



Effects of high hydrostatic pressure treatment on structural, allergenicity, and functional properties of proteins from ginkgo seeds

Hao Zhou^a, Chengzhang Wang^{a,*}, Jianzhong Ye^a, Hongxia Chen^a, Ran Tao^a, Fuliang Cao^b

^a Institute of Chemical Industry of Forest Products, CAF, Nanjing, Jiangsu 210042, China

^b College of Forestry, Nanjing Forestry University, Nanjing, Jiangsu 210037, China

ARTICLE INFO

Article history:

Received 4 November 2015

Received in revised form 4 February 2016

Accepted 4 February 2016

Available online 13 February 2016

Keywords:

High hydrostatic pressure treatment

Structure

Allergenicity

Functional properties

Ginkgo seed proteins

ABSTRACT

The effects of high hydrostatic pressure (HHP) treatment on the structural, allergenicity and functional properties of ginkgo seed protein (GSP) were investigated. GSP was treated under pressures of 100, 200, 300, 400, 500, 600 and 700 MPa. Western blotting and ELISA assay revealed that HHP treatment at pressure ranges from 300 up to 700 MPa can significantly reduce the allergenicity of GSP. Meanwhile, SDS-PAGE, MALDI-TOF-MS, CD spectra, fluorescence spectra and UV absorption spectra analysis revealed that, after HHP treatment, the GSP disintegrated into proteins with small molecular weights ranging from 4 to 30 kDa; the secondary structures of α -helices and β -sheet were largely destroyed and turned into random coil; and the UV absorption intensity, surface hydrophobicity and sulfhydryl group (SH) content of GSP were significantly increased. Moreover, the HHP-treated GSP showed markedly improved heat stability and emulsifying properties compared to the untreated GSP.

Industrial relevance: As a traditional food and medicine source, the seeds of *Ginkgo biloba* were used in china for several thousand years. Ginkgo seeds could be added to desserts, glazed fruit, beverages and tippie, also ginkgo seeds, as a traditional Chinese medicinal material, have been recorded in the Compendium of Materia Medical. Research shows that ginkgo seeds have a relatively high content (10%–15%) of proteins, which possess rich and reasonable composition of amino acids, and much higher content of essential amino acids than the recommended FAO/WHO standard, belonging to the high-quality protein. Ginkgo seeds proteins (GSP) have many biological activities, including anti-oxidation, anti-aging, anti-tumor, and anti-bacterial, so it has high value of nutrition and medicine. However, eating GSP could result in allergic reaction to humans, infant, or children are more sensitive to GSP, the clinical symptoms such as nausea, vomiting, bellyache, diarrhea, dysphoria, exanitation, convulsions, dyspnea, and mydriasis, and sometimes even death can occur.

In the study, we used the method of high hydrostatic pressure (HHP) treatments, which can significantly improve the functional properties and reduce the antigenicity of GSP. After the HHP treatment, GSP can be transformed into proteins with small molecular weights ranging from 4 to 30 kDa, the antigenicity of GSP can be reduced by more than 95%, and the heat stability and emulsifying properties of GSP can be markedly improved. Thus, this study is very helpful for designing the hypoallergenic and improved functional GSP, which could have a relevant practical application for their use as base ingredients of food.

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1. Introduction

Ginkgo biloba, one of the oldest species of tree, has existed on the earth for two hundred million years, and 70% is from China (Deng et al., 2011). The seeds of *G. biloba* have been used in China as a traditional food and medicine source for several thousand years (Huang et al., 2010). Ginkgo seeds could be added to desserts, glazed fruit, beverages, and tippie. In addition, as a traditional Chinese medicinal material, ginkgo seeds have been recorded in the Compendium of Materia Medica (Tredici, 1991). Research shows that ginkgo seeds have a

relatively high protein content (10%–15%), with a rich and reasonable composition of amino acids and a much higher content of essential amino acids than the recommended FAO/WHO standard, thus qualifying as high-quality protein (Zhou, Chen, Wang, Ye, & Chen, 2012). Yang et al. (2011) reported the molecular weights of Ginkgo seed protein (GSP) ranges from 10 to 100 kDa, exhibiting five major protein bands of high intensity with molecular weights of approximately 10, 21, 32, 38, and 48 kDa. GSP has many biological activities, including anti-oxidation, anti-aging, anti-tumor, and anti-bacterial properties, so it has high nutritional and medicinal value (Huang, Xie, Wang, Yang, & Luo, 2004). However, eating GSP can result in allergic reactions in humans. Infants and children are more sensitive to GSP, and clinical symptoms such as nausea, vomiting, bellyache, diarrhea, dysphoria,

* Corresponding author. Tel./fax: +86 25 85482471.
E-mail address: wangczl@sina.com (C. Wang).

examination, convulsions, dyspnea, and mydriasis, and sometimes even death can occur (Yang & Wu, 2009). Till now, research on how to reduce and eliminate GSP allergy remains lacking, which limits the development of hypoallergenic GSP and the suitable use of this precious protein source in the food industry.

Food proteins are often subjected to different processing methods to improve their safety, stability, functionality, and suitability for specific product applications (Sathe & Sharma, 2009). The types of modifications that food proteins may undergo during processing include protein unfolding and aggregation and chemical modification (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). These modifications could decrease food allergenicity by hiding, destroying, or disclosing allergenic epitopes (Cabanillas et al., 2010) and could improve functional properties, including heat stability and emulsification, which are important for preparing a stable formulation (Kilara & Panyam, 2003). Traditional food processing methods mainly include heat treatment and enzymatic hydrolysis. Heat treatment, usually conducted at high temperature, has been described as having denaturing effects that can enhance texture and flavors or ensure the microbiological safety of proteins; however, this processing method has poor effects on reducing the allergenicity of food proteins (Sathe, Teuber, & Roux, 2005). Although enzymatic hydrolysis is generally considered to be an effective method to reduce or eliminate the allergenicity of food proteins, such treatment usually results in a degradation of the processed proteins' characteristics, as well as a deterioration in the taste, for example, the development of bitterness or an unpleasant odor (Yamamoto et al., 2010).

High hydrostatic pressure (HHP), an emerging technology for food processing, is a valuable treatment to reduce the immunoreactive properties of food proteins (Peñas, Préstamo, Baezac, Marti'nez-Moleroc, & Gomez, 2006). HHP can modify the secondary, tertiary, and quaternary structures of proteins and alter some of the epitope structures, which can reduce the allergenicity and improve the functional properties of food proteins (Pereda, Ferragut, Buffa, Guamis, & Trujillo, 2008). Compared with other food processing methods, HHP can not only effectively reduce the allergenicity of food proteins but also offer chemical preservation with less destruction of factors associated with fresh-like character in food proteins, so that the original color, flavor, and nutrients can be preserved (Johnson et al., 2010). Recently, HHP treatment has been widely used to produce food proteins with lower residual antigenicity, improved functional properties, excellent organoleptic quality, and high nutritional value. Many studies on the effects of HHP treatment on the allergenicity and functional properties of food proteins have been reported. Hu et al. stated that high pressure induces conformational changes in peanut proteins, and according to their results, the binding of peanut allergens to IgE decreased with increasing pressure (Hu et al., 2011). Li et al. reported that the allergenicity of soybean proteins was reduced by more than 50% after HHP treatment (300 to 500 MPa, 20 min), and the proteins that underwent HHP treatment showed a higher hydrophobicity character compared with intact proteins (Li, Zhu, Zhou, & Peng, 2012). Hildebrandt et al. demonstrated that after HHP treatment (400 to 700 MPa, 20 min), the immunoreactivity of egg proteins decreased with increasing pressure, and changes to protein structure and functional properties including foaming and emulsification were positively correlated with pressure (Hildebrandt et al., 2010). Chicón et al. found that the binding of whey protein allergens to IgE significantly decreased after HHP treatment, and the heat stability of whey proteins was obviously increased (Chicón, Belloque, Alonso, & López-Fandiño, 2009). However, to date, no information has been available on the effects of HHP treatment on GSP in relation to allergenicity, structural, and functional properties.

