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Effects of high hydrostatic pressure treatment on allergenicity and structural properties of soybean protein isolate for infant formula

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ABSTRACT

The objective of this study is to investigate the influences of HHP treatment on the allergenic properties of SPI for infant formula. The processing parameters, including HHP pressure and duration time, could significantly influence the allergenicity reducing efficiency. At 300 MPa and 15 min, the allergenicity decreased 48.6%, compared to the native SPI. In the ranges of 200–300 MPa and 5–15 min, the free SH content and hydrophobicity of SPI significantly increased. Meanwhile, at the levels above 300 MPa and 15 min, the two interactions progressively decreased. Whatever HHP pressure and time, the maximum emission wavelength indicated blueshifts. At 300 MPa and 15 min, there was an 11.5-fold increase in fluorescence intensity and the maximum emission wavelength shifted from 516 to 466 nm. After HHP treatment, the helix1 and turns content, significantly increased and the strand1 and unordered content considerably decreased; whereas the amount of the helix2 and strand2 did not indicate any obvious change. The average length of helices significantly increased, while the helices (per 100 residues) did not strikingly change after HHP modification. However, both the strand (per 100 residues) and the average length of strands clearly decreased. Some documents indicate that the epitopes of SPI allergens could be closely related to the secondary structure of α -helix and β -sheet. These interactions and secondary structure results can provide direct evidence, or explanation, for HHP-induced modification of soy proteins, which could alter the allergenicity of SPI and enhance the security of SPI for cow milk allergic babies.

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1. Introduction

Soy proteins have been widely utilised in the food industry as a major ingredient due to their highly nutritious and desirable functional properties (Torrezan, Thamb, Bell, Frazier, & Cristianini, 2007; Wang et al., 2008). Soy based infant formula is an important area for soy proteins application, whose major target consumers are cow milk allergic (CMA) babies (El-Agamy, 2007). Researches have been published supporting the use of soy based infant formula (Maldonado, Gil, Narbona, & Molina, 1998), indicating that a significant allergic improvement is produced, while normal growth is maintained. However, 17–47% of milk allergic children can have adverse reactions to soy. Therefore, around 53–83% of CMA infants can tolerate soy-based formula (El-Agamy, 2007). Thus, soy formulas should be the prudent choice for treating allergies to cow milk proteins.

Nowadays, non-thermal techniques, including high hydrostatic pressure (HHP), are regarded with special interest by the field of food science. The effects of high pressure are currently being investigated at different levels, ranging from proteins, enzymes and

viruses, to microorganisms, mammalian cells and tissues (Liu, Powers, Swanson, Hill, & Clark, 2005; Messens, van Camp, & Huyghebaert, 1997). The main goal was to achieve the decontamination of foods, while preserving their organoleptic characteristics. As far as soybeans are concerned, the studies mainly relate to three aspects including microbial inactivation, proteins and enzymes (Estrada-Girón, Swanson, & Barbosa-Cánovas, 2005). The effects of high hydrostatic pressure on microbial inactivation will depend on several factors, such as type of microorganism, extent and duration of the high pressure treatment, temperature, and composition of suspension media or food (Préstamo, Lesmes, Otero, & Arroyo, 2000). The influence of various process parameters (e.g., pressure, time, temperature, protein concentration, pH, presence of salts) on proteins has been investigated (Messens et al., 1997). The research aim mainly focused on the functional and structural properties (Molina, Papadopoulou, & Ledward, 2001; Puppo et al., 2004; Tang & Ma, 2009; Torrezan, Thamb, Bell, Frazier, & Cristianini, 2007; Wang et al., 2008; Zhang, Li, Tatsumi, & Kotwal, 2003). Additional information is available in a more extensive context about the effects of this technology on the inactivation of pure soybean lipoxigenase (Messens et al., 1997).

Reducing allergenicity of some food resources by high hydrostatic pressure processing has been ascertained by some research-

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ers. Hajós et al. stated that high pressure induces conformational changes in the pork batter's proteins, with alteration of some of the epitope structures. According to their results, 600 MPa HHP treatment effectively modifies the IgE immunoreactivity of the proteins in the sausage batter (Hajós, Polgár, & Farkas, 2004). In studies conducted by Kato, Katayama, Matsubara, Omi, and Matsuda (2000), rice grains (*Oryza sativa* L. Japonica var. Akitakomachi) immersed in distilled water exhibited solubilisation and subsequent release of ice allergenic proteins, in the range 100–400 MPa. In this range of pressure considerable amount of proteins, 0.2–0.5 mg per gram of rice, was released with maximum amounts obtained in the pressure range 300–400 MPa. The application of HHP (100–300 MPa for 15 min) to soy “whey”, a by-product from the manufacture of tofu, reduced the immunoreactivity of soy proteins to the antibodies against Gly m 1, an important allergen of soybean that causes allergy by inhalation (Peñas, Préstamo, Polo, & Gomez, 2006). Soy sprouts obtained from HHP-treated seeds showed significantly lower immunoreactivity than those obtained from untreated seeds. These results suggest higher susceptibility of HHP-treated soybean seed proteins to the enzymatic hydrolysis during germination (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011). Dairy whey hydrolysates obtained by pepsin and trypsin in combination with HHP treatment could be used as a source of peptides in hypo-allergenic infant formula (Peñas, Préstamo, Baeza, Martínez-Moleroc, & Gomez, 2006). Few studies have been conducted on the effects of HHP on the allergenic properties of soy protein isolate special for infant formula.

