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Structural Rearrangement of Ethanol-Denatured Soy Proteins by High Hydrostatic Pressure Treatment

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ABSTRACT: The effects of high hydrostatic pressure (HHP) treatment (100−500 MPa) on solubility and structural properties of ethanol (EtOH)-denatured soy β-conglycinin and glycinin were investigated using differential scanning calorimetry, Fourier transform infrared and ultraviolet spectroscopy. HHP treatment above 200 MPa, especially at neutral and alkaline pH as well as low ionic strength, significantly improved the solubility of denatured soy proteins. Structural rearrangements of denatured β-conglycinin subjected to high pressure were confirmed, as evidenced by the increase in enthalpy value (ΔH) and the formation of the ordered supramolecular structure with stronger intramolecular hydrogen bond. HHP treatment (200−400 MPa) caused an increase in surface hydrophobicity (F_{max}) of β-conglycinin, partially attributable to the exposure of the Tyr and Phe residues, whereas higher pressure (500 MPa) induced the decrease in F_{max} due to hydrophobic rearrangements. The Trp residues in β-conglycinin gradually transferred into a hydrophobic environment, which might further support the finding of structural rearrangements. In contrast, increasing pressure induced the progressive unfolding of denatured glycinin, accompanied by the movement of the Tyr and Phe residues to the molecular surface of protein. These results suggested that EtOH-denatured β-conglycinin and glycinin were involved in different pathways of structural changes during HHP treatment.

KEYWORDS: high hydrostatic pressure, ethanol denaturation, β-conglycinin, glycinin, structural rearrangement, hydrophobicity

INTRODUCTION

Soy proteins as functional ingredients have been extensively used in food products owing to their high nutritional quality and excellent functional properties. β-Conglycinin and glycinin are the two main protein fractions of soy proteins, accounting for about 70% of the total seed protein. β-Conglycinin is a trimer with a molecular mass of approximately 180−200 kDa, composed of three subunits: α (~67 kDa), α′ (~71 kDa), and β (~50 kDa). The α and α′ subunits contain the core regions and the extension regions, while the β subunit consists of only the core region.[1] Glycinin is an oligomeric protein with a molecular mass of approximately 350 kDa. The individual glycinin monomer consists of an acidic polypeptide (38 kDa) and a basic one (20 kDa) linked by a single disulfide bridge.[1] Soy protein concentrate (SPC), as one of the main industrial soy protein ingredients, is predominantly obtained by soaking defatted flakes with 60−70% aqueous alcohol. This process can significantly improve color and flavor of SPC.[2] Unfortunately, alcohol-washed SPC generally has low protein solubility (nitrogen solubility index, < 12%) due to protein denaturation, which greatly limits its application in liquid food system, such as dairy products and plant protein drink.[2]

Using thermal analysis, the loss of denaturation enthalpy value (ΔH) of β-conglycinin (94.8%) is higher than that of glycinin (48.1%) during SPC preparation.[3] Conformational transitions of both β-conglycinin and glycinin to α-helix structure occur during EtOH treatment at acidic and alkaline pH.[4,5] In fact, different from heat denaturation, EtOH is generally considered to weaken noncovalent bonds in protein, and simultaneously to enhance local hydrogen bonds because of its lower dielectric coefficient.[6,7] Thus, the formation of an α-helical conformation appears to be a generic alcohol-denatured behavior for many globulin proteins.[8] Moreover, the exposure of hydrophobic side chains and the burying of polar amide groups through the formation of helix structure may be the reasons for the insolubility of EtOH-denatured proteins.[9]

High hydrostatic pressure (HHP) treatment, as an athermal technology, has been widely applied to modify structural and physicochemical properties of food proteins.[10,11] The HHP modifications of soy proteins greatly depend on the system conditions (e.g., pressure level, pH, type of protein and protein concentration).[12−14] Depending on the pressure levels, HHP treatment leads to the denaturation of β-conglycinin and glycinin in soy milk and the dissociation of soy proteins into subunits.[15] Puppo et al.[12] reported that, at neutral and acidic pH, soy proteins treated above 200 MPa showed more disordered structures, accompanied by the formation of aggregates, especially for glycinin. The establishment of hydrophobic interactions and disulfide bonds may be responsible for the formation of soluble aggregates from the different subunits of β-conglycinin and glycinin.[13,16] In addition, at neutral pH, an increase in surface hydrophobicity and a reduction in free sulfhydryl content have been showed for soy protein isolated (SPI) after HHP treatment.[12,14] In terms of functional properties, HHP treatment significantly improves the emulsifying activity index of SPI by strengthening

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protein adsorption at the oil/water interface, especially for the β-subunit of β-conglycinin and the acidic polypeptides of glycinin.[17]

Recently, it has been demonstrated that HHP treatment can promote protein dissolution and refolding from insoluble aggregates and inclusion bodies to reconstitute the native structure of protein, and even to recover biological activity.[18] High refolding yields (>98%) were found for some biotherapeutic proteins, such as endostatin and recombinant human growth hormone.[19,20] High pressure refolding of protein probably results from the disruption of noncovalent interactions and the reformation of hydrogen bonds at high pressure condition, which take place with subsequent refolding in a single process step.[18,20]

HHP modifications of soy proteins have been extensively studied, especially for proteins with native structure. However, to date, the molecular interactions and the conformational changes of denatured food proteins subjected to high pressure have not been characterized. Therefore, the present work focused on investigating whether HHP treatment could rearrange structure of EtOH-denatured β-conglycinin and glycinin during the dissolution of insoluble soy proteins. The changes in structural properties, including thermal behavior, secondary structure and distribution of aromatic acid residues, were investigated using differential scanning calorimetry, Fourier transforms infrared and ultraviolet spectroscopy.

