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Functional properties and *in vitro* trypsin digestibility of red kidney bean (*Phaseolus vulgaris* L.) protein isolate: Effect of high-pressure treatment

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ABSTRACT

The effects of high-pressure (HP) treatment at 200–600 MPa, prior to freeze-drying, on some functional properties and *in vitro* trypsin digestibility of vicilin-rich red kidney bean (*Phaseolus vulgaris* L.) protein isolate (KPI) were investigated. Surface hydrophobicity and free sulfhydryl (SH) and disulfide bond (SS) contents were also evaluated. HP treatment resulted in gradual unfolding of protein structure, as evidenced by gradual increases in fluorescence strength and SS formation from SH groups, and decrease in denaturation enthalpy change. The protein solubility of KPI was significantly improved at pressures of 400 MPa or higher, possibly due to formation of soluble aggregate from insoluble precipitate. HP treatment at 200 and 400 MPa significantly increased emulsifying activity index (EAI) and emulsion stability index (ESI); however, EAI was significantly decreased at 600 MPa (relative to untreated KPI). The thermal stability of the vicilin component was not affected by HP treatment. Additionally, *in vitro* trypsin digestibility of KPI was decreased only at a pressure above 200 MPa and for long incubation time (e.g., 120 min). The data suggest that some physiochemical and functional properties of vicilin-rich kidney proteins can be improved by means of high-pressure treatment.

1. Introduction

Kidney bean (*Phaseolus vulgaris* L.) is the most widely produced and consumed food legume in Africa, India, Latin America and Mexico (FAO, 1993). This bean usually contains 20–30% protein on a dry basis, and the protein has a good amino acid composition but is low in sulfur-containing amino acids (notably methionine) and tryptophan (Gueguen & Cerletti, 1994; Sathe, 2002). Vicilin, sometimes also named as phaseolin (or G1 globulin), is the major storage protein of this bean. It is an oligomeric protein consisting of three polypeptide subunits α-, β- and γ-phaseolin with molecular weight distribution from 43 to 53 kDa (Bollini & Vitale, 1981; Hall, McLeester, & Bliss, 1977; Romero, Sun, McLeester, Bliss, & Hall, 1975). This kind of protein shows pH-dependent association–dissociation behavior between tetrameric, protomeric, and polypeptide forms of the molecule (Romero et al., 1975; Sun, McLeester, Bliss, & Hall, 1974), and its subunits also display molecular heterogeneity, attributed to differential degrees of glycosylation (Paaren, Slightom, Hall, Inglis, & Blagrove, 1987). In our recent study, it was shown that the protein isolate (rich in vicilin component) from kidney bean had much higher subunit homogeneity and gelation ability, relative to those from other *Phaseolus* legumes (Tang, 2007). This seems to be closely related with its unique structural peculiarity, e.g. least susceptible to trypsin digestion, as compared to other vicilin components (Di Lollo, Alli, Biliarderis, & Barthakuri, 1993; Jivotovskaya, Vitalyi, Vitalyi, Horstmann, & Vaintraub, 1996).

High static pressure (HP), a promising technology, has been widely applied to modify food proteins, since this treatment has little influence on the nutritional compositions or flavor of related foods. Pressure-induced modification of protein is a complex phenomenon, concerning the disruption of interactive forces (especially hydrophobic bonds and electrostatic interactions) and changes in structural conformation (Huppertz, Fox, De Kruif, & Kelly, 2006; Messens, van Camp, & Huyghebaert, 1997; Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002). The degree of modification of the target properties (e.g. emulsifying activities) of proteins greatly depend on the protein system (e.g., type of protein, pH and ionic strength), applied pressure level and duration time of pressure treatment. When compared to milk proteins and food emulsions, information on the application of HP treatment to modify seed storage proteins is limited.

To date, HP-induced changes in physicochemical and/or functional properties have been reported for soybean protein or its purified protein components (Molina, Papadopoulou, & Ledward, 2001; Puppo et al., 2004, 2005; Wang et al., 2008) and lupin protein (Chapleau & De Lamballerie-Anton, 2003). On the whole, the

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emulsifying activity is the functional property mostly influenced by HP treatment. In some cases, the protein solubility is also negatively influenced by HP treatment, due to HP-induced protein aggregation. The literature about the application of HP technique to modify other seed storage proteins is very limited.