Food technology aims to reduce allergenicity, either by irreversible removal of allergens, or by modifying the allergen structure in such a way that the allergenic epitopes are no longer recognised by the immune system. The objective of this study is to investigate the influences of HHP treatment on the allergenic properties of SPI. Allergenicity could be removed by using processes that alter the structure of the allergens, making them less available to antibody receptors. Therefore, the effect of HHP treatment on structural properties of SPI (free SH content, surface hydrophobicity and secondary structure) will also be studied.

2. Materials and methods

2.1. Materials

Soy protein isolate (model: special for infant formula) were purchased from Henan Anyang Mantianxue Food Co. Ltd. (China). According to Chinese National Food Safety Standard–infant formula, SPI for infant formula must be qualified with following requirements: protein content (on dry basis) $\geq 90\%$, nitrogen soluble index (NSI) $\geq 80\%$, made from non-genetically modified soybean cultivars, negative urease activity. Its protein content on dry basis and NSI are, respectively 91.61% and 82.8%, mainly made from non-genetically modified soybean cultivars Zhonghuang35 and Beidou10. Its urease activity is negative.

5,5-Dithiobis(2-bitrobenzoic acid) (DTNB) (Sigma D-8130) was purchased from Sino-American Biotechnology Co. (China). 1,8-Anilino-naphthalenesulfonate (ANS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 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 Shengzheng Biotech. Co. Ltd. (China). Soybean allergen Elisa Fastkit was purchased from Nippon Meat Packers Inc., R&D Center. All other chemicals used were of analytical grade.

2.2. High hydrostatic pressure (HHP) treatment

A high pressure equipment (model UHPF-750 MPa-3 L; maximum pressure 750 MPa; KEFA Hitech Food Machine Co. Ltd., Bao-

tu, China) with a hydraulic type cell with an inner capacity of 3 litres (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. Temperature during treatment was controlled to avoid overheating. The 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 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 unpressurised products were used as experimental controls.

2.3. Allergenicity determination

2.3.1. Soy protein isolate allergen extraction

One gramme of the prepared sample was weighed into a plastic centrifugal tube. Nineteen millilitres of the sample extracting medium, for soybean, was added into the tube. It was well mixed to prepare a uniform dispersion. The centrifugal tube was placed in a shaking machine and shaken overnight (at least 12 h), at room temperature, to obtain an extract. The extract was centrifuged at 3000g and room temperature for 20 min to collect the supernatant. If no sedimentation occurred, the centrifuged extract was passed through a filter. Samples were filtered through a 0.45 μ m HA Milipore membrane prior to analysis. A 1/20 dilution of the collected supernatant, or filtrate, using the dilution buffer was prepared and used as a measurement solution.

2.3.2. Sandwich Enzyme-linked immunoabsorbent assay (ELISA)

The antibody immobilized plate was warmed to room temperature in the original aluminium pouch. It was removed from the pouch immediately before use. Hundred microlitres of the diluted standard was added and measurement solutions into the wells. In the wells the solution was lightly agitated and allowed to stand for 1 h at room temperature (20–25 °C) for reaction. After the reaction was completed, the standard and measurement solutions were discarded. Two hundred and fifty microlitres of the diluted washing solution was added to each well and discarded, and repeated five times. Hundred microlitres of the prepared Biotin-labelled antibody solution was added to each well. The solution in the wells was agitated and allowed to stand for 1 h at room temperature (20–25 °C) for reaction. After the reaction was completed, the Biotin-labelled antibody solution was discarded and the plate washed, as described in the above step. Hundred microlitres each of the prepared Enzyme-labelled streptavidin solution was added to each well. The solution in the wells was lightly agitated and allowed to stand for 30 min at room temperature (20–25 °C) for reaction. After the reaction was completed, the Enzyme-labelled streptavidin solution was discarded and the plate washed, as described in the above step. The Chromogenic substrate was warmed to room temperature and 100 μ l of the reagent added to each well. The reagent in the wells was lightly agitated and allowed to stand for 20 min at room temperature (20–25 °C) to make the reagent produce a colour. This step should be performed under a light-shielded condition to increase data reproducibility. The stop solution was warmed to room temperature and 100 μ l of the solution added to each well. The solution was lightly agitated to stop colour development. After agitation, the absorbance at the primary wavelength of 450 nm and secondary wavelength of 600–650 nm was measured. The absorbance at 450 nm was determined on a microtitre automated ELISA plate reader (SH-1000 Lab Microplate read-

er, Corona electric, Japan). The standard curve equation was as follows: $Y = 0.0469X + 0.0372$, $R^2 = 0.991$, where Y is the soy allergen content (mg/g protein), X is the absorbance of sample extraction solution at 450 nm. The concentration of the proteins was determined by the Bradford method (Bradford, 1976) using BSA as the standard. Each sample was analysed in duplicate.

2.4. Determination of Free sulfhydryl (SH) content

The SH content was determined according to the method of Beveridge, Toma, and Nakai (1974). About 8 mg of SPI sample was solubilised in 8 ml of 0.086 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 SPI 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-2800 spectrophotometer (Unico Shanghai Instrument Co. Ltd., China), 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. Each sample was determined in triplicate.

2.5. Surface Hydrophobicity (Ho) Determination

Ho of SPI was determined according to the method of Wang et al. (2008), using 1-anilinonaphthalene-8-sulphonic acid (ANS) as the fluorescence probe. Protein dispersions were diluted (0.005–0.2% w/v) in 0.01 M phosphate buffer (pH 7.0). Then, aliquots (20 μ l) of ANS (8.0 mM in the same buffer) were added to 4 ml of sample. Fluorescence intensity was measured with a Hitachi F7000 fluorescence spectrometer (Tokyo, Japan), at wavelengths of 390 nm (excitation) and 470 nm (emission). The initial slope of fluorescence intensity versus protein concentration plot was used as an index of Ho. Measurements were performed in triplicate.

