus, in present study, associating near-UV CD spectra with the solubility, WHC, EAI and ESI, FC, and FS, the much higher Tyr band magnitude of SPI treated at 500 MPa and 15 min, to a great extent, indicated much lower flexibility in quaternary conformation than the control and the other HHP-treated SPIs. This alteration trend of Tyr bond magnitude is consistent with the findings by Tang et al.[25] Moreover, the much higher Phe band magnitude of SPI treated at 300 MPa and 15 min may reveal higher flexibility in quaternary conformation than native SPI and the other HHP-treated SPIs. The molecular weights of Phe, Tyr, and Trp were 165.2, 181.2, and 204.2,[43] respectively, which will provide some proof for the relative higher flexibility in quaternary conformation with higher magnitude of Phe. Consequently, SPI, treated at 300 MPa and 15 min, owned the highest EAI and FC.

SDS-PAGE. Using SDS-PAGE electrophoresis, differences in subunit composition in native SPI and HHP-treated SPI were observed. Figure 9 summarizes the electrophoretic migration of the various subunits in the samples before and after HHP processing. Glycinin and β-conglycinin fractions showed different alteration trends (Figure 9A). With the increase of pressure and elongation of time, the bands of α′, α, β, AB, A_1, A_2, and A_4 became darker than the native sample, whereas the bands of A_3 and B showed no obvious difference. Moreover, the fraction densities of α′, α, and β indicated apparent changes at 300 MPa and 10 min. The fraction densities of AB, A1, A_2, and A_4 gave obvious changes at 300 MPa and 15 min. This discrepancy of HHP treatment time verified that β-conglycinin was more pressure labile than glycinin. This finding is consistent with Molina et al.[7]

Figure 9B indicated the nonreducing electrophoretic patterns of native SPI and HHP-treated SPIs. The solubility of SPIs in nonreducing sample buffer is lower than that of reducing patterns, by comparing the densities of the bands. As compared to the reducing electrophoretic patterns, native sample and SPIs (treated at 300 MPa and 20 min and 500 MPa and 15 min), showed the similar alteration tendency. Two bands of high molecular weight (HMW) subunits appeared, verifying that the

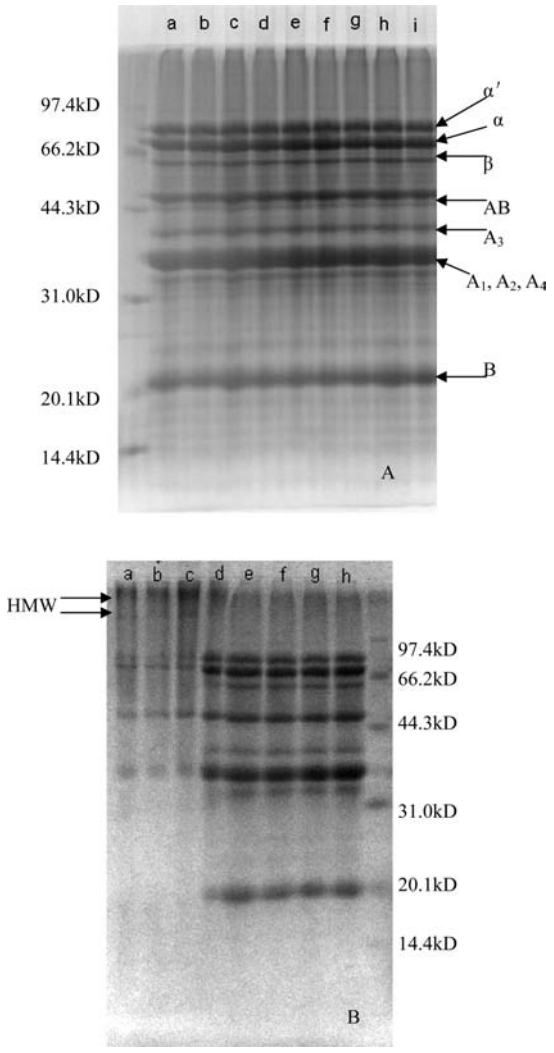

Figure 9. SDS-PAGE of control SPI and HHP-treated SPI. (A) Lane distribution: a, control; b, 300 MPa, 5 min; c, 300 MPa, 10 min; d, 300 MPa, 15 min; e, 300 MPa, 20 min; f, 500 MPa, 15 min; g, 400 MPa, 15 min; h, 300 MPa, 15 min; and i, 200 MPa, 15 min. (B) Lane distribution: a, control, without 2-ME; b, 300 MPa, 20 min, without 2-ME; c, 500 MPa, 15 min without 2-ME; d, 300 MPa, 15 min, without 2-ME; e, 300 MPa, 20 min, with 2-ME; f, 500 MPa, 15 min, with 2-ME; g, control, with 2-ME; and h, 300 MPa, 15 min, with 2-ME.

new aggregates associated via disulfide linkage. However, the SPI treated at 300 MPa and 15 min exhibited the similar subunit distributions in reducing and nonreducing conditions. According to Messens et al.,[15] high pressure effects on protein are primarily related to the rupture of noncovalent interactions within protein molecules and to the subsequent reformation of intra- and intermolecular bonds within or between protein molecules. The van der Waal's interactions and hydrogen bonds are insensitive to pressure.[15] HHP exhibited no obvious influence on ζ-potential describing the electrostatic interactions. Therefore, it could be drawn a sound conclusion that HHP mainly rendered the changes of SPI conformation at 300 MPa and 15 min via the hydrophobicity. This is further confirmed by the hydrophobicity determination (data not shown). Wang et al.[11] asserted that SDS-PAGE analyses of untreated and HHP-treated SPI in the presence or absence of β-mercaptoethanol had confirmed the relative contribution of the formation of SS bonds to aggregates formed after HHP treatment. On the basis of the above electrophoretic analyses, these findings could provide some explanations for the effects of HHP treatment on the solubility, EAI, ESI, FC, FS, and gel properties.

The mechanism was verified from the three aspects, including molecular interaction forces, tertiary structure, and subunit distribution. Although HHP exhibited no action on the ζ-potential of SPI, this technology can exert its application via its influences on the hydrophobicity, disulfide bonds, and tertiary conformations. These results suggest that HHP could be used to modify the properties of SPI by appropriate selection of pressure and time levels.

Apart from the above aspects, our research team is conducting the effects of HHP on reducing the allergenicity of SPI for infant formula. The processing cost of SPI is approximately 10−20% of whey protein isolate processing costs.[44] Our results provide direct evidence for the potential utilization of HHP in infant formula processing, especially in developing countries, where some families cannot afford the milk-based infant formula. However, they can choose soy-based infant formula for a relatively low cost.

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