With the aim to produce GSP-based ingredients that could be used in hypoallergenic foods, we have now tested whether HHP treatment yields ginkgo seed proteins that combine reduced IgE binding with acceptable functional properties. As allergenicity could be reduced through processes that alter the structure of the allergens, making them less available to antibody receptors, the effect of HHP treatment

on the structural properties of GSP (molecular weight, secondary structure, free SH content, surface hydrophobicity, and UV absorbance) will also be addressed.

2. Materials and methods

2.1. Plant materials

GSP was obtained from seeds of *G. biloba* purchased from the Taixin Market of Jiangsu province. Ginkgo seeds were freeze-dried, crushed, and then defatted by petroleum ether at 4 °C for 10 h. The defatted flour was air-dried, then homogenized in Tris–HCl buffer (TBS, 0.2 mol/L, pH 7.4). The homogenate was centrifuged at 5000 rpm at 4 °C for 15 min, and then ammonium sulfate was added to the supernatant to 80% saturation to precipitate proteins. After stirring overnight, the proteins were collected by centrifugation at 5000 rpm for 30 min, then dialyzed with a dialysis bag of 3.5 kDa molecular weight cutoff. The dialysate was freeze-dried to obtain the purified GSP. GSP powder was dissolved in MilliQ water at the concentration of 50 g/L and used for HHP treatment.

2.2. HHP treatment

A high-pressure instrument (model UHPF-750 MPa-3 L; KEFA Hitech Food Machine Co. Ltd., Baotou, China) with a hydraulic type cell with an inner capacity of 3 L (100 mm in diameter and 300 mm in height) and a water jacket for temperature control was used in this study. Prior to pressure processing, solutions of GSP with a suitable volume were vacuum conditioned in a polyethylene bag. Temperature during treatment was controlled to avoid overheating. Water was used as the pressure-transmitting medium in the machine, and its temperature was maintained at 20 °C during the pressure processing. Distilled water was used as the solvent. Solutions of GSP at 5% (w/v) and pH 8.0 were subjected to HHP treatment at 100, 200, 300, 400, 500, 600, and 700 MPa for 20 min. The target pressure was achieved within 1–2 min, held for 20 min, and released to atmospheric pressure within 1–2 min. After HHP treatment, the solutions were freeze-dried. The unpressurized products were used as experimental controls.

2.3. SDS–PAGE analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Huang et al. (2010), using a PhastSystem apparatus (Amersham Bioscience, Piscataway, NJ) with ready-made slab gels (PhastGel Gradient 4–20%) and ready-made buffer strips (PhastGel SDS Buffer Strips). Prior to electrophoresis, each sample was diluted to a concentration of 1 mg/mL with 0.01 M Tris–HCl buffer (pH 8.0) containing 2.5% SDS, 20% glycerol, 5% mercaptoethanol, and 0.5% bromophenol blue and heated in a boiling water bath for 10 min. After loading 20 µL samples, gels were run at 70 V for 30 min and then at a constant voltage of 120 V for 45–60 min. A low molecular weight marker ranging from 200 to 10 kDa (Sigma-Aldrich, Madrid, Spain) was used. After running, the gels were stained with 0.1% PhastGel Blue R solution (Merck, Darmstadt, Germany) containing 20% methanol and 10% acetic acid and destained using this solvent.

2.4. MALDI-TOF-MS analysis

The molecular weights of raw and HHP-treated GSP were analyzed using MALDI-TOF-MS as described previously (Wang & Yang, 2001). The apparatus was set up as follows: nitrogen laser, wavelength 337 nm; linear-flight distance, 1.2 m; acceleration voltage, 20 kV; detector voltage, 25 kV. α -Cyano (α -cyano-4-hydroxybenzoic acid) was used as the matrix as follows: α -Cyano was placed in a tube and mixed by vortexing with 40–100 µL 0.1% TFA/50% acetonitrile solution. The tube was then centrifuged, leaving a small amount of insoluble α -

Cyano at the bottom of the tube under ideal conditions. The resulting supernatant, a saturated solution, was used for the experiments. Internal and external standards were prepared using distilled water. The samples were dissolved in deionized water to 40 pmol/μL. One microliter of sample was mixed with the same volume of matrix solution. The matrix-embedded sample was dried on a stainless steel target for analysis. Ten picomoles of CytoC were used for internal calibration.

2.5. Western blotting assay

Western blotting was conducted on a Trans-blot electrophoretic transfer cell according to the method of Shimakura, Tonomura, Nagashima, and Shiomi (2005). The proteins and hydrolysates separated by SDS–PAGE were electrotransferred from the gel to the PVDF membrane (Millipore). The membrane was washed with Tween-PBS (0.01 M phosphate buffer, pH 7.0, containing 0.15 M NaCl, and 0.05% Tween 20) and blocked with 2% skim milk in Tween-PBS at 37 °C overnight. After washing with Tween-PBS, the membrane was incubated for 2 h at 37 °C with mouse anti-ginkgo seed protein serum (obtained from Professor Yin Lu, Nanjing University of Traditional Chinese Medicine; diluted 1:100 with Tween-PBS) as the primary antibody, washed in TBS containing 0.5 mL/L Tween 20 (TBST), and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgE (Sigma-Aldrich, St Louis, MO; diluted 1:500 with Tween-PBS) as the secondary antibody for 2 h at 37 °C. Finally, immunoblots were detected by immersing the membrane in 20 mL of TBS containing 12 mg of 4-chloro-1-naphthol, 4 mL of ethanol, and 12 μL of 300 mL/L H₂O₂ for 5 min at 37 °C, and the membrane was imaged and analyzed using a gel scanner (Sharp JX-330, Pharmacia Biothec).