2.6. Extrinsic emission fluorescence spectroscopy

Extrinsic emission fluorescence spectra of various SPIs treated at various pressure and time levels were determined according to the method of Wang et al. (2008), using 1-anilinonaphthalene-8-sulphonic acid (ANS) as the fluorescence probe in a Hitachi F7000 fluorescence spectrometer (Tokyo, Japan). Protein dispersions (0.20 mg/ml) were prepared in 10 mM PBS (pH 7.0). Protein solutions were excited at 390 nm, and emission spectra were recorded from 400 to 650 nm, at a constant slit of 5 nm for both excitation and emission. Measurements were performed in triplicate.

2.7. Circular dichroism (CD) spectra determination

Far-UV CD spectra were obtained using a MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The far-UV CD spectroscopic measurements were performed according to the method of Tang and Sun (2011), in a quartz cuvette of 2 mm, with a protein concentration around 0.1 mg/ml in 10 mM PBS (pH 7.0). The sample was scanned from 190 to 250 nm. The spectra were an average of eight scans. The following parameters were used: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 0.5 nm; sensitivity, 100 mdeg. The cell was thermostated with a Peltier element at 25 °C. The concentration of the proteins was determined by the Bradford method (Bradford, 1976) using BSA as the standard. 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 expressed as mean residue ellipticity (θ) in deg cm² dmol⁻¹. The secondary structure compositions of the samples were estimated from the far-UV CD spectra using the DICHROWEB proce-

dures (Whitmore & Wallace, 2004). Each data was the means of duplicate measurements.

2.8. Statistical analysis

Data were analysed using SPSS for Windows (version 11.5, SPSS Inc., Chicago, IL, USA) following an analysis of variance (ANOVA) one-way linear model. Mean comparisons were performed using the Duncan test, and the significant level was established for $P < 0.05$.

3. Results and discussion

3.1. High hydrostatic pressure induced allergenicity changes in soy protein isolate

The results about effects of HHP treatment pressure and time on the allergenicity of SPI were shown in Fig. 1. ELISA was adopted to assess the immunochemical reactivity in untreated and HHP-treated SPI. Fig. 1A shows a sharp decrease in allergenicity obtained from HHP treated SPI at 200–300 MPa, in comparison to raw SPI ($P < 0.05$). However, in the range of 400–500 MPa, the allergenicity of SPI was not significantly different from that of 300 MPa. The HHP treatment pressure of 300 MPa gave the highest decline of the allergenic potency, which was employed in subsequent experiments. Fig. 1B indicates that HHP treatment at various times led to a reduction in allergenicity of HHP treated SPI, in comparison to control sample. The data suggests that 15 min was the optimal HHP treatment time to reduce the allergenic potency.

Similar phenomenon has been reported by Omi, Katto, Ishida, Kato, and Matsuda (1996), who confirmed the release of basic 7S globulin, an important allergenic protein, in soybean seeds soaked in water and pressurised at 300 MPa and 20 °C for 25 min. These results are in accordance with that obtained by (Peñas, Préstamo, Polo, & Gomez, 2006) for soybean whey, who found a decrease in the allergen Gly m 1 content, after utilisation of HHP at 100, 200 and 300 MPa. A rice product with a lower allergenic potential is on the market in Japan, based on the preferential release of allergenic proteins by high pressure treatment. When polished rice grains were immersed in water and pressurised at 100–400 MPa, a considerable amount of protein was released. The major proteins released were identified as a 16 kDa albumin and a 33 kDa globulin, which are major rice allergens (Kato et al., 2000). These alteration trends of the allergenicity of SPI treated by HHP, could attribute to the protein conformation changes caused by high pressure. The tertiary and secondary structures of proteins can be strikingly modified at pressures >200 MPa (Messens et al., 1997). It has been reported that HHP induces structural unfolding and protein denaturation, and even dissociation of some proteins into subunits, and aggregation of the subunits or precipitation (Wang et al., 2008). Basing on these ideas, the effects of HHP treatment on covalent and non-covalent interactions in SPI and the conformation of SPI were conducted in subsequent experiments, which could further verify our findings.

3.2. Effects of high hydrostatic pressure on covalent and non-covalent interactions in soy protein isolate

The free SH content of SPI samples was significantly influenced by HHP treatment (Fig. 2). In the range 200–300 MPa, the free SH content of SPI significantly ($P < 0.05$) increased. Meanwhile at pressure above 300 MPa, the free SH content progressively and significantly ($P < 0.05$) decreased (Fig. 2A). The pressure of 300 MPa gave the maximum increase. At 300 MPa, a significant increase ($P < 0.05$) of the free SH content in the range of 5–15 min was observed, whereas a significant decrease ($P < 0.05$) of the free SH con-

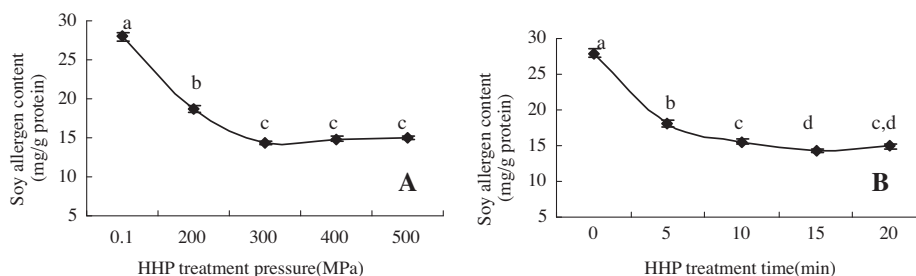


Fig. 1. Effects of HHP treatment pressure and time on allergenicity of SPI (A: HHP treatment time is fixed at 15 min; B: HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