MATERIALS AND METHODS

Materials. Defatted soy flour was provided by Shandong Yuwang Industrial & Commercial Co., Ltd. (Shandong, China). Bovine serum albumin (BSA) was obtained from Fitzgerald Industries International Inc. (Concord, MA). Potassium bromide (KBr) and 1-anilinonaphthalene-8-sulfonic acid (ANS) reagents were purchased from Sigma-Aldrich (St. Louis, MO). Bovine thyroid (669 kDa), rabbit muscle (158 kDa) and chicken egg white (75 kDa) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). All other reagents and chemicals in the work were of analytical or better grade.

Preparation of EtOH-Denatured Soy β-Conglycinin and Glycinin. The native β-conglycinin and glycinin were prepared according to the method of Nagano et al.[21] The protein contents of β-conglycinin and glycinin were 88.42 ± 2.17% and 94.08 ± 0.36%, respectively (determined by micro-Kjeldahl method, $N \times 5.71$, wet basis). The native proteins were soaked in freshly prepared 65% (v/v) EtOH/water mixtures (1:15, w/v), and gently stirred at 25 °C for 4 h. The slurry was filtered by filter paper, and the resultant cake was air-dried at room temperature. The dried materials (EtOH-denatured β-conglycinin and glycinin) were lightly ground using a mortar and pestle to obtain fine powder, and stored at room temperature prior to use.

HHP Treatment. Denatured β-conglycinin and glycinin were dispersed (5 mg/mL) in 50 mM Tris-HCl buffer (pH, 6.4−8.4). This buffer was selected for its relatively low pH dependency on pressure because of a small volume of ionization (4.3 ± 0.5 $cm^3 \cdot mol^{-1}$).[22] Sodium chloride (at a concentration of up to 200 mM) was used to adjust the ionic strength of the buffers. Soy protein dispersions were vacuum-conditioned in a polyethylene bag after hydration overnight at 4 °C, and then subjected to a range of pressure for 30 min at ambient temperature in a 5 L reactor unit of Datong Pressure system (KEFA Hitech Food Machine Company Co. Ltd., China) using oil as the pressure medium. The rates of pressure increase and release were about 100 MPa/min and 300 MPa/min, respectively. The pressurized samples were kept at room temperature for 6 h after decompression to restore protein structure.

The partial pressurized dispersions were centrifuged at 10000*g* for 20 min in a CR22G centrifuge (Hitachi Co., Japan) to evaluate the HHP-induced solubilization of denatured proteins. The protein concentrations of supernatant were measured by Lowry's method,[23] with BSA as standard using a UV2300 spectrophotometer (Techcomp, Shanghai, China). Because of the low solubility of denatured proteins, the rest of the pressurized protein dispersions were directly dialyzed against distilled water at 4 °C overnight, freeze-dried and then stored at −20 °C prior to further analysis to obtain good comparability among all samples. After freeze-drying, no obvious changes in protein solubility and structure were observed, as evidenced by circular dichroism and fluorescence spectra.

Gel Permeation Chromatography (GPC). GPC analysis was performed on a Waters Breeze system equipped with a Waters 1525 pump and Waters 2487 UV detector (Waters, USA). Native and HHP-treated proteins (2 mg) were dissolved in 0.5 mL of 50 mM sodium phosphate (pH 7.2) containing 50 mM NaCl, centrifuged at 10000*g* for 20 min, and filtered through 0.45 μm filters (Millipore, Fisher Sci.). The same buffer was used as mobile phase, and aliquots (20 μL) were injected into a prepacked TSK G4000SWxl column (TOSOH, Japan). The flow rate was 0.5 mL/min. The elution was monitored by absorbance at 280 nm. Bovine thyroid (669 kDa), rabbit muscle (158 kDa) and chicken egg white (75 kDa) were used as the standard proteins for calibration.

Differential Scanning Calorimetry (DSC). The denaturation degree and thermal stability of proteins were determined by DSC measurements performed using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE 19720, USA). The calorimeter was calibrated using an indium standard. Native, denatured and HHP-treated proteins (2 mg) were weighed into coated aluminum pans, and 10 μL of 10 mM phosphate buffer (pH 7.2) was added. In all cases, the sealed pans containing protein samples and buffers were equilibrated at 25 °C for more than 4 h. The samples were analyzed at a rate of 5 °C/min from 25 to 100 °C. An empty aluminum pan was used as a reference. Denaturation temperature (T_d) and denaturation enthalpy (ΔH) were analyzed from the thermograms by the Universal Analysis 2000 software, Version 4.1D (TA Instruments-Waters LLC).

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra of proteins were measured with a Vector33 model Fourier transform infrared instrument (Bruker Co., Germany) at 25 °C. Typically, 256 interferograms covering a spectral range from 4000 to 400 cm^{-1} region at a 2 cm^{-1} interval were collected for each spectrum. A total of 32 scans were performed at 4 cm^{-1} resolution and were averaged. Protein samples were prepared using a KBr pellet method.[24] Native, denatured and HHP-treated proteins (1 mg) were mixed with 100 mg of ground KBr, and pressed in a pellet at 10 tons and 1 min. Reference spectra were recorded under identical conditions except that the media (KBr) contained no protein.