2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was applied to determine antigenicity as an indirect competitive assay according to the method of Julià et al. (2007). To estimate the IgE-binding, high-binding polystyrene microtiter plates (Corning, Cambridge, MA, USA) were used as the solid support. The individual wells of a 96-well microtiter plate were coated with 300 μL/well of antigen of native GSP (2 μg/mL) in coating buffer (0.015 mol/L Na₂CO₃, 0.035 mol/L NaHCO₃, pH 9.6), left overnight at 4 °C. Afterwards, the plate was washed three times with 300 μL/well of PBS containing 0.05% Tween 20 (PBST) and subsequently blocked with 50 mL/L skimmed milk in phosphate-buffered saline (PBS) for 1 h at 37 °C. After three times washing, 100 μL of the samples was added per well: those consisting of native GSP standards (200 μg/mL, 100 μg/mL, 20 μg/mL, 2 μg/mL, 1 μg/mL, and 20 ng/mL) and HHP-treated samples (50 μg/mL) at different pressure. Immediately after adding the samples to the wells, 100 μL of mouse anti-ginkgo seed protein serum (diluted to 1:400 in PBS) was added to each well. After incubation for 1 h at 37 °C, the plate was washed three times and 200 μL of goat anti-mouse IgE conjugated with horseradish peroxidase (diluted to 1:1000 in PBS) was added to each well, followed by incubation for 1 h at 37 °C. Three more washings were done, and then 100 μL of substrate solution (0.5 mg/mL o-phenylene diamine, 0.3 mL/L H₂O₂ in 0.1 mol/L citrate buffer, pH 5.5) was added to each well and incubating for 15 min at 37 °C. The reaction was terminated by adding 50 μL of 2 mol/L H₂SO₄. The absorbance at 490 nm was read using a Bio-Rad 1860 microplate reader (Bio-Rad, CA, USA).

2.7. Structure analysis

The conformational changes of ginkgo seed proteins after HHP treatment were defined as changes in secondary structure, sulfhydryl group content, protein surface hydrophobicity, and UV absorbance based on far-ultraviolet circular dichroism (Far-UV CD) spectra, fluorescence spectra, and UV absorption spectra, respectively. All the experiments

were measured three times, and the results of each sample were averaged.

2.7.1. Circular dichroism (CD) spectrum determination

Far-UV CD spectra were obtained using a JASCO J810 spectropolarimeter (Jasco, Groß-Umstadt, Germany) at room temperature. The far-UV CD spectroscopic measurements were performed according to the method of Tang and Sun (2011). Protein concentrations of 0.2 mg/mL in 10 mM PBS (pH 7.0) were used for analysis. Samples were scanned from 190 to 250 nm. The following parameters were used: scan speed at 100 nm per min, bandwidth of 1 nm, response time of 1–4 s. The CD spectra were measured three times, and the results of each sample were averaged, followed by the molar residue ellipticity (θ) being expressed as deg cm²·dmol^{−1}. The contents of different secondary structures were calculated using the JASCO secondary structure software.

2.7.2. Surface hydrophobicity (Ho) determination

The Ho values of raw and HHP-treated GSP were determined according to the method of Wang et al. (2008), using 8-anilino-1-naphthalenesulfonate (ANS) as the fluorescence probe. Sample concentrations of 0.1 mg/mL in 0.01 M phosphate buffer (pH 7.0) were used for analysis. Then 20 μL of ANS solution (8.0 mM in the same buffer) was added to 4 mL of each protein sample solution. Fluorescence intensity was measured using a Hitachi F7000 fluorescence spectrometer (Tokyo, Japan). The excitation wavelength was 390 nm, and the emission wavelength was from 400 to 650 nm at a scanning speed of 25 nm/s. Measurements were performed in triplicate.

2.7.3. Determination of free sulfhydryl (SH) content

The SH content on the surface of raw and HHP-treated GSP was determined according to the method of Ellman (1959). Approximately 8 mg of each sample was solubilized in 8 mL of 0.1 M Tris buffer (pH 8.0) containing 0.09 M Gly, 0.004 M EDTA and 8 M urea. An aliquot (1.0 mL) of sample solution was then mixed with 40 mL Ellman's reagent (4 mg/mL in methanol). The absorbance of the mixture was measured at 412 nm in a UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and the buffer was used as the blank. The SH contents were obtained by dividing the absorbance value by the molar extinction coefficient of 13,600 M^{−1} cm^{−1} and expressed as μmol/g of protein. Each sample was determined in triplicate.

2.7.4. UV absorption spectrum analysis

UV absorption spectrum analysis was performed using a UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The UV absorption spectra were scanned from 220 to 420 nm.

2.8. Functional properties

To estimate heat stability, 0.5 g native GSP or HHP-treated GSP were respectively dissolved in 100 mL distilled water at pH 8.0 and maintained at different temperatures (room temperature, 50, 65, 80, and 95 °C) for 15 min. Samples were then cooled to room temperature and centrifuged for 5 min at 10 °C and 3500 rpm/min. The UV absorbance (280 nm) of the supernatant was measured to estimate the protein concentration. Heat stability was expressed as the percentage of soluble protein compared to the corresponding unheated sample at the same pH.

To determine the emulsifying activity index (EAI), different concentrations (from 0.25 to 1.5 mg/mL) of aqueous protein solutions were prepared at pH 8.0. To make the emulsions, 3 mL of sample and 1 mL of corn oil (Φ , volume fraction of the dispersed phase = 0.25) were homogenized using an Ultra-turrax Type 18/10 (Staufen, Baden-Württemberg, Germany) for 3 min at maximum power. After homogenization, 20 μL of emulsion was added to 10 mL of 0.1% SDS, giving a protein dilution of 1/500, and the absorbance was immediately measured

at 500 nm. Absorbance was converted into EAI units (m^2/g) following Pearce and Kinsella (1978). Duplicate emulsions were prepared and analyzed twice.

2.9. Statistical analysis

The SPSS program (SPSS 11.5 for windows, SPSS Inc. Chicago, IL, USA) was used for statistical analysis of the results. Analysis of variance (ANOVA) one-way linear model was performed, and the significance level was established for $p \leq 0.05$. Mean comparisons were conducted using the Duncan test.

3. Results and discussion

3.1. SDS-PAGE and MALDI-TOF-MS profiles of HHP-treated GSP

To observe the effects of HHP treatment on the molecular weight changes of GSP, the proteins were analyzed by SDS-PAGE and MALDI-TOF-MS, and the results are shown in Fig. 1 and Table 1. With regard to the SDS-PAGE profile, the electrophoretogram of raw GSP showed bands with apparent molecular masses ranging from 5 to 100 kDa, exhibiting major protein bands of high intensity with molecular weights of approximately 5, 10, 22, 30, 39, and 46 kDa. The same molecular weights of proteins have been reported for ginkgo seeds in the literature (Yang et al., 2011). When the GSP was treated by HHP at a pressure of 100 or 200 MPa, most of the protein bands still remained, and only the protein bands at 39 kDa disappeared. When protein samples were treated under pressure up to 300 or 400 MPa, the protein bands at 5, 10, 22, and 46 kDa still remained, while the protein bands at 30 and 39 kDa disappeared; moreover, compared with intact protein, some new protein bands with molecular weights of approximately 28 and 90 kDa appeared. When protein samples were treated under pressure up to 500, 600, or 700 MPa, most of the protein bands disappeared, and only the protein bands at 10 kDa still remained, while new protein bands at 26–28 kDa appeared.