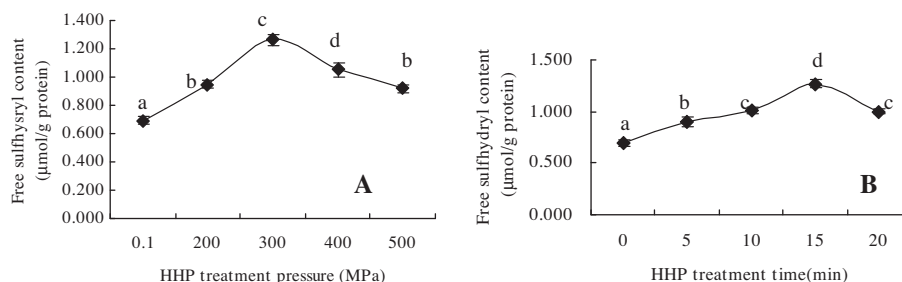


Fig. 2. Effects of HHP treatment pressure and time on free sulphydryl content of SPI (A: HHP treatment time is fixed at 15 min; B: HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

tent was found in the range of HHP treatment time 15–20 min (Fig. 2B). The HHP treatment time of 15 min gave the highest free SH content. These results further verify that the occurrence of HHP-induced protein unfolding and subsequent aggregation/re-association of unfolded proteins. The increase in free SH content of SPI treated at 300 MPa for 15 min may reflect the disruption of SS bonds within the protein molecule. At this pressure and time level, free SH groups seems to be stable with relatively low extent of aggregation, whereas at higher pressure and longer time levels, aggregation of unfolded proteins would become prominent due to hydrophobic interactions and/or sulphydryl-disulphide exchange reactions, thus enhancing re-formation of SS bonds.

These results are consistent with those reported by Wang et al. (2008), who observed similar change tendency of free SH content of SPI under HHP treatment. The highest free SH content was obtained at 200 MPa, which is lower than our result (300 MPa). This difference could result from raw SPI made by different soybean cultivar and extraction methods. Similar influences of HHP treatment on free SH content were observed in 1% SPI at pH3 and 8 (Puppo et al., 2004). Zhang et al. (2003) revealed gradual increase of free SH content in HHP-treated glycinin in the range of 0–600 MPa, but the concentration of glycinin solution utilised for HHP processing was not given.

However, there are some contrary opinions with our results. Torrezan et al. (2007) observed the effects of HHP on functionality of SPI, who employed over two pH ranges (2.66–4.34 and 5.16–6.84) with SPI concentration (0.32–3.68%) and pressure (198–702 MPa) as the other independent variables. After high-pressure treatment, free SH contents are lower in all the samples in comparison with initial SPI. Puppo et al. (2004) suggested that this occurred due to the formation of SS bonds through SH/SS interchange reactions. The discrepancy between our results and those reported by those authors could be related to different pH and pressure ranges, which have strong effects on the aggregation of HHP treated samples (Puppo et al., 2004).

Surface hydrophobicity was used as a probe for the determination of conformational difference between native and HHP-modified SPI. HHP treatment resulted in gradual and significant

($P < 0.05$) increases of Ho in SPI when the pressure increased in the ranges of 200–300 MPa and 5–15 min, meanwhile a significant increase in Ho was observed in SPI treated at 300 MPa and 15 min, when compared to the control (Fig. 3).

These results indicate that HHP treatment resulted in unfolding of the protein's native structure, leading to the exposure of hydrophobic regions to the exterior of the protein molecules. Actually, HHP processing on Ho also reflects the difference in protein aggregation and unfolding/refolding extent at different pressure levels. At high pressure (400–500 MPa), the increase in the extent of association between denatured proteins with high Ho may lead to the increase in the formation of aggregates with high molecular weight, and subsequently resulting in decrease of total Ho. The observed surface hydrophobicity could be caused by the balance between aggregation and partial unfolding. At lower protein concentrations, e.g., less than 3%, protein–protein association among unfolded globulins may not be dominant (the unfolded globulins are stable in the medium), and the extent of exposure of hydrophobic groups may be mainly dependent on the applied pressure level (Wang et al., 2008). Puppo et al. (2004) used 1% SPI (pH 3 and 8) for HHP treatment, and observed a significant increase in Ho at 400 MPa compared to other pressure levels (including 600 MPa). In this study, no explanation was provided on why the Ho of SPI treated at 400 MPa was higher than that treated at 600 MPa. Molina et al. (2001) used 10% SPI solutions (pH 7.5 and 6.5) for HHP treatment, and showed that SPI treated at 400 MPa exhibited the lowest Ho value, attributed to partial denaturation and subsequent aggregation of hydrophobic groups. In Molina et al. (2001) study, 7S globulin showed its highest surface hydrophobicity after treatment at 400 MPa whereas 11S globulin showed its highest surface hydrophobicity at 200 MPa. It is suggested that pressure at 400 MPa dissociated the 7S of the SPI into partially or totally denatured monomers that enhanced the surface activity but at the same time, the unfolding of the polypeptides of the 11S within the hexamer led to aggregation, negatively affecting the surface hydrophobicity of the SPI. Therefore, the ratio of 7S–11S is one crucial aspect about HHP treatment on the surface hydrophobicity, which relates to soybean cultivar and SPI extrac-