The subtractions and Fourier self-deconvolutions (FSD) by application of a Lorentzian line shape were performed to study the amide I region of proteins using Peak Fit 4.12 software (SeaSolve Software, Inc., CA). Full-width at half-maximum (fwhm) was 10.5 cm^{-1} and was kept constant for all peaks during deconvolution. The second-derivative spectra were used to ensure that the spectra were not over-deconvoluted by comparing the number and positions of the bands with those in the deconvoluted spectra. Band assignments in the amide I region (1600−1700 cm^{-1}) and quantitative estimation of secondary structure components were executed according to the method of Byler et al.[25] The areas of the bands were calculated by integration of the corresponding fitted band.

Surface Hydrophobicity. Protein surface hydrophobicity was determined by titration with ANS according to the method of Liu et al.,[26] with modifications as described below. Native and HHP-treated proteins were dissolved in 10 mM sodium phosphate (pH 7.2) and centrifuged at 10000*g* for 20 min. The protein concentrations of all samples were adjusted to 0.2 mg/mL. Fluorescence of the samples was

measured as a blank before ANS titration. The solutions (1 mL) were placed in the cell of an F7000 fluorescence spectrophotometer (Hitachi Co., Japan), and then aliquots (10 μL) of ANS (5.0 mM in 10 mM phosphate buffer, pH 7.2) were titrated to reach a final concentration at 50 μM for ANS. The molar coefficient (5000 M^{-1} cm^{-1} at 350 nm) was used to calculate ANS concentration.[26] The relative fluorescence intensity (F) was measured at 390 nm (excitation, slit width 5 nm) and 470 nm (emission, slit width 5 nm). All determinations were conducted three times. Data were elaborated using the Lineweaver−Burk equation:

$$1/F = 1/F_{max} + (K_d/L_0)(1/F_{max})$$

where L_0 is the fluorescent probe concentration (μM), F_{max} is the maximum fluorescence intensity (at saturating probe concentration) and K_d is the apparent dissociation constant of a supposedly monomolecular protein/ANS complex. F_{max} and K_d can be obtained by standard linear regression fitting procedures. The ratio F_{max}/K_d, corrected for the protein content, represents the protein surface hydrophobicity index (PSH), taking into account both the number and affinity of hydrophobic sites.

Ultraviolet (UV) Spectroscopy. Native and HHP-treated proteins were dissolved in 10 mM sodium phosphate (pH 7.2) and centrifuged at 10000g for 20 min. The protein concentrations of all samples were adjusted to 0.3 mg/mL. Baseline corrected UV spectra were recorded between 250 and 300 nm at medium speed in the double beam mode using a UV2300 spectrophotometer (Techcomp, China). The data interval was 0.1 nm. Second-derivative UV spectra were analyzed using Origin 8.0 software (Origin-Lab Corp., Northampton, MA).

Statistics. An analysis of variance (ANOVA) of the data was performed using the SPSS 13.0 statistical analysis system, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

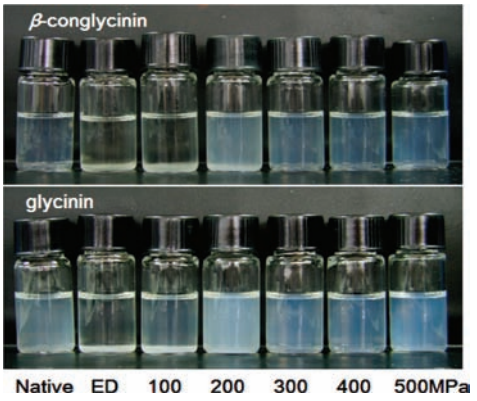

Figure 1. The images of native, EtOH-denatured (ED) and HHP-treated (100−500 MPa) β-conglycinin and glycinin dispersions at pH 8.0.

RESULTS AND DISCUSSION

The Solubilization of EtOH-Denatured Soy Globulins. As shown in Figure 1, the β-conglycinin precipitates appeared at the bottom of the flasks after EtOH treatment, indicating the insolubility of protein. HHP treatment caused the gradual disappearance of these precipitates with increasing pressure from 200 to 500 MPa. However, these precipitates were not dissolved by HHP treatment at 100 MPa. Similar phenomena were observed for glycinin. These observations may suggest that HHP treatment above 200 MPa can be used to recover the solubility of EtOH-denatured soy proteins. Figure 2 shows the solubility of native, EtOH-denatured and HHP-treated soy proteins. After EtOH treatment, the solubility of native β-conglycinin and glycinin significantly ($p < 0.05$) decreased,