The main objective of this study was to investigate the effects of additional HP treatment prior to freeze-drying on some physicochemical and functional properties of KPI at neutral pH. Additionally, the influence on *in vitro* trypsin digestibility was evaluated. The protein isolate from kidney bean was chosen for the experiments, because of the two following reasons: (1) this protein is rich in vicilin (a representative 7S globulin), and its nutritional characteristics have been well recognized and (2) this protein is important for the diet in many countries, and thus the related conclusions would be of importance for the commercial utilization of this bean.

2. Materials and methods

2.1. Materials

Red kidney bean (*Phaseolus vularis* L.) seeds, cultivated in Gangshou Province of China were purchased from a local supermarket (Guangzhou, China). The seeds were stored at −20 °C, before use. Trypsin powder (from porcine pancreas; 96 catalog no. T4799, 1000–5000 BAEE units/mg solid), 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB), Folin & Ciocalteu's Phenol Reagent (F-9252), Guanidinium thiocyanate and 1,8-anilinonaphthalenesulfonate (ANS) reagents were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Fitzgerald Industries International Inc. (Concord, MA, USA). All other chemicals used were of analytical or better grade.

2.2. Preparation of protein isolates

The seeds were soaked in de-ionized water for 12 h at 4 °C and de-hulled manually. The de-hulled seeds were freeze-dried, ground and defatted by Soxlet extraction with hexane. Protein isolates were extracted according to the procedure of Fan and Sosulski (1974), with some modifications. Defatted flours were dispersed in de-ionized water (1:20, w/v), and the pH of the dispersions was adjusted to 8.0 with 2 N NaOH. The resultant dispersions were gently stirred at room temperature for 2 h, and then centrifuged at 8000*g* at 20 °C for 30 min in a CR22G centrifuge (Hitachi Co., Japan). The pellets were discarded, and the supernatants were adjusted to pH 4.5 with 2 N HCl to precipitate the proteins. Last, the precipitates obtained by centrifugation at 5000*g* for 20 min were re-dispersed in de-ionized water. The dispersions were homogenized and adjusted to pH 7.0 with 2 N NaOH. For HP processing, the protein concentration was adjusted to 30 g/L (determined by micro-Kjeldahl method, using a nitrogen conversion factor of 6.25).

2.3. High-pressure processing

HP processing was carried out in a 5 L reactor unit (Datong Pressure Systems, KEFA 75 Hitech Food Machine Company Co. Ltd., Baotou, China), equipped with temperature and pressure regulation. Prior to pressure processing, aliquots of KPI solutions were packaged in a polyethylene bag (with a size of about 50 × 150 cm) under vacuum condition. Temperature during HP treatment was controlled to avoid overheating. Protein solutions were subjected to HP treatment at 200 ± 10, 400 ± 10 and 600 ± 10 MPa for 20 min, respectively. The target pressure was reached at a rate of about 250 MPa/min, and released at a rate of about 300 MPa/min. The dioctyl sebacate oil was used as the pressure transmitting medium in the vessel, and its temperature was kept at 25 ± 2 °C during processing. Both untreated (0.1 MPa) and HP-treated KPI solutions were freeze-dried in the same process. All the protein samples were obtained from duplicate experiments.

2.4. Protein solubility (PS)

PS was determined according to the method of Petruccelli and Añón (1994), with minor modification. One hundred milligrams of untreated and HP-treated KPIs (freeze-dried) were dispersed and stirred in 10 mL of de-ionized water for 1 h at room temperature. Then, the dispersions were centrifuged at 12,000*g* for 20 min at 20 °C in a CR22G centrifuge (Hitachi Co., Japan). Protein content of the supernatants was determined by micro-kjeldahl method (N × 6.25). The PS was expressed as gram of soluble protein per 100 gram of total protein. All determinations were conducted in triplicate.