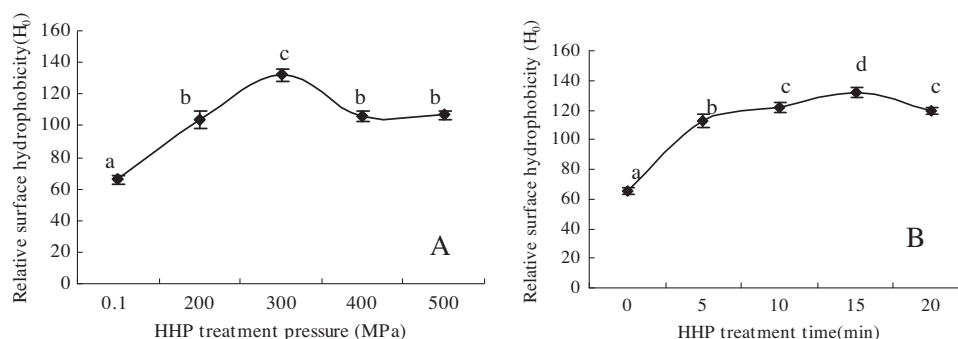


Fig. 3. Effects of HHP treatment pressure and time on relative surface hydrophobicity of SPI (A: HHP treatment time is fixed at 15 min; B: HHP treatment pressure is fixed at 300 MPa). Means with different letters in the same line are significantly different at the 5% level.

tion methods. Our results do not agree with those reported by Wang et al. (2008) who concluded that the surface hydrophobicity of 1% SPI indicated a gradual increase at 200, 400 and 600 MPa, compared to control sample. This discrepancy could be related to the various soybean cultivar and SPI extraction parameters.

Fig 4 illustrates the fluorescence spectra of ANS at various high pressure and time processed SPI in solutions at pH 7.0 phosphate buffer. The data showed raw SPI exhibited the lowest level of relative fluorescence intensity. HHP treatment time is fixed at 15 min level. After processing at low pressures (200–300 MPa), the relative fluorescence intensity peak increased significantly compared with that of control. When pressure increased to 400 MPa, relative fluorescence intensity peak of SPI decreased, slightly being higher than that of SPI treated at 200 MPa. When pressure kept increasing to 500 MPa, relative fluorescence intensity peak decreased continuously, slightly being lower than that of SPI treated at 200 MPa. The change tendency of relative fluorescence intensity of SPI after high pressure treatment revealed that when processed at relatively low pressure (200–300 MPa), hydrophobic regions of SPI increased; however, when processed at high pressure (400–500 MPa), hydrophobic regions decreased. Our results do not agree with those reported by Wang et al. (2008), who concluded that the fluorescence peak (460–480 nm) of 1% SPI indicated a gradual increase from 0.1 to 600 MPa, compared to control. This inconsistency could attribute to the different soybean cultivar and SPI extraction methods. HHP treatment pressure is fixed at 300 MPa level. The fluorescence peaks of HHP treated SPI increased in 5–15 min, however it decreased at 20 min level, being lower than that of 5 min and higher than that of control. The decreases of fluorescence intensity of SPI by HHP treatment at higher pressure and longer time levels suggested that the exposed hydrophobic groups may re-associate or aggregate to form a more stable structure.

A blue shift of the maximum emission wavelength was observed after HHP treatment, which indicates SPI binded ANS in a less polar environment compared to native SPI (Liu, Powers, Swanson, Hill, & Clark, 2005). Whatever HHP pressure and time, the maximum emission wavelength indicated blueshifts (Fig. 4). At 300 MPa and 15 min, there was an 11.5-fold increase in fluorescence intensity and the maximum emission wavelength shifted from 516 to 466 nm. Further HHP treatment time and pressure did not cause further changes in the maximum emission wavelength, indicating major changes in SPI binding ANS environment during pressurisation occurred at 200 MPa and within 5 min. The 50 nm blue shift and the increases in the fluorescence intensity of SPI after HHP treatments are consistent with the results of HHP treatment of β -LG (Yang, Dunker, Powers, Clark, & Swanson, 2001). A reasonable explanation, consistent with pressure studies on other proteins, is that unfolding is more or less complete but that partially folded, perhaps molten globular forms, remain after HHP treatment (Yang et al., 2001).

Overall, HHP treatment significantly influenced the free SH content and hydrophobicity of SPI, which are closely associated with the allergenicity of SPI. The Kunitz family of soybean trypsin inhibitors is one of the many families of proteinase inhibitors, containing two disulphide bridges. The low-molecular-weight proteins are cysteine rich and have similar 3-dimensional structures that are rich in α -helices (Breitenede & Radauer, 2004). According to our result of SDS-PAGE electrophoresis (data shown in Supplementary materials), the above allergens belonging to 2S albumin still existed after HHP treatment. Therefore, HHP would influence the interactions such as free SH content and hydrophobicity, and interfere with the structure of soy allergen epitopes, thus reducing the allergenicity of SPI.