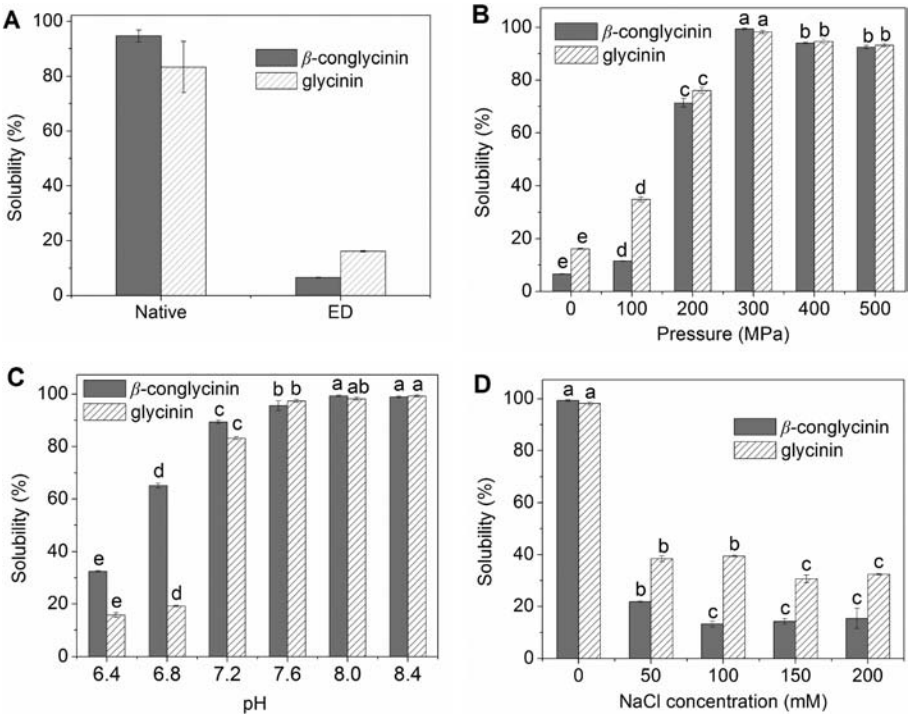

Figure 2. (A) Effects of EtOH on solubility of β-conglycinin and glycinin at pH 8.0. Effects of pressure, pH and ionic strength on HHP-induced solubilization yields of denatured β-conglycinin and glycinin incubated at 25 °C for 30 min. At the same time that pressure, pH and ionic strength were varied, and all other process variables were held constant. (B) Pressure (at pH 8.0); (C) pH (at 300 MPa); (D) ionic strength (at pH 8.0 and 300 MPa). Different letters (a−d) on the top of a column indicate significant ($p < 0.05$) differences among samples treated under different levels.

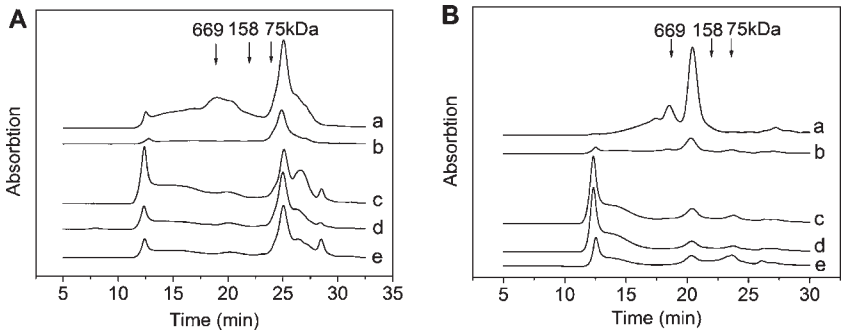

Figure 3. Gel permeation chromatography profiles of native (a) and HHP-treated β-conglycinin (A) and glycinin (B) at 200 (b), 300 (c), 400 (d) and 500 MPa (e), respectively. Denatured proteins were not tested due to low solubility.

and the values were only 6.63 ± 0.11% and 16.15 ± 0.21%, respectively. It appears that β-conglycinin shows a higher solubility loss (88.04%) than glycinin (67.24%). HHP treatment significantly ($p < 0.05$) improved the solubility of denatured soy proteins depending on the pressure conditions. The solubility of both β-conglycinin and glycinin reached a maximum value (>90%) at 300 MPa, and then decreased when pressure was further increased to 400 or 500 MPa (Figure 2B). These results revealed that the moderate pressure (300 MPa) was effective in dissociating insoluble proteins.

Structural transitions of proteins upon HHP treatment are generally concomitant with the formation of charged residues due to deprotonation.[18,27] Thus, the effects of pH and ionic strength during HHP treatment at 300 MPa on the protein solubility were investigated to further explore if electrostatic interactions contribute to the dissolution of denatured soy proteins (Figure 2C,D). The solubility of denatured β-conglycinin and glycinin significantly ($p < 0.05$) increased with increasing pH, and leveled off (>90%) when pH of buffer was increased to 7.2 (Figure 2C). In particular, after HHP treatment at 300 MPa and pH 6.8, the solubility of denatured β-conglycinin increased from 5.15 ± 0.17% to 65.15 ± 0.94%, while that of denatured glycinin only increased from 6.92 ± 0.42% to 19.16 ± 0.34% (Figure 2C). This difference in solubility improvement may be related to the differences in protein surface charges due to the exposure/formation of charged residues during HHP treatment. The absolute value of the zeta potential of β-conglycinin and glycinin dispersions distinctly increased after HHP treatment at 300 MPa (data not shown), suggestive of the increase of protein surface charges. It could be speculated that the disruption of electrostatic interactions under high pressure may be bound up with the dissolution of denatured soy proteins. This hypothesis was further supported by the observations that HHP-induced solubilization could be evidently inhibited by the addition of salt due to strong electrostatic screening. The solubility of β-conglycinin and glycinin subjected to HHP of 300 MPa at pH 8.0 and ionic strength of 50 mM only reached 21.84 ± 0.23% and 38.43 ± 1.14%, respectively (Figure 2D). As a result, the changes in protein charged residues upon pressure may be responsible for the increase in solubility of denatured proteins by inhibiting protein reaggregation.

Aggregation State of Pressurized Proteins. GPC was performed to better understand the aggregation state of the HHP-treated proteins. The elution profiles of various soy proteins are shown in Figure 3. The elution profiles of the native β-conglycinin (Figure 3A) and glycinin (Figure 3B) showed major eluting peaks appearing at 25 and 20 min, respectively. In contrast to native soy proteins, large soluble aggregates caused by HHP treatment (200−500 MPa) were found for β-conglycinin and glycinin, as evidenced by the appearance of a peak eluting at 12.5 min and a decrease of the typical peak areas (at 25 and 20 min). Previous studies reported that heat treatment induced the formation of soluble aggregates from insoluble SPC.[3,28] The proportion of soluble β-conglycinin aggregates decreased when pressure was increased from 300 to 500 MPa, accompanied by an increased relative area of peak eluting at 25 min (Figure 3A). The peaks eluting after 25 min, indicative of smaller molecular mass molecules, were also observed for β-conglycinin treated above 300 MPa. These results indicated that HHP treatment above 300 MPa dissociated soluble aggregates derived from β-conglycinin into smaller molecular mass molecules, such as monomer or subunits.