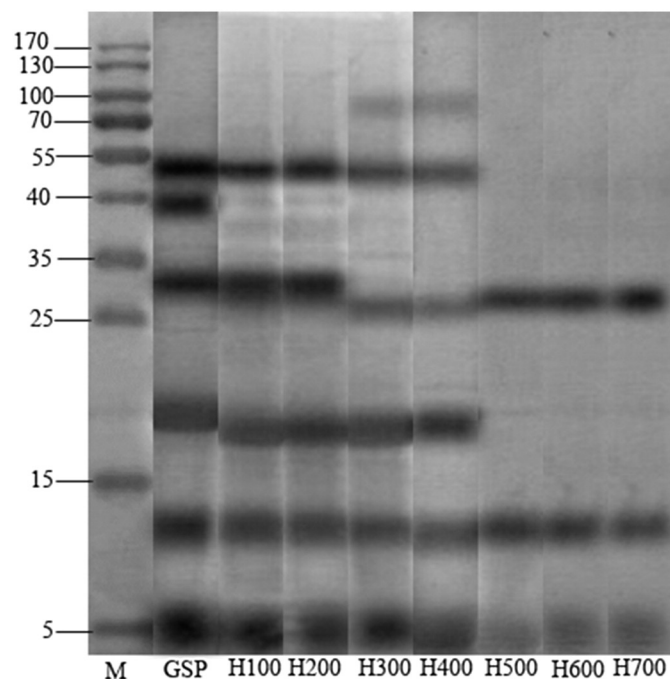


Fig. 1. SDS-PAGE of native and HHP-treated GSP. M: Molecular weight standard solution; GSP: ginkgo seed proteins; H100: HHP treatment at 100 MPa; H200: HHP treatment at 200 MPa; H300: HHP treatment at 300 MPa; H400: HHP treatment at 400 MPa; H500: HHP treatment at 500 MPa; H600: HHP treatment at 600 MPa; H700: HHP treatment at 700 MPa.

Table 1
MALDI-TOF-MS spectral data of native and HHP-treated GSP.

Native GSP	Measured mass [M + H] ⁺						
	100 MPa	200 MPa	300 MPa	400 MPa	500 MPa	600 MPa	700 MPa
5308	5395	5396	4661	5396	4329	4329	4371
9315	9326	9327	9327	9328	9327	9328	9329
22,489	22,904	22,897	22,839	22,046	–	–	–
			27,869	27,816	27,844	27,855	27,854
			29,020	29,037	29,010	29,034	29,018
30,658	30,551	30,744	–	–	–	–	–
39,391	–	–	–	–	–	–	–
45,111	45,890	45,814	46,028	46,888	–	–	–
			92,346	93,728			

–, not detected (compared to native GSP).

MALDI-TOF-MS analyses can determine the accurate molecular weight and content of GSP. The MS spectra of ginkgo seed protein showed a mass range of 5 to 100 kDa, with major protein peaks at masses of 5.3, 9.3, 22.5, 30.6, 39.4, and 45.1 kDa, the results of molecular weights analysis of GSP by MALDI-TOF-MS are consistent with the results analyzed by SDS-PAGE. When protein samples were treated at pressures of 100 and 200 MPa, most of the protein peaks with masses of 5.3, 9.3, 22.9, 30.5, and 45.8 kDa were still remained, and only the protein peak with a mass of 39.4 kDa disappeared. When protein samples were treated under pressures up to 300 or 400 MPa, the protein peaks with masses of 5.3, 9.3, 22.9, and 45.8 kDa still remained, while the protein peaks with masses of 30.6 and 39.4 kDa disappeared; moreover, the protein peak at 45.8 kDa showed reduced content, and new protein peaks at 27.8, 29.0, and 93.7 kDa appeared compared with the raw protein. When protein samples were treated under pressures up to 500, 600, and 700 MPa, only the protein peaks with masses of 9.3 kDa remained, while the protein peaks with masses of 5.3, 22.9, 30.6, 39.4, and 45.1 kDa disappeared, and new protein peaks with masses of 27.8 and 29.0 kDa appeared in comparison with the native protein. In addition, compared with the protein samples treated under pressures of 300 or 400 MPa, the protein peaks at 45.8 kDa completely disappeared, and the new protein peaks at 27.8 and 29.0 kDa showed much higher content. The disappearance or appearance of protein peaks of GSP treated at different pressure analyzed by MALDI-TOF-MS are basically consistent with the results analyzed by SDS-PAGE.

It has been reported that the primary, secondary, tertiary, and quaternary structures of proteins result from different types of interactions. The primary structure consists of an amino acid sequence connected by covalent bonds; the polypeptide chain forms α -helices or β -sheets/strands by intra- or intermolecular hydrogen bonds, creating the secondary structure of proteins; the tertiary structure, which is also called the protein subunit, reflects how the secondary structure domains fold into a 3-dimensional configuration as a consequence of noncovalent interactions among amino acid side chains; and the quaternary structure describes the spatial arrangement of the subunits, held together by noncovalent bonds (Yaldagard, Mortazavi, & Tabatabaie, 2008). Generally, hydrogen and noncovalent bonds can be affected by HHP; therefore, HHP has a substantial impact on the secondary, tertiary, and quaternary structures of the protein, which can induce changes in molecular weight. In this study, it was found that the molecular weights of GSP decreased with increasing pressure from 100 to 700 MPa, but at a pressure of 300 or 400 MPa, some new proteins with large molecular weights of 90 kDa appeared that were not observed in the raw protein. A similar phenomenon has been reported by Peñas et al., who found that dairy whey protein showed new large protein bands in the SDS-PAGE analysis when treated at pressures of 300 to 400 MPa (Peñas, Préstamo, Polo, & Gomez, 2006). A possible explanation is that HHP treatment can induce protein unfolding and create new free thiol residues, some of which aggregate and recombine to produce

sulfhydryl–disulfide exchange reactions or new sulfhydryl–disulfide binding through oxidation and aggregation (Wang et al., 2008). It was also found that when GSP was treated under pressures from 300 to 700 MPa, the proteins with molecular weights of 46 kDa gradually decreased and then disappeared, while new proteins with molecular weights of 26–28 kDa appeared and showed rapidly increasing content, probably due to proteins with large molecular weights of 46 kDa transformed into proteins with small molecular weights of 26–28 kDa. Moreover, when protein samples were treated under pressures up to 500, 600, and 700 MPa, most of the proteins with molecular weights above 10 kDa disappeared or turned into proteins with relatively low molecular weights. A reasonable explanation, consistent with pressure studies on other proteins, is that unfolding is less complete and the proteins partially folded when pressure is lower, whereas at higher pressure, unfolding becomes prominent and more hydrogen bonds, noncovalent bonds, and disulfide (S–S) bonds are broken, turning proteins with large molecular weights into peptides with relatively low molecular weights (Yang, Dunker, Powers, Clark, & Swanson, 2001).