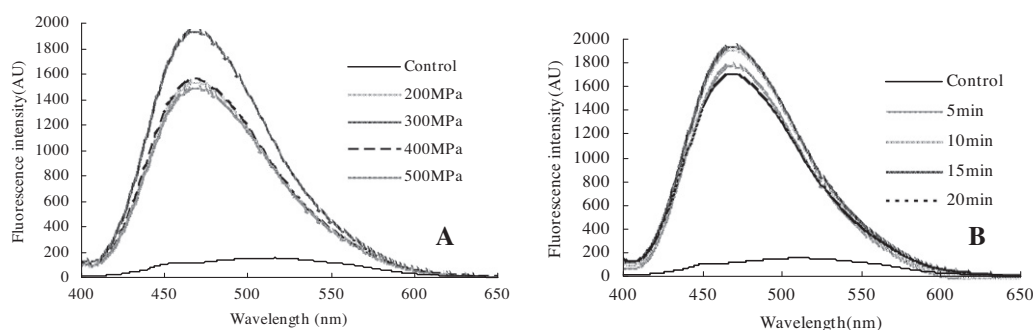


Fig. 4. Emission fluorescence spectra of SPI treated by HHP (A: HHP treatment time is fixed at 15 min; B: HHP treatment pressure is fixed at 300 MPa) (using ANS as a fluorescence probe). (A): Peak values of each curve were as follows: 0.1 MPa–516 nm, 154.3 ± 0.2 AU; 200 MPa–469 nm, 1536 ± 2 AU; 300 MPa–466 nm, 1936 ± 2 AU; 400 MPa–469 nm, 1553 ± 2 AU; 500 MPa–469 nm, 1486 ± 2 AU. (B): Peak values of each curve were as follows: 0 min–516 nm, 154.3 ± 0.2 AU; 5 min–468 nm, 1774 ± 2 AU; 10 min–467 nm, 1911 ± 1 AU; 15 min–466 nm, 1936 ± 2 AU; 20 min–468 nm, 1701 ± 1 AU.

3.3. Effects of high hydrostatic pressure on secondary structure of soy protein isolate

Far-UV CD was used to obtain information about the changes occurring at secondary folding level. CD spectra of native and HHP modified soy protein (shown in [Supplementary materials](#)) show that upon HHP the negative extreme in far-UV CD spectrum shifts to a higher wavelength. Upon HHP, intensity of the negative peak of 208 nm steady increased, indicating an increase of α -helical structure with a concomitant decrease of β -sheet structure as the loss of intensity of negative shoulder at 217 nm. The phenomena demonstrated that modification by HHP resulted in an increase of α -helical structure and a loss of the β -sheet structure.

The secondary structure content was estimated using DICHRO-WEb procedure, which is an online server for protein secondary structure analyses from circular dichroism spectroscopic data ([Whitmore & Wallace, 2004](#)). The results ([Table 1](#)) deduced from the CD spectra revealed that secondary structure of native soy protein was composed of four different conformations including α -helix, β -turn, β -sheet and random structure, in agreement with the measurement by [Li, Chen, and Mo \(2007\)](#). After HHP treatment, the helix1 and turns content significantly increased and the strand1 and unordered content significantly decreased; whereas the amount of the helix2 and strand2 did not indicate any obvious change. [Table 2](#) shows the parameters of helix and strand structure in detail. As far as helices are concerned, the average length of helices significantly increased, while the helices (per 100 residues) did not strikingly change after HHP modification. However, both the strand (per 100 residues) and the average length of strands clearly decreased. This alteration of helices indicates HHP processing unfolds the helices and compresses the strands. At 300 MPa and 15 min, the secondary structure of SPI obtained the maximum alterations, compared to native SPI and other two HHP treated SPIs. The detailed alteration information were as follows: the content of helix1 and turns, respectively increased by 195% and 13.0%; while the content of strand1 and unordered, respectively decreased by 472% and 18.7%. The average length of helices increased by 68.9%, whereas the strand (per 100 residues) and the average length of strands, respectively decreased by 15.5% and 57.0%. [Puppo et al. \(2004\)](#) used circular dichroism spectra (far UV-CD) to investigate the influence of HHP treatment, and pointed out that α -helix content decreased, and random coil content increased with increase in pressure level. However, the α -helix content of untreated SPI in this work was estimated as about 37% at pH 3 and 40% at pH 8, much higher than that previously predicted or reported for 11S seed globulins ([Zhang et al., 2003](#)). [Zhang et al. \(2003\)](#) also pointed out that some ordered structures of α -helix

and β -structure of glycinin were destroyed and converted to random coil after processed at 500 MPa. These discrepancies between our results and those opinions could result from different HHP treatment parameters such as pH and concentration.

Connecting the effects of HHP modification on the allergenicity of SPI with its secondary structures, these alterations of secondary structures suggest that the allergenicity of SPI might be associated with the helix1 and strand1. The α subunit, Gly m Bd 60 K, is one of the major allergenic proteins in the soybean 7S-globulin fraction ([Ogawa, Bando, Tsuji, Nishikawa, & Kitamura, 1995](#)), which includes 16.5% α -helix, 47.6% β -sheet, 35.9% turn in aqueous 50 mM phosphate buffer pH 7.5 ([Zhao, Chen, Xue, & Lee, 2008](#)). The 11S fraction of soybean proteins consists almost entirely of glycinin. Native glycinin is a 350-kd hexamer composed of different combinations of the five subunits G1 to G5. IgE epitopes of the acidic chain of G1 (A_1) have been found to be similar to IgE epitopes of the peanut glycinin Ara h 3. Each basic chain (B) of the five soybean glycinin subunits reacted with IgE from individuals with soybean allergy ([Breitenede & Radauer, 2004](#)). The 11S fraction of soybean proteins contained 17.0% α -helix, 47.3% β -sheet, 35.8% turn in aqueous 50 mM phosphate buffer pH 7.5 ([Zhao et al., 2008](#)). P34/Gly m Bd 30 K is a major allergen from soybean seed storage vacuoles. Its structures are stabilized by three conserved disulphide bonds and composed of two domains, one predominantly α -helix and the other containing a β -barrel ([Breitenede & Radauer, 2004](#)). These documents indicate that the epitopes of SPI could be closely related to the secondary structure of α -helix and β -sheet. Using SDS-PAGE electrophoresis, subunit composition in native and HHP treated SPI were observed. The allergens of α subunit, A_1 , B and other allergens still existed after HHP treatment. According to the measurement principle of ELISA Kit Instruction Manual, the immobilized antibody captures soybean proteins ($s_1, s_2, \dots$) in the measurement solution to form complexes. In other words, the determinants of soy allergen were complexes in current study. Therefore, our results can provide direct evidence or explanation for HHP-induced modification of soy proteins, which could alter the allergenicity of SPI and improve the security of SPI for CMA babies.