It should be pointed out that the total peak area of HHP-treated glycinin considerably decreased with increasing pressure from 400 to 500 MPa, which seemed to be in contrast with the high solubility of glycinin treated at 500 MPa (Figure 2B). In fact, the additional filtration through 0.45 μm filters before GPC analysis may be a rational explanation for this decrease. The turbidity of glycinin dispersion was evidently increased after HHP treatment at 500 MPa (data not shown). Consequently, it probably suggested that HHP treatment at 500 MPa led to the formation of larger glycinin aggregates. These findings are well in agreement with the previous studies on native glycinin.[12]

Structural Rearrangement of EtOH-Denatured Soy Globulins. To understand if HHP-induced solubilization of denatured soy globulins is involved in a pathway of structural rearrangement, it was attempted to evaluate the structural changes and intermolecular forces of various pressurized proteins using DSC and FTIR techniques.

Thermal Behavior. In the DSC thermograms of native β-conglycinin (Figure 4A) and glycinin (Figure 4B), typical endothermic peaks were observed at approximately 74 and 89 °C, respectively. The denaturation peak temperature (T_d) can be used to monitor the thermal stability of protein, and the enthalpy value (ΔH) is correlated with the proportion of undenatured protein or the extent of ordered protein structure.[29] The endothermic peak of denatured β-conglycinin was completely disappeared, indicating the evident structural destruction of protein caused by EtOH treatment (Figure 4A). In contrast, the ΔH value of glycinin only decreased from 14.19 to 10.03 J/g (Figure 4B). It seems possible that glycinin is not so sensitive to EtOH washing, and only shows partial denaturation. These phenomena coincided with previous results for SPC.[3]

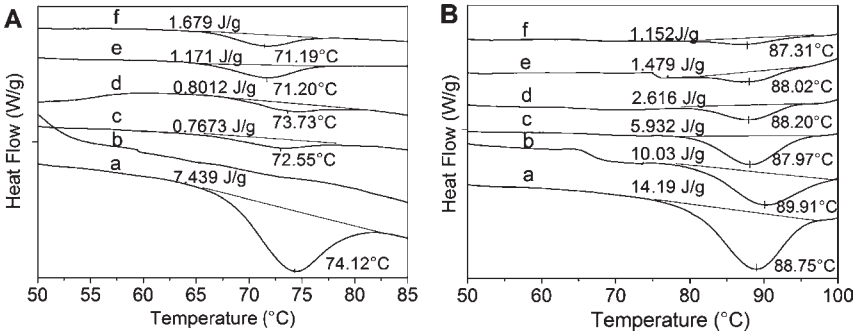

Figure 4. DSC curves of native (a), denatured (b) and HHP-treated β-conglycinin (A) and glycinin (B) at 200 (c), 300 (d), 400 (e) and 500 MPa (f), respectively.

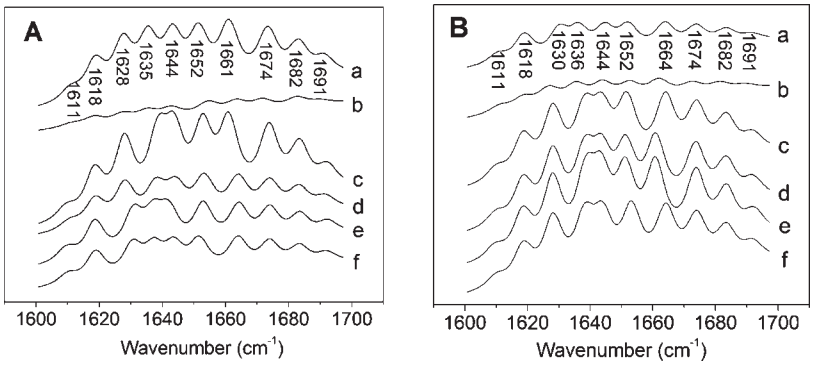

Figure 5. Deconvoluted FTIR spectra in the amide I region of native (a), denatured (b) and HHP-treated β-conglycinin (A) and glycinin (B) at 200 (c), 300 (d), 400 (e) and 500 MPa (f), respectively.

The remarkable observation was the finding that the typical endothermic peak of denatured β-conglycinin (approximately 74 °C) reappeared after HHP treatment at 200 MPa. Moreover, the ΔH value of β-conglycinin gradually increased from 0.7673 to 1.679 J/g as pressure was increased from 200 to 500 MPa (Figure 4A). A decrease in T_d of β-conglycinin caused by HHP treatment at 300 MPa was also observed compared to treatment at 200 MPa. These results clearly indicate an increase in ordered structures of β-conglycinin, but a loss of protein thermal stability. A possible explanation for these phenomena is that HHP treatment probably triggers the structural rearrangement of denatured β-conglycinin through unfolding, refolding and/or reassociation of protein, accompanied by the formation of ordered supramolecular structures. In the case of glycinin, however, opposite changes in the thermal behavior were observed (Figure 4B). The ΔH value of typical endothermic peak of glycinin (approximately 89 °C) gradually decreased from 5.932 to 1.192 J/g with increasing pressure from 200 to 500 MPa, consistent with the changes in ΔH value of native glycinin.[14] There were no distinct changes in T_d. These phenomena suggested a progressive unfolding of denatured glycinin at high pressure, leading to more disordered structures without the loss of thermal stability.