3.2. HHP induced allergenicity changes in GSP

Allergens of food proteins have certain site(s) that can be recognized by a specific antibody to provoke an immune response, called epitope(s). Epitopes are either linear or conformational and may consist of a few amino acids along the primary structure or a unique three-dimensional motif of the protein structure. In general, conformational epitopes play a greater role in causing immunoreactivity (Sathe et al., 2005). It has been demonstrated that HHP processing can destroy the structure of conformational epitopes, which ultimately reduces the allergenicity of food proteins (Mills et al., 2009). In our study, the allergenicity of GSP after HHP treatment was determined by western blotting and enzyme-linked immunosorbent assays.

Western blotting was performed using a serum pool from mice with an actual allergy to ginkgo seed protein, and the results are shown in Fig. 2. The raw GSP showed allergenic proteins with molecular masses of 22, 30, and 46 kDa. After HHP treatment, a reduction of the total IgE-reactive bands was seen in GSP. When protein samples were treated under the pressures of 100 and 200 MPa, all the immunoreactive bands at 22, 30, and 46 kDa still remained; after HHP treatment under pressures up to 300 and 400 MPa, the bands at 30 kDa disappeared, but additional immunoreactive bands were observed at 90 kDa. When protein samples were treated under pressures up to 500, 600, or 700 MPa, no immunoreactive bands were detected.

Clinical studies have confirmed that, among all abnormal immune responses caused by food proteins, the proportion of the ones regulated by IgE is the highest (Mekore, 1996). Therefore, investigations of allergies in food proteins have primarily focused on food allergies regulated by IgE. In our study, to further assess the immunochemical reactivity in HHP-treated GSP, the IgE-binding activities were tested by ELISA assays, and the results are shown in Fig. 3. On the whole, the immunoreactivity of the HHP-treated protein samples was lower than in raw proteins, and the immunoreactivity showed a decreasing trend as the pressure increased from 100 to 700 MPa. Antigenicity reductions of 5%, 10%, and 42% were observed for protein samples treated at 100, 200, and 300 MPa, respectively. However, after HHP treatment under pressure up to 400 MPa, the protein sample presented a small increase in immunochemical reactivity. After treatment at pressure above 400 MPa, it showed a greater decrease in antigenicity: an antigenicity reduction of 95% was observed in protein samples treated at 500 MPa, and a complete loss of allergenicity was observed in protein samples treated at 600 and 700 MPa.

The results of this study suggest that HHP treatment can effectively reduce the antigenicity of GSP. However, the allergenicity of GSP was studied using mouse anti-GSP serum for immunoblots and ELISA assay, not humans, so this antigenicity study is an estimated approach to evaluate the effects of HHP treatment on in vitro immunoreactivity

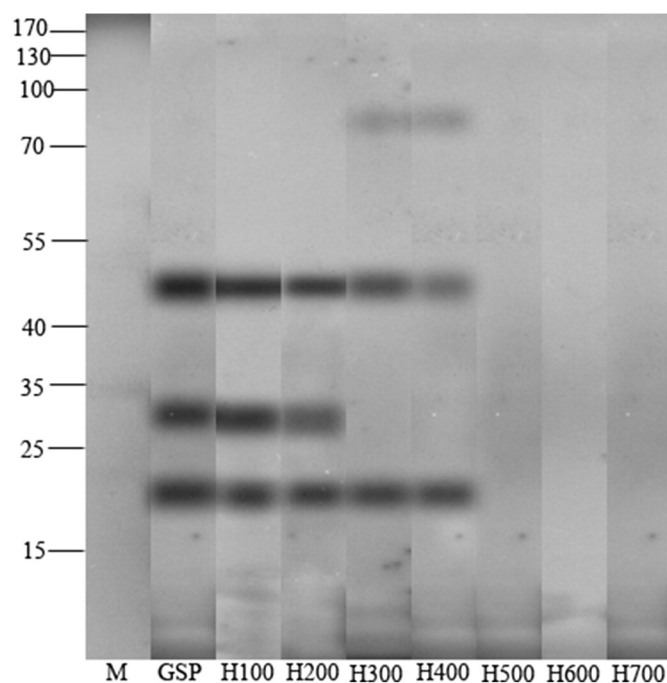


Fig. 2. Western blotting of native and HHP-treated GSP. M: Molecular weight standard solution; GSP: ginkgo seed proteins; H100: HHP treatment at 100 MPa; H200: HHP treatment at 200 MPa; H300: HHP treatment at 300 MPa; H400: HHP treatment at 400 MPa; H500: HHP treatment at 500 MPa; H600: HHP treatment at 600 MPa; H700: HHP treatment at 700 MPa.

of GSP. It has been demonstrated that HHP induces a reversible or irreversible structural modification in the proteins, leading to protein denaturation, aggregation, or gelatinization; therefore, the structure of the allergen epitope binding site for IgE might be destroyed or altered by HHP and thus no longer recognized by IgE antibodies or able to stimulate an immune response, ultimately reducing the allergenicity (Shriver & Yang, 2011). It is worth noting that during the HHP treatment, an increase in the allergenicity of the HHP-treated proteins was found at 400 MPa in comparison with 300 MPa. This increase in antigenicity might result from the unfolding of the structure, which could expose hydrophobic groups, intermolecular disulfide bonds, and more antigenic epitopes buried inside the native molecule on the surface of the molecule, leading to increased allergenicity (Sathe et al., 2005). However, when the pressure was increased to 500, 600, and 700 MPa, complete loss of allergenicity was observed in the protein samples, most likely because the structures of the proteins were deeply destroyed and more conformational epitopes were eliminated at higher pressure, resulting

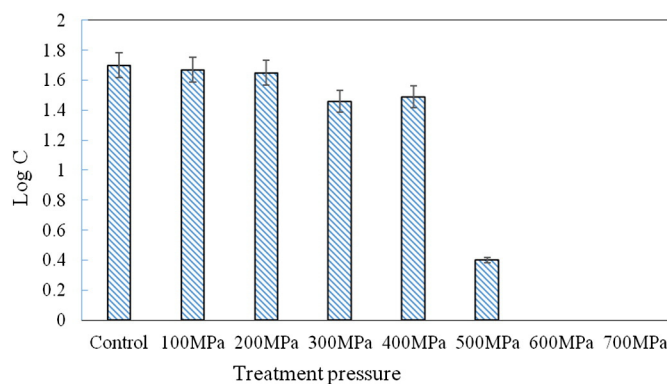


Fig. 3. The IgE-binding activities determined by ELISA assay, of GSP untreated (control) and treated by HHP (100, 200, 300, 400, 500, 600, or 700 MPa, for 20 min at 20 °C). Different letters above the bars for each enzyme indicate significant differences ($p \leq 0.05$).

in the sweeping reduction of the allergenicity of GSP. Many studies have demonstrated HHP treatment can effectively reduce the allergenicity of food proteins, such as bovine globulin, soybean protein, peanut protein, and so on (Hu et al., 2011; Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011; Yamamoto et al., 2010), but some researches showed that the HHP treatment is not effective to alter the immunoreactivity of hazelnut proteins or walnut allergens (Cabanillas et al., 2014; Prieto et al., 2014), these different effects are probably related to the type of antigen epitope in proteins because HHP treatment can mainly destroy conformational epitopes but cannot destroy linear epitopes, so if the antigen epitope in protein is mainly composed of linear epitopes, their allergenicity will not be affected by HHP treatment.