Additionally, our research team compared HHP method with the microwave, ultrasound and high pressure homogenisation methods in the aspect of reducing allergenicity of soy protein isolate for infant formula. At 300 MPa and 15 min, the allergen content decreased 48.6% compared to native SPI. [Crevel et al.](#) illustrated the relationship between the dose distributions in the whole allergic population and the proportion of reaction. If the dose of allergen decreased 50%, the proportion of reaction will also decrease 50% ([Crevel et al., 2008](#)). Therefore, HHP is a valid novel

Table 1
Effects of HHP on secondary structure composition of SPI.

Treatment	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
Control	0.075 $\pm$ 0.003 ^a	0.145 $\pm$ 0.003 ^c	0.166 $\pm$ 0.001 ^d	0.069 $\pm$ 0.002 ^g	0.193 $\pm$ 0.003 ^h	0.385 $\pm$ 0.004 ^k	1.033 $\pm$ 0.003 ^m
300 MPa, 15 min	0.221 $\pm$ 0.004 ^b	0.141 $\pm$ 0.002 ^c	0.029 $\pm$ 0.002 ^e	0.058 $\pm$ 0.003 ^g	0.218 $\pm$ 0.002 ^j	0.313 $\pm$ 0.003 ⁱ	0.981 $\pm$ 0.003 ^m
300 MPa, 20 min	0.212 $\pm$ 0.005 ^b	0.140 $\pm$ 0.003 ^c	0.043 $\pm$ 0.001 ^f	0.068 $\pm$ 0.004 ^g	0.236 $\pm$ 0.003 ⁱ	0.318 $\pm$ 0.002 ⁱ	1.017 $\pm$ 0.003 ^m
500 MPa, 15 min	0.210 $\pm$ 0.002 ^b	0.139 $\pm$ 0.002 ^c	0.044 $\pm$ 0.003 ^f	0.069 $\pm$ 0.002 ^g	0.237 $\pm$ 0.004 ⁱ	0.317 $\pm$ 0.003 ⁱ	1.017 $\pm$ 0.003 ^m

Means with different letters in the same column are significantly different at the 5% level.

Table 2
Effects of HHP on parameters of helices and strands of SPI.

Treatment	Helices (per 100 residues)	Average length of helices	Strands (per 100 residues)	Average length of strands
Control	3.617 $\pm$ 0.003 ^a	6.086 $\pm$ 0.003 ^b	3.453 $\pm$ 0.002 ^d	6.803 $\pm$ 0.002 ^g
300 MPa, 15 min	3.527 $\pm$ 0.003 ^a	10.279 $\pm$ 0.003 ^c	2.917 $\pm$ 0.003 ^f	2.992 $\pm$ 0.003 ⁱ
300 MPa, 20 min	3.511 $\pm$ 0.004 ^a	10.041 $\pm$ 0.004 ^c	3.398 $\pm$ 0.003 ^e	3.258 $\pm$ 0.003 ^h
500 MPa, 15 min	3.466 $\pm$ 0.002 ^a	10.068 $\pm$ 0.002 ^c	3.451 $\pm$ 0.003 ^e	3.287 $\pm$ 0.003 ^h

Means with different letters in the same column are significantly different at the 5% level.

processing for enhancing the safety of SPI for infants. Moreover, *in vitro* digestibility, at the end of pepsin and trypsin digestion after HHP treated SPI at 300 MPa and 15 min, significantly ($P < 0.05$) increased 6.02% and 7.45% (data not shown), respectively, compared to native SPI, which may benefit infant development.

Currently, we are conducting studies HHP assisted enzymatic method to further decrease allergenicity of special SPI and further structure–function studies of SPI allergens, adopting 2-dimensional electrophoresis and western blotting methods, to arrive at a clearer understanding of the features of a protein that result in allergenicity for Chinese infants and children.

4. Conclusions

HHP considerably reduced the allergenicity of soy protein isolate for infant formula. The processing parameters including HHP pressure and duration time could significantly influenced the allergenicity reducing efficiency. At 300 MPa and 15 min, the allergenicity decreased 48.6% compared to native SPI. In the ranges of 200–300 MPa and 5–15 min, free SH content and hydrophobicity of SPI significantly increased. Meanwhile at the levels above 300 MPa and 15 min, two interactions progressively decreased. Whatever HHP pressure and time, the maximum emission wavelength indicated blueshifts. At 300 MPa and 15 min, there was an 11.5-fold increase in fluorescence intensity and the maximum emission wavelength shifted from 516 to 466 nm. Further HHP treatment time and pressure did not cause further changes in the maximum emission wavelength, indicating major changes in SPI binding ANS environment during pressurisation occurred upon 200 MPa and within 5 min. After HHP treatment, the helix1 and turns content significantly increased and the strand1 and unordered content significantly decreased; whereas the amount of the helix2 and strand2 did not indicate any obvious change. The average length of helices significantly increased, while the helices (per 100 residues) did not strikingly change after HHP modification. However, both the strand (per 100 residues) and the average length of strands clearly decreased. Some documents indicate that the epitopes of SPI allergens could be closely related to the secondary structure of α -helix and β -sheet. Our results can provide direct evidence or explanation for HHP-induced modification of soy proteins, which could alter the allergenicity of SPI and improve the security of SPI for CMA babies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.11.040.

References

- Beveridge, T., Toma, S. J., & Nakai, S. (1974). Determination of SH- and SS-groups in some food proteins using Ellman's reagent. *Journal of Food Science*, 39, 49–51.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, 72, 248–254.