Secondary Structure. FTIR as a tool to characterize the conformation of protein can be applied to liquid, semisolid and solid samples, particularly suitable for studying proteins with limited solubility.[30] Ten major bands, attributed to the C═O stretching vibration and to C−N stretching vibration of the peptide bonds, were observed in the amide I region of deconvoluted curve of native β-conglycinin and glycinin (Figure 5).

Table 1. Assignments of Deconvoluted Amide I Bands in the FTIR Spectrum of Soy Proteins

wavenumber (cm^{-1})	band assignments
1611	side chain vibrations
1618	antiparallel β-sheet
1618	aggregated strands
1628−1630	β-strand
1635−1636	β-sheet
1644	random coil
1652	α-helix
1661−1664	β-turns
1674	β-strand
1682	antiparallel β-sheet
1682	aggregated strands
1691	β-type structure

Table 1 shows the assignments of deconvoluted amide I bands, and the secondary structure compositions of soy globulins are summarized in Table 2.

The EtOH-induced strengthening of intermolecular hydrogen bonds of β-conglycinin were characterized by the red-shifts of bands attributed to α-helix (1652 cm^{-1}) and β-turns (1660 cm^{-1}) (Figure 5A), meaning more disorder conformations.[31] The increases in α-helix and antiparallel β-sheet aggregates and a decrease in β-sheet were also found for β-conglycinin (Table 2), further indicating the protein unfolding and the formation of

Table 2. Estimation of the Secondary Structure of β-Conglycinin and Glycinin by FTIR Analysis[a]

	β-conglycinin (%)					glycinin (%)				
protein samples	α-helix	β-sheet	β-turns	random coil	aggregated strands	α-helix	β-sheet	β-turns	random coil	aggregated strands
native	12.82	37.27	14.30	12.54	16.59	12.56	35.64	12.19	12.30	19.79
denatured	14.76	32.50	12.49	11.88	20.27	11.72	37.16	12.85	13.96	15.61
200 MPa	13.43	37.96	14.16	12.31	16.45	14.00	37.74	13.96	11.59	16.66
300 MPa	13.88	37.19	13.02	11.46	17.53	12.96	38.48	14.31	11.47	16.73
400 MPa	13.80	34.89	12.17	11.92	19.30	13.26	38.30	14.40	12.05	16.26
500 MPa	13.24	35.05	12.80	11.54	19.69	14.24	37.99	13.34	12.35	16.22

[a] α-Helix: band at 1652 cm^{-1}. β-Sheet: bands at 1628, 1635, and 1674 cm^{-1}. β-Turns: band at 1661 cm^{-1}. Random coil: band at 1644 cm^{-1}. Aggregated strands: bands at 1618 and 1682 cm^{-1}.

antiparallel β-sheet aggregates. In the case of denatured glycinin, the observation that the band at 1630 cm^{-1} shifted to 1628 cm^{-1} suggested that the intermolecular hydrogen bond strength of β-sheet structure was enhanced due to partial unfolding (Figure 5B).[13] Such unfolding possibly led to the increases in random coil and β-sheet structure of native glycinin at the expense of antiparallel β-sheet structure (Table 2).

β-Conglycinin treated at 200 MPa showed similar locations of the infrared bands and the secondary structure content with native protein (Figure 5A, Table 2), further confirming the structural rearrangement, as previously evidenced by DSC data (Figure 4A). This rearrangement is accompanied by the transformations of EtOH-induced α-helix and antiparallel β-sheet aggregated strands to β-sheet structure (Table 2). The gradual blue-shifts of β-sheet band (1638 cm^{-1}) were also observed for β-conglycinin with increasing pressure (200−500 MPa) (Figure 5A), indicating that HHP treatment induced a strengthening of intramolecular hydrogen bond of β-sheet structure. This probably could be a reason for the increase in ΔH value (Figure 4A). The band at 1628 cm^{-1} is indicative of intermolecular β-sheet type structure.[32] Compared to HHP treatment at 200 MPa, a red-shift of this band and an amplitude decrease for β-conglycinin treated above 300 MPa demonstrated the transformation of intermolecular hydrogen-bonded β-sheet to intramolecular hydrogen-bonded β-sheet structure (Figure 5A).[33] In practice, turn structure is considered to be a product of the protein unfolding of any higher order structures.[25] HHP treatment at 300 MPa caused a decrease of β-turn content of β-conglycinin and a red-shift of β-turn band at 1661 cm^{-1} (Table 2, Figure 5A). These results revealed that the protein unfolding was also involved in the β-conglycinin structural rearrangement.[13] Additionally, the antiparallel β-sheet aggregated strand content of β-conglycinin gradually increased at the expense of β-sheet and random coil contents with increasing pressure (Table 2). Complying with these results, it could be concluded that the unfolded β-conglycinin at 200 MPa is prone to rearrange into the native-like supramolecular structures by competing with reaggregation, whereas higher pressure (300−500 MPa) results in a reassociation process of secondary structures, with the involvement of the protein unfolding and the formation of antiparallel β-sheet aggregates.

In the case of glycinin, HHP treatment at 200 MPa caused the increases in α-helix, β-turns and antiparallel β-sheet, as well as a decrease in random coil of denatured protein (Table 2). Moreover, obvious red-shifts (1636, 1664, and 1674 cm^{-1}) and a blue-shift of band at 1644 cm^{-1} were also observed at 200 MPa (Figure 5B), indicating the unfolding of protein molecules.[13] Unlike β-conglycinin, glycinin treated at different pressures (200−500 MPa) had similar deconvoluted curves except the shift of β-turn band (1664 cm^{-1}) (Figure 5B), revealing that the unfolding process dominated in structural changes of denatured glycinin at high pressure. The increase of random coil structure at pressures above 400 MPa also supported this viewpoint (Table 2).