3.3. Effect of HHP treatment on the structure of GSP

3.3.1. Effect of HHP treatment on CD spectra

The secondary structure of native GSP and HHP-treated GSP was analyzed by CD spectroscopy, and the far-UV spectra are shown in Fig. 4. The percentages of α -helix, β -sheet, and random coil are shown in Fig. 5. The negative peak at 200–220 nm is characteristic of the α -helical configuration, while the positive peak at 190–198 nm is characteristic of the β -sheet configuration (Chang, Wu, & Yang, 1978). The estimated secondary structure of native GSP using spectral analysis revealed 25.2% α -helix, 53.7% β -sheet, and 21.1% random coil. The CD spectrum of GSP treated at 100 and 200 MPa, however, was almost identical to the spectrum of the untreated protein. When GSP was processed at pressures up to 300 or 400 MPa, the intensity of the negative molar residue ellipticity peak began to decrease, and the positive molar residue ellipticity peaks began to increase, reflecting a decrease in α -helices in favor of β -sheet formation. The α -helix content decreased by 12.4% for 300 MPa and 15.8% for 400 MPa, while the β -sheet content increased by 6.5% for 300 MPa and 8.6% for 400 MPa, respectively. When GSP was treated at pressures of 500, 600, or 700 MPa, the intensity of both the negative and positive molar residue ellipticity peaks significantly decreased, indicating a decrease in both α -helix and β -sheet: the α -helix content dropped to 5.3% for 500 MPa and 0 for 600 or 700 MPa, and the β -sheet content dropped to 20.2%, 13.4%, and 12.5% for 500, 600, and 700 MPa, respectively. By contrast, the random coil content increased to 74.5%, 86.6%, and 87.5% for 500, 600, and 700 MPa, respectively. This result showed that under pressures of 500 MPa or more, the ordered structures of both the α -helices and β -sheets of GSP can be destroyed and converted to random coil. Similar observations have been made in soybean glycinin: Zhang et al. used

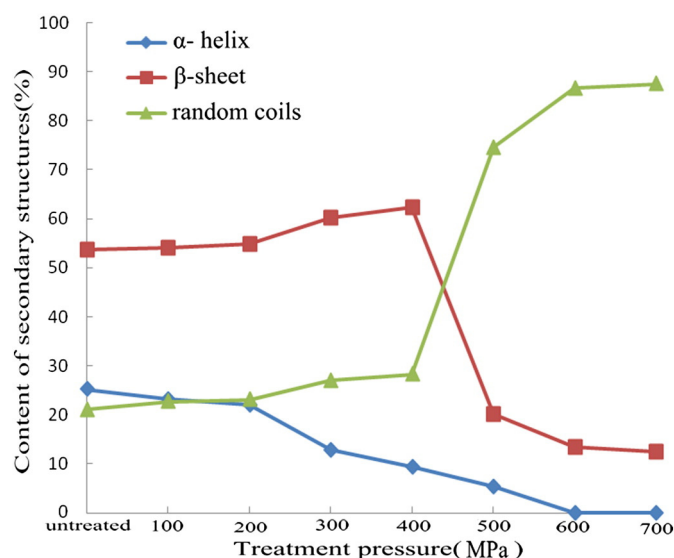


Fig. 5. The content of each secondary structure of untreated and HHP-treated GSP at different pressure.

circular dichroism spectra (far-UV CD) to investigate the influence of HHP treatment on soybean glycinin and reported that α -helix and β -sheet content markedly decreased and random coil content increased after processing at 500 MPa or more (Zhang, Li, Tatsumi, & Kotwal, 2003).

After HHP treatment, the secondary structure of GSP changed and α -helices and β -sheets could be destroyed; however, the extent of these alterations depended on the pressure value. Interestingly, we found that the reduction rates of antigenicity matched well with the changes in secondary structure. For example, after 300 MPa treatment, the secondary structure of GSP changed significantly, and the percentage of α -helices decreased; after 500 MPa treatment, the percentages of both α -helix and β -sheet decreased greatly. At the same time, the percentage of random coil increased considerably, which corresponded with a significant decrease in antigenicity. The α -helix and β -sheet content decreased with the reduction in antigenicity, indicating that IgE-binding epitopes are probably localized within the α -helix and β -sheet structures of GSP.

3.3.2. Effect of HHP treatment on sulfhydryl groups

The disulfide bonds of GSP play an important role in defining its secondary and tertiary structure. Based on literature data, a decrease in free SH can indicate protein cross-linking, while an increase generally accounts for both protein unfolding and backbone fragmentation (Sen et al., 2002). Fig. 6 shows the changes in the sulfhydryl group (SH) content of GSP resulting from HHP treatment. In the range 100–300 MPa, the free SH content of GSP showed a progressive increase with increased pressure. Meanwhile, at 400 MPa, the free SH content showed a slight decrease compared with 300 MPa. However, when the pressure was increased to 500–700 MPa, the free SH content progressively and significantly ($P < 0.05$) increased. The pressure of 700 MPa gave the maximum increase, but the difference between 600 and 700 MPa was not significant.

These results suggest that the structural modification induced by HHP may be rather complex, potentially involving more than one mechanism of denaturation. According to Wu, Sheng, Xie, and Wang (2008), HHP can break disulfide bonds, which will directly disturb the protein structure and lead to its partial unfolding. The newly generated and unstable free thiol groups would react to form intermolecular S–S bonds as well as protein radicals. Based on these considerations, it can be inferred that the decrease in free SH concentration of GSP under 400 MPa treatment could be attributed to partial unfolding and the formation of S–S bonds. However, disulfides have also been reported to further dissociate,

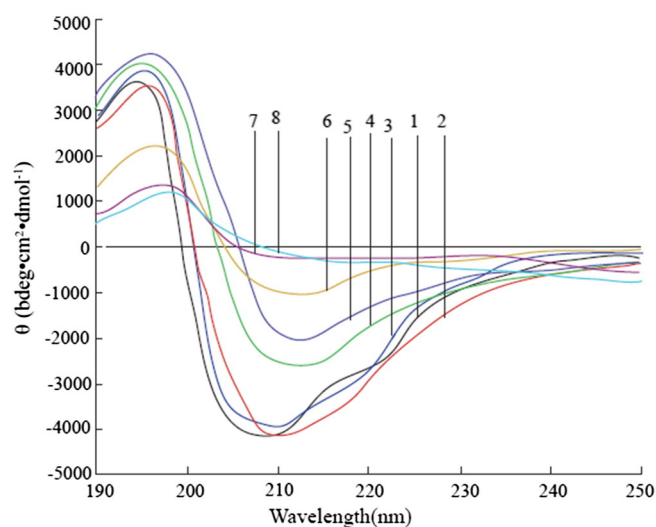


Fig. 4. Far-UV CD spectra of untreated and HHP-treated GSP. Curves 1–8 represent samples of untreated GSP and of HHP-treated GSP under pressure of 100, 200, 300, 400, 500, 600, and 700 MPa, respectively.