2.5. Emulsifying activity index (EAI) and emulsion stability index (ESI)

EAI and ESI were determined according to the method of Pearce and Kinsella (1978), with minor modifications. For emulsion formation, 15 mL of 0.1% (w/v) untreated or HP-treated dispersions in de-ionized water (pH 7.0) and 5 mL of corn oil were homogenized in FJ-200 High-Speed Homogenizer (Shanghai Specimen Model Co., China) for 1 min at the maximum velocity (close to 10,000 rpm). Fifty micro-liters of emulsion were taken from the bottom of the homogenized emulsion, immediately (0 min) or 10 min after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) SDS solution. After shaking in a vortex mixer for 5 s, the absorbance of dilute emulsions was read at 500 nm using a spectrophotometer (Spectrumlab 22PC, Shanghai Lengguang Technol. Ltd. Co., Shanghai, China). EAI and ESI values were calculated using the following equations:

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{c \times \phi \times (1-\theta) \times 10,000},$$

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

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

2.6. Differential scanning calorimetry (DSC)

DSC experiments were performed on a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE), according to the procedure of Meng and Ma (2001), with some modifications. Approximately 2.0 mg of untreated or HP-treated KPI samples were accurately weighed into aluminum liquid pans, and 10 μL 50 mM phosphate buffer (pH 7.0) was added. The pans were hermetically sealed and heated in the calorimeter from 20 to 120 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. Peak or denaturation temperature (T_d), enthalpy change of denaturation (ΔH) and width at half peak height of endothermic peak ($\Delta T_{1/2}$) were computed from the thermograms by the universal analyzer 2000, version 4.1D (TA Instrument-Waters LLC, USA). All experiments were conducted in triplicate.

2.7. Free sulfhydryl (SH) content

Sulfhydryl groups of protein isolates were determined according to the method of Ellman (1959), as modified by Beveridge, Toma, and Nakai (1974), with some modifications. Ellman's

reagent was prepared by dissolving 4 mg of DTNB reagent in 1 mL of Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, pH 8.0). Total and exposed SH contents for the proteins were obtained by suspending 15 mg of protein samples in 5.0 mL of reaction buffer, Tris-glycine buffer with (total SH) or without 8 M urea (exposed SH), respectively. Then, 50 μL of the Ellman's reagent were added. The resultant suspensions were incubated for 1 h at room temperature (25 ± 1 °C), with occasional vibrating, and then centrifuged at 12,000*g* for 10 min. The absorbance of the supernatant was determined at 412 nm with the reagent buffer as the blank. The protein contents of isolates were determined by micro-Kjeldahl method ($N \times 6.25$). The SH contents were calculated by use of the extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm, 13,600 M^{-1} cm^{-1} (Ellman, 1959), and expressed as μmol/g of protein.

2.8. Determination of disulfide bond (SS) content

Synthesis of 2-nitro-5-thiosulfobenzoate (NTSB) was performed according to the method of Thannhauser, Konishi, and Scheraga (1984), as modified by Petruccelli and Añón (1995). First, DTNB (0.1 g) was dissolved in 10 mL of 1 M Na_2SO_3. Then, the pH of the reaction mixture was adjusted to 7.5, and 50 μL of a 0.1 M ammoniacal solution of $CuSO_4$ was added. The reaction was magnetically stirred at 38 °C; it was ended when more than 99% of DTNB was transformed into NTSB (the reaction was followed by measuring the concentration of NTB by its absorbance at 412 nm). The remaining NTB was determined by measuring absorbance at 412 nm. The stock solution was stored at −20 °C. The test NTSB solution was prepared by diluting the stock solution (1:100) with a fresh 0.2 M Tris-base buffer containing 0.1 M Na_2SO_3, 10 mM EDTA, and 3 M guanidinium thiocyanate. This solution was adjusted to pH 9.5 with l N HC1.

Determination of disulfide bonds was carried out according to the method of Thannhauser et al. (1984). Protein isolates (25 mg/mL) were previously dispersed in Tris-base buffer. Then, 100 μL of the protein solution was mixed with 3 mL of the NTSB test solution prepared just prior to use. Absorbance at 412 nm was determined using the test NTSB solution as the reference. The extinction coefficient of NTB used to transform absorbance values into concentration values was 13,600 M^{-1} cm^{-1}.