In brief, FTIR and DSC data suggested that denatured β-conglycinin and glycinin displayed different structural changes during HHP treatment. The increasing pressure led to the conformational transition of denatured β-conglycinin into ordered supramolecular structure with the stronger intramolecular hydrogen bond, accompanied by the partial unfolding and aggregation. However, denatured glycinin showed more disordered structure with increasing pressure.

Surface Hydrophobicity. The surface hydrophobicity of protein was previously used to identify the structural changes of proteins.[12] Table 3 summarizes the surface hydrophobicity of various samples. The overall surface hydrophobicity index (PSH) of all HHP-treated proteins was significantly ($p < 0.05$) higher than that of native proteins, suggesting more hydrophobic clusters on the molecular surface of protein after EtOH and HHP treatment.

The fluorescence intensity at saturating concentrations of the probe (F_{max}) represents an overall surface hydrophobicity. The F_{max} of β-conglycinin gradually increased as a function of increasing pressure (200−400 MPa), with an increase of the apparent dissociation constant (K_d) (Table 3). These observations suggested the generation of new binding sites on the β-conglycinin's surface for ANS and the decrease of the binding affinity of ANS to the protein. These results may result from the structural modifications of denatured protein by weakening hydrophobic interactions at high pressure. On the other hand, the dissociation of large β-conglycinin aggregates due to the rupture of hydrophobic interaction is also responsible for this increase in F_{max}. However, both F_{max} and K_d values of β-conglycinin treated at 500 MPa significantly ($p < 0.05$) decreased (Table 3), revealing a decrease in the accessibility of the binding sites for ANS. Hence, most likely these sites are used for structural rearrangement of β-conglycinin upon pressure at 500 MPa, as demonstrated by the DSC and FTIR data (Figure 4, 5). The previous studies have shown that the surface hydrophobicity of SPI treated at 400 MPa was higher than that treated at 600 MPa.[12,14] Therefore, it is reasonably speculated that the unfolded β-conglycinin upon pressure above 400 MPa could easily be involved in hydrophobic rearrangement.

The F_{max} and K_d values of glycinin treated at 400 MPa significantly ($p < 0.05$) increased compared to protein treated at 200 MPa, indicating the formation of new hydrophobic sites on the glycinin's surface with affecting the overall affinity of these sites for

Table 3. Protein Surface Hydrophobicity (PSH, Assessed by Titration with ANS as the Hydrophobic Probe) of Native and HHP-Treated β-Conglycinin and Glycinin[a]

	β-conglycinin			glycinin		
protein samples	F_{max}	K_d (μM)	PSH (F/mg μM)	F_{max}	K_d (μM)	PSH (F/mg μM)
native	244.99 ± 4.89 d	30.66 ± 0.19 d	39.95 ± 0.56 c	261.75 ± 8.28 c	43.47 ± 2.62 b	30.14 ± 0.99 d
200 MPa	591.34 ± 19.51 c	39.32 ± 1.79 c	75.24 ± 1.26 b	442.25 ± 20.18 b	41.16 ± 2.84 b	53.79 ± 1.35 c
300 MPa	726.11 ± 10.80 b	48.48 ± 1.12 b	74.95 ± 2.85 b	714.97 ± 44.02 a	45.58 ± 4.43 ab	78.62 ± 2.99 a
400 MPa	985.33 ± 39.76 a	64.34 ± 3.80 a	76.63 ± 1.45 ab	702.80 ± 20.25 a	51.57 ± 3.46 a	68.27 ± 3.04 b
500 MPa	728.03 ± 24.93 b	44.83 ± 2.05 b	81.31 ± 4.82 a	743.88 ± 34.95 a	51.39 ± 5.14 a	72.73 ± 6.20 ab

[a] Different letters (a−d) in the column indicate significant ($p < 0.05$) differences among samples. F_{max}: the maximum fluorescence intensity (at saturating probe concentration). K_d: the apparent dissociation constant of the protein−ANS complex. PSH: protein surface hydrophobicity index (PSH = F_{max}/K_d · [protein concentration], fluorescence intensity/mg μM). The protein concentration is 0.2 mg/mL.