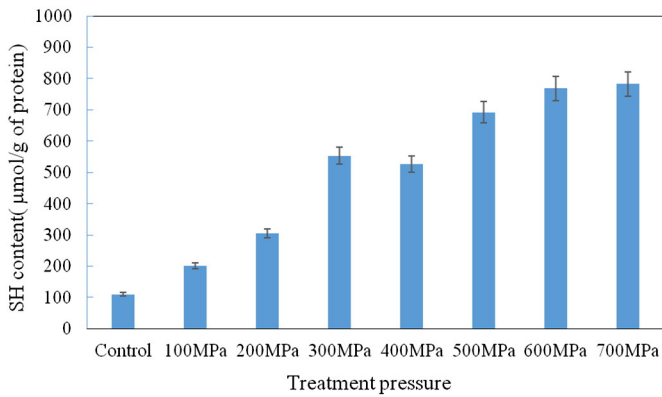


Fig. 6. Sulfhydryl group (SH) content on surface of untreated and HHP-treated GSP at different pressure.

in a reversible reaction, to give free SH groups (Davies, 2003). Moreover, there have been indications that disulfide bonds play an important role in the integrity of the IgE-binding epitopes (Marzban et al., 2009), which could be related to the allergenicity of OVT. In this study, with increasing pressure, the disulfide bonds were cleaved, resulting in an increase in the sulfhydryl group content on the surface of GSP accompanied by conformational changes in GSP, resulting in a decrease in the allergenicity of GSP. Therefore, we can hypothesize that HHP treatment may reduce the allergenicity of GSP by changing its conformation.

3.3.3. Effect of HHP treatment on hydrophobicity and UV absorption spectra

Hydrophobic interactions play an important role in the stabilization of the tertiary and quaternary structure of proteins, and ANS has been widely used as a fluorescent probe to measure protein surface hydrophobicity. Because ANS shows a low quantum yield of fluorescence in aqueous solution, a large increase in fluorescence is observed when it binds to the accessible hydrophobic regions of proteins (Maleki et al., 2003). Fig. 7 shows the ANS-induced fluorescence spectra of GSP samples treated under different pressures. It can be seen that HHP treatment resulted in gradual increases of Ho in GSP when the pressure increased in the range of 100–300 MPa, while a significant increase ($P < 0.05$) in Ho was observed in GSP treated at 400–700 MPa compared to the control. The highest Ho in GSP was obtained at 700 MPa, but this value was only slightly higher than the value for GSP treated at 600 MPa, and the difference between them was not significant.

UV absorption spectra depend mainly on the UV absorption of Tyr and Trp residues located in side-chain groups and to some extent on

the UV absorption of Phe, His, and Cys (San Martin, Barbosa Canovas, & Swanson, 2002). The effects of HHP treatment on the UV absorption spectra of GSP are presented in Fig. 8. The maximum UV absorption peak of unheated OVT was found at approximately 275 nm, indicating that the UV absorption spectrum of OVT was mainly dependent on tyrosine and tryptophan residues. It can be seen that HHP treatment resulted in gradual increases in UV absorption in GSP when the pressure increased in the range of 100–300 MPa, while a significant increase ($P < 0.05$) in UV absorption was observed in GSP treated at 400–700 MPa compared to the control. The highest UV absorption in GSP was obtained at 600 MPa, but the UV absorption of GSP treated at 700 MPa was only slightly lower than at 600 MPa, and the difference between them was not significant.

Conformational changes of the protein can also be inferred based on alterations to the surface hydrophobicity and UV absorption of GSP. Because the increased intensity of fluorescence indicated that the protein was unfolded, exposing more hydrophobic regions after HHP treatment, the hydrophobicity of the HHP-treated GSP increased. This finding illustrated that the tertiary and quaternary structure of GSP were changed by HHP. Moreover, the UV absorption intensity of HHP-treated GSP samples increased with increasing pressure, and the increase in the absorption intensity suggested that Tyr and Trp residues were exposed on the surface of the protein molecule as a consequence of the unfolding of the structure. A slight blue shift of the absorption peak was also observed as the pressure increased, which showed that the polarity of the microenvironment of Tyr and Trp had changed after HHP, and peptide bonds might be destroyed, which could change the primary structure of GSP. In addition, the results of this study demonstrate that HHP leads to an irreversible change in the tertiary structure, as detected by measuring surface hydrophobicity and UV absorption. Some researchers have reported that the tertiary structure change in allergen proteins greatly affected the reduced allergenicity (Nogami et al., 2006), and this study agrees with these results. Based on the results here, it is clear that HHP affected the structure of GSP, particularly the tertiary structure. Therefore, the lower IgE-specific binding activity and allergenicity of GSP after HHP were probably related to the changes in the tertiary structure. Furthermore, our studies suggest that the pressure-induced modification of the tertiary structure of GSP was confirmed in regions that might include Tyr and Trp residues. These results suggest that the epitopes of GSP might contain Tyr and Trp residues.

3.4. Effect of HHP treatment on the functional properties of GSP

To understand whether the changes in GSP structure upon HHP could be associated with modifications in the functional properties,

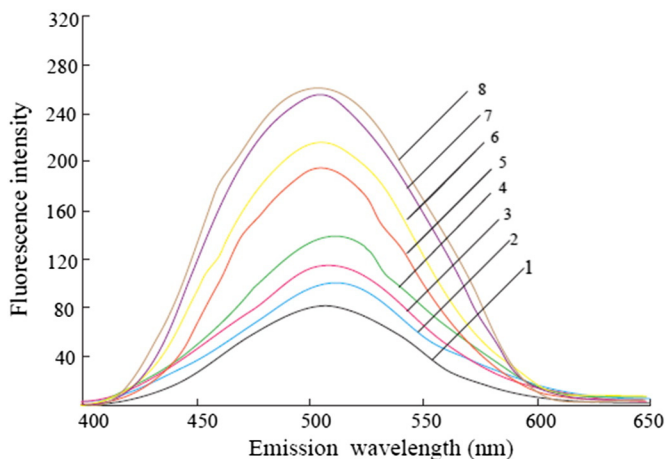


Fig. 7. Fluorescence emission spectra of untreated and HHP-treated GSP. Curves 1–8 represent samples of untreated GSP and of HHP-treated GSP under pressure of 100, 200, 300, 400, 500, 600, and 700 MPa, respectively.

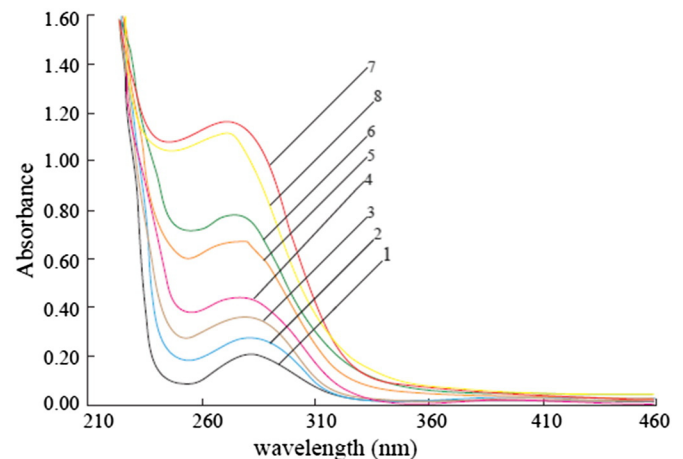


Fig. 8. UV absorption spectra of untreated and HHP-treated GSP. Curves 1–8 represent samples of untreated GSP and of HHP-treated GSP under pressure of 100, 200, 300, 400, 500, 600, and 700 MPa, respectively.

samples exposed to HHP were also analyzed for heat stability and emulsifying properties.