- Breitenede, H., & Radauer, C. (2004). A classification of plant food allergens Molecular mechanisms in allergy and clinical immunology. *Journal of Allergy and Clinical Immunology*, 113, 821–830.
- Crevel, R. W. R., Ballmer-Weber, B. K., Holzhauser, T., Hourihane, J. O'B., Knulst, A. C., Mackie, A. R., Timmermans, F., & Taylor, S. L. (2008). Thresholds for food allergens and their value to different stakeholders. *Allergy*, 63, 597–609.
- El-Agamy, E. I. (2007). The challenge of cow milk protein allergy. *Small Ruminant Research*, 68, 64–72.
- Estrada-Girón, Y., Swanson, B. G., & Barbosa-Cánovas, G. V. (2005). Advances in the use of high hydrostatic pressure for processing cereal grains and legumes. *Trends in Food Science & Technology*, 16, 194–203.
- Hajós, G., Polgár, M., & Farkas, J. (2004). High-pressure effects on IgE immunoreactivity of proteins in a sausage batter. *Innovative Food Science & Emerging Technologies*, 5, 443–449.
- Kato, T., Katayama, E., Matsubara, S., Omi, Y., & Matsuda, T. (2000). Release of allergic proteins from rice grains induced by high hydrostatic pressure. *Journal of Agricultural and Food Chemistry*, 48, 3124–3126.
- Liu, X. M., Powers, J. R., Swanson, B. G., Hill, H. H., & Clark, S. (2005). Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. *Innovative Food Science and Emerging Technologies*, 6, 310–317.
- Li, Y. Q., Chen, Z. X., & Mo, H. Z. (2007). Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *LWT – Food Science and Technology*, 40, 1167–1175.
- Maldonado, J., Gil, A., Narbona, E., & Molina, J. A. (1998). Special formulas in infant nutrition: a review. *Early Human Development* (53 Suppl.), S23–S32.
- Messens, W., van Camp, J., & Huyghebaert, A. (1997). The use of high pressure to modify the functionality of food proteins. *Trends in Food Science and Technology*, 8, 107–112.
- Molina, E., Papadopoulou, A., & Ledward, D. A. (2001). Emulsifying properties of high pressure treated soy protein isolates and 7S and 11S globulins. *Food Hydrocolloids*, 15, 263–269.
- Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., & Kitamura, K. (1995). Alpha-subunit of beta-conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic dermatitis. *Bioscience, Biotechnology, and Biochemistry*, 59, 831–833.
- Omi, Y., Katto, T., Ishida, K. I., Kato, H., & Matsuda, T. (1996). Pressure-induced release of basic 7S globulin from cotyledon dermal tissue of soybean seeds. *Journal of Agricultural and Food Chemistry*, 44, 3763–3767.
- Peñas, E., Gomez, R., Frias, J., Baeza, M. L., & Vidal-Valverde, C. (2011). High hydrostatic pressure effects on immunoreactivity and nutritional quality of soybean products. *Food Chemistry*, 125, 423–429.
- Peñas, E., Préstamo, G., Baezac, M. L., Martínez-Moleroc, M. I., & Gomez, R. (2006). Effects of combined high pressure and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *International Dairy Journal*, 16, 831–839.
- Peñas, E., Préstamo, G., Polo, F., & Gomez, R. (2006). Enzymatic proteolysis, under high pressure of soybean whey: Analysis of peptides and the allergen Gly m 1 in the hydrolysates. *Food Chemistry*, 99, 569–573.
- Préstamo, G., Lesmes, M., Otero, L., & Arroyo, G. (2000). Soybean vegetable protein (tofu) preserved with high pressure. *Journal of Agricultural and Food Chemistry*, 48, 2943–2947.
- Puppo, M. C., Chapleau, N., Speroni, F., de Lamballerie-Anton, M., Michel, F., Afón, C., et al. (2004). Physicochemical modifications of high-pressure-treated soybean protein isolates. *Journal of Agricultural and Food Chemistry*, 52, 1564–1571.
- Tang, C. H., & Ma, C. Y. (2009). Effect of high pressure treatment on aggregation and structural properties of soy protein isolate. *LWT – Food Science and Technology*, 42, 606–611.
- Tang, C. H., & Sun, X. (2011). A comparative study of physicochemical and conformational properties in three vicilins from *Phaseolus* legumes: Implications for the structure–function relationship. *Food Hydrocolloids*, 25, 313–324.
- Torrezan, R., Thamb, W. P., Bell, A. E., Frazier, R. A., & Cristianini, M. (2007). Effects of high pressure on functional properties of soy protein. *Food Chemistry*, 104, 140–147.
- Wang, X. S., Tang, C. H., Li, B. S., Yang, X. Q., Li, L., & Ma, C. Y. (2008). Effects of high pressure treatment on some physicochemical and functional properties of soy protein isolates. *Food Hydrocolloids*, 22, 560–567.
- Whitmore, L., & Wallace, B. A. (2004). DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Research*, 32, W668–W673.
- Yang, J., Dunker, A. K., Powers, J. R., Clark, S., & Swanson, B. G. (2001). β -Lactoglobulin molten globule induced by high pressure. *Journal of Agricultural and Food Chemistry*, 49, 3236–3243.
- Zhang, H. K., Li, L. T., Tatsumi, E., & Kotwal, S. (2003). Influence of high pressure on conformational changes of soybean glycinin. *Innovative Food Science and Emerging Technologies*, 4, 269–275.
- Zhao, X. Y., Chen, F. S., Xue, W. T., & Lee, L. T. (2008). FTIR spectra studies on the secondary structures of 7S and 11S globulins from soybean proteins using AOT reverse micellar extraction. *Food Hydrocolloids*, 22, 568–575.