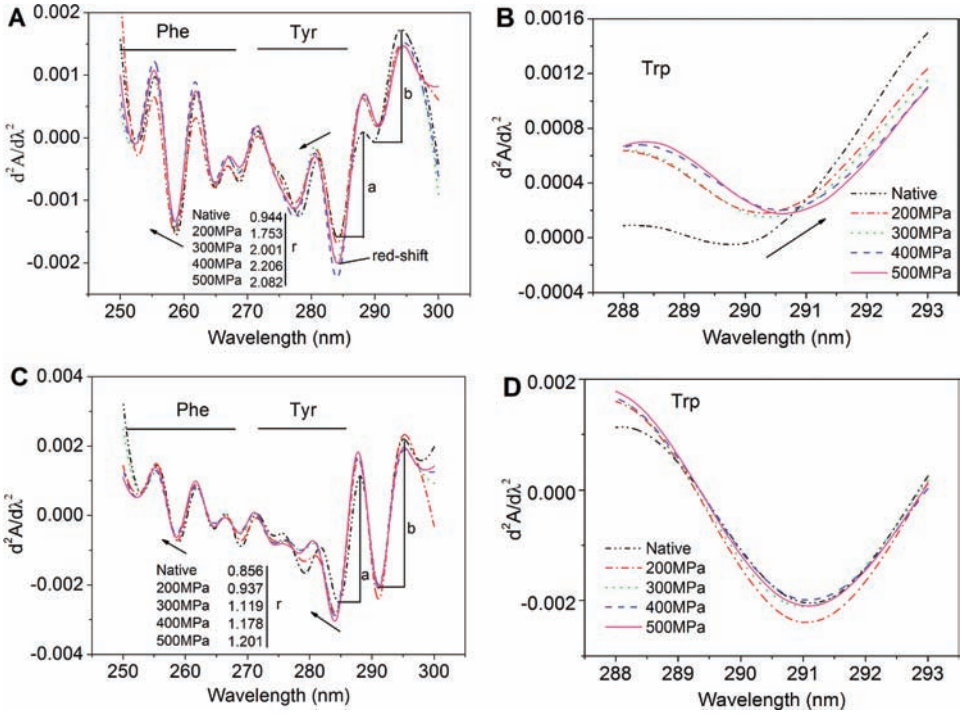

Figure 6. Second-derivative UV spectra of native and HHP-treated (200−500 MPa) β-conglycinin (A, B) and glycinin (C, D). Denatured proteins were not tested due to low solubility. All labels in profiles are same. The insets show changes in the "r" values ($r = a/b$) of native and HHP-treated β-conglycinin and glycinin.

ANS, when pressure was increased from 200 to 400 MPa. These results are in accordance with the previous studies on the effect of HHP treatment on native glycinin.[34]

Distribution of Aromatic Acid Residues. β-Conglycinin and glycinin contain all types of aromatic acid residues, such as Phe, Tyr and Trp.[35] The second-derivative UV spectroscopy was thereby selected to critically delineate the distribution of aromatic acid residues in HHP-treated proteins. Figure 6 shows the second-derivative UV spectra of native and HHP-treated soy proteins (200−500 MPa). The position and amplitude of the derivative spectral bands can be used as sensitive probes to characterize the polarity of microenvironments. Generally, a decrease of the solvent polarity results in red-shifts of the derivative bands.[36] The changes in amplitude, described by calculating the ratio ($r = a/b$) of the two peak-to-trough values marked in Figure 6, can also be used to assess the environments of the Tyr residues.[36] Compared with native β-conglycinin and glycinin, HHP-treated soy proteins showed distinct distributions among aromatic acid residues (Phe, Tyr and Trp) after EtOH and HHP treatment, as shown in Figure 6.

The slight blue-shifts of bands in the Phe residue region (250−270 nm) with increasing pressure (200−500 MPa) occurred for β-conglycinin (Figure 6A), indicating a gradual transition to more polar environments. The UV spectra of β-conglycinin showed the continuous blue-shifts of bands in the Tyr residue region (270−285 nm) and a gradual increase in r value with increasing pressure from 200 to 400 MPa. A slight red-shift of bands in the Tyr residue region and a decrease in r value were found when pressure was increased up to 500 MPa (Figure 6A). Interestingly, these results are in good agreement with surface hydrophobicity data (Table 3). These findings may not only confirm that the generation of new hydrophobic sites in the molecular surface of β-conglycinin at moderate pressure (200−400 MPa) is partially attributed to the exposure of the Phe

and Tyr residues but also demonstrate that pressure up to 500 MPa causes a hydrophobic buried effect of some Tyr residues by hydrophobic arrangement.

The gradual red-shifts of bands in the Trp residue region (above 290 nm) for β-conglycinin with increasing pressure (200−500 MPa) indicated that these residues were in more hydrophobic regions in soluble protein or aggregates (Figure 6B). It is very important to note that the Trp residues are located only in the N-terminal extensions of the α and α′ subunits (2 in α subunit and 3 in α′ subunit).[37] Therefore, the increase in hydrophobicity of the Trp residues may suggest an increase in the interactions of the Trp residues in the extended regions with the core regions, probably screening some hydrophobic sites on the protein surface. These results may further support the finding of structural rearrangements for denatured β-conglycinin upon HHP treatment. It is well-known that the extension regions of the α and α′ subunits are rich in charged acidic amino acids.[1] Considering the solubility and structure data (Figures 2, 4, 5), it could be indirectly speculated that the conformational changes of the extension regions of the α and α′ subunits may be closely related to the EtOH-induced insolublization of β-conglycinin and the HHP-induced dissolution of denatured protein. This hypothesis is supported by the structure−function relationships of β-conglycinin. Based on the work of successful cloning for recombinant subunits of β-conglycinin, it has been proposed that the extension regions of the α and α′ subunits contribute to high solubility and emulsifying abilities, and that the core regions determine thermal stability and surface hydrophobicity.[1]

In the case of glycinin, the slight blue-shifts of bands in the Phe and Tyr residue region, with a gradual increase in r value from 0.937 to 1.201, were observed as a function of increasing pressure (200−500 MPa) (Figure 6C). These results indicated that the Phe and Tyr residues shifted to a polar environment, contributive to the increase in F_{max} upon pressure (Table 3). However, there were no obvious differences in the environments of the Trp residues in HHP-treated glycinin (Figure 6D). In structural terms, it is difficult to obtain definite answers for HHP-induced solubilization due to the multiplicity of glycinin's genetic variants, and some more detailed work is needed.

In conclusion, HHP treatment was confirmed to be an efficient method to dissolve insoluble proteins, and to modify the surface hydrophobicity of denatured soy proteins. The increase in hydrophobic surface of HHP-recovered soluble proteins may be beneficial to the application of soy proteins as emulsifiers or emulsion stabilizers. Thus, further study will be conducted to investigate its emulsifying properties, and to further elucidate structure−function relationships.

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