To estimate the thermal stability, 0.5 g native GSP or HHP-treated GSP at pressure of 100 to 700 MPa were respectively dissolved in 100 mL distilled water, their absolute protein concentrations separately are 5.0, 5.0, 4.8, 4.5, 4.6, 4.2, 4.0, 4.1 mg/mL, these samples were heated at different temperatures (from 25 to 95 °C), and the results are showed in Fig. 9. The solubility of the native GSP showed a temperature-dependent decrease, and its stability was considerably reduced upon heating at 80 and 95 °C. It is well known that some proteins undergo irreversible denaturation at temperatures higher than 65–70 °C (Iametti, DeGregori, Vecchio, & Bonomi, 1996). However, compared to the native GSP, at temperatures from 25 to 95 °C, samples treated at 100 or 200 MPa showed slightly improved heat stability, while a significant improvement ($P < 0.05$) in heat stability was observed in GSP treated at 300–700 MPa, and the best heat stability was obtained at 600 or 700 MPa, where samples retained their solubility even at the highest temperatures applied. Moreover, it was found that the heat stability of GSP treated at 400 or 500 MPa was slightly worse than for GSP treated at 300 MPa.

In this study, HHP treatment resulted in substantially increased thermal stability and solubility of GSP; it was probably because HHP could avoid or prevent the thermal-induced instability of proteins. The different water binding properties of these particles might have affected the thermal stability and solubility of GSP. The thermal stability of GSP showed a pressure-dependent increase, except that GSP treated at 400 or 500 MPa showed slightly worse heat stability than GSP treated at 300 MPa, possibly because the significant disruption of GSP upon HHP treatment may have exposed some reactive sites of the soluble polymer molecules, making them sensitive to newly induced heat reaggregation (Iordache & Jelen, 2003). Native GSP showed poor thermal stability, which limits its technological application for food systems. However, HHP-treated GSP resulted in proteins that retain their solubility even at very high temperatures, which might be advantageous in the manufacture of GSP-enriched nutritional supplements that are not vulnerable to fouling during thermal processing.

The emulsifying activity index (EAI) was assessed for the native GSP and the HHP-treated samples over a range of concentrations (0.25–1.5 mg mL⁻¹), and the results are shown in Fig. 10. Compared to native GSP, at concentrations from 0.25 to 1.5 mg mL⁻¹, samples treated at 100 or 200 MPa showed slightly improved emulsion capacity, while a significant improvement ($P < 0.05$) in emulsion capacity was observed in GSP treated at 300–700 MPa. The best emulsion capacity was obtained at 700 MPa, but the EAI value of GSP treated at 700 MPa was only slightly higher than at 600 MPa, and the difference between them was not significant.

The emulsifying capacity of GSP can be significantly improved by HHP. It has been reported that HHP treatment can cause the exposure

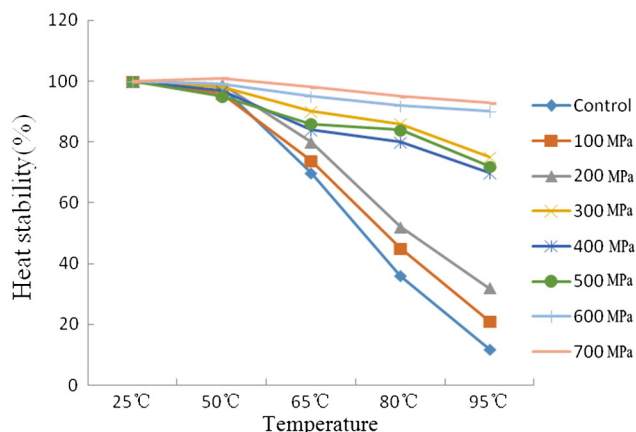


Fig. 9. Heat stability of untreated and HHP-treated GSP at different pressure.

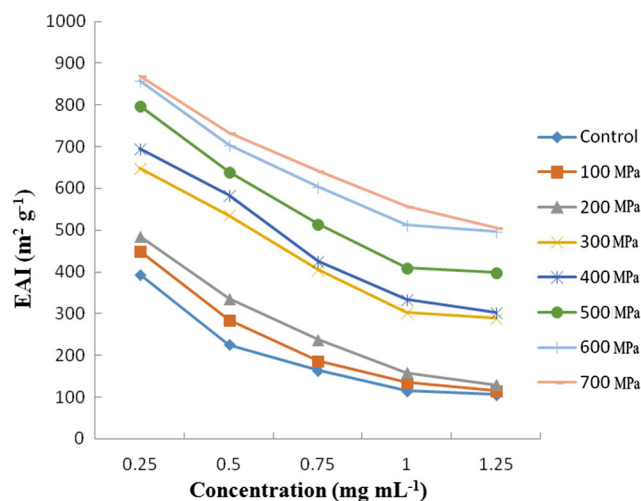


Fig. 10. Emulsifying activity index (EAI) of untreated and HHP-treated GSP at different pressure.

of hydrophobic groups or changes in molecular flexibility, so it is possible to obtain peptides with a structure and distribution of polar and hydrophobic residues that results in better emulsifying properties than in the untreated protein (Huang, Catignani, & Swaisgod, 1996). It is also important that the apparent molecular weight of the proteins is related to emulsifying properties. The presence of high molecular weight material may hinder the emulsification performance of the proteins (Van der ven, Gruppen, de Bont, & Voragen, 2001). In this work, HHP treatment can promote certain cleavages that release fragments with suitable molecular mass and improved emulsifying properties.

4. Conclusions

This work describes the allergenicity, structural, and functional properties of GSP obtained under HHP treatment. The results suggest that HHP treatment with pressure ranges from 300 to 700 MPa can considerably reduce the allergenicity of GSP. After HHP treatment, the intermolecular and intramolecular disulfide bonds that originally stabilized the native protein structure were disrupted and the molecular weights, secondary, tertiary, and quaternary structures of GSP were significantly changed. These structural changes in GSP are closely related to the decrease of its antigenicity by either destroying or burying the conformational IgE-binding epitopes. In addition, HHP treatment can improve the functional properties of GSP: the HHP-treated GSP showed improved heat stability and EAI values compared to the untreated GSP, suggesting that the HHP-treated GSP retained the ability to efficiently form emulsions. These results suggest that HHP could constitute an important technological approach for the industrial production of hypoallergenic and improved-function GSP for potential use as base ingredients in food.

Acknowledgements

The authors gratefully acknowledge the Foundation Research Project of Jiangsu Province (The Natural Science Fund, BK20141073) and the financial support by the Sanxin Forestry Project of Jiangsu Province (LYSX[2015]25).

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