2.9. High-performance size-exclusion chromatography (HPSEC) combined with multi-angle laser light scattering (MALLS)

The HPSEC and the MALLS systems were the same as those described by Zhao, Mine, and Ma (2004). Two TSK columns (G4000 PW_{XL} + TSK G6000 PW_{XL}) were connected in series (TOSOH Corp., Montgomeryville, PA). The fractionation range of these two columns was 2000–8,000,000 for proteins. The mobile phase (50 mM phosphate buffer, pH 7.4, containing 0.05 M NaCl) was filtered through 0.2 μm (Whatman International Ltd., Maidstone, England) and then 0.02-μm filters (Millipore Corp., Bedford, MA). The flow rate was 0.8 mL/min.

A Dawn EOS photometer (Wyatt Technology Corp., Santa Barbara, CA) was used. Two auxiliary analogue inputs enabled interfacing to external detectors such as RI and UV detectors. The instrument was placed directly before the RI detector and after the SEC columns and UV detector to avoid backpressure on the RI cell. Dynamic light scattering measurement was performed on-line in the flow cell using a QELS meter (Wyatt Technology Corp., Santa Barbara, CA). An optical fiber receiver was mounted in the read head of one of the MALLS detectors (detector 13 in our works). Chromatographic data were collected and processed by the ASTRA software (Wyatt Technology Corp.). The M_w of protein eluting in small and individual slices of the SEC chromatogram was determined, based on the Debye plot (Zhao et al., 2004). The d*n*/d*c* is reckoned to be constant (±0.185 mL/g) across the sample peak, and nearly independent of its amino acid composition, where *n* and *c* present the refractive index and sample concentration for each data slice. Bovine serum albumin monomer (Sigma, St. Louis, MO) was used for normalizing various detectors' signals relative to the 90° laser light detector signal.

2.10. Intrinsic fluorescence emission spectra

Intrinsic emission fluorescence spectra of protein samples were determined in a RF-5301 PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan). Protein solutions (0.15 mg/mL) were prepared in 10 mM phosphate buffer (pH 7.0). To minimize the contribution of tyrosyl residues to the emission spectra, protein solutions were excited at 290 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission.

2.11. Measurement of surface hydrophobicity (H_0)

H_0 was determined using ANS, according to the method of Kato and Nakai (1980), as modified by Haskard and Li-Chan (1998). In brief, stock solutions of 8×10^{-3} M ANS^-, and 1.5% (w/v) protein were prepared in 10 mM phosphate buffer (pH 7.0). To successive samples containing 4 mL of buffer and 20 μL of ANS^- stock solution were added 10, 20, 30, 40, and 50 μL of 1.5% protein solution. The mixtures were shaken in a vortex mixer for 5 s. Fluorescence intensity (FI) was measured at wavelengths of 390 nm (excitation) and 470 nm (emission) using the same spectrofluorometer at 20 ± 0.5 °C, with a constant excitation and emission slit of 5 nm. The FI for each sample with probe was then computed by subtracting the FI attributed to protein in buffer. The initial slope of the FI versus protein concentration plot was calculated by linear regression analysis and used as an index of H_0.

2.12. In vitro trypsin digestibility

The *in vitro* trypsin digestibility of protein samples was determined as follows: 5 mL of protein dispersions (1%, w/v) in 10 mM phosphate buffer (pH 8.0) was mixed with 1 mg of trypsin powder, and the mixtures were incubated at 37 °C for 30 or 120 min. The reactions were stopped by adding an equal volume of 20% (w/v) trichloroacetic acid, and the protein precipitates were removed by centrifugation at 10,000*g* for 20 min. The TCA-soluble nitrogen in the supernatants was determined by micro-Kjeldahl method ($N \times 6.25$). The %*N* release during the digestion was calculated as % N release = $(N_S - N_0) \times 100/N_{tot}$, where N_S is TCA-soluble nitrogen in supernatant phase, N_0 (mg) TCA-soluble nitrogen at 0 min, and N_{tot} (mg) total nitrogen of protein.

2.13. Statistics

An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) or Tamhane's with a confidence interval of 95 or 99% was used to compare the means.

3. Results and discussion

3.1. SEC–MALLS–RI analysis

Typical SEC elution profiles of untreated (0.1 MPa) KPI, combined with MALLS (at 90°) and RI detection are shown in Fig. 1. In the RI profile, one major peak (peak 3) eluting at 20.6 mL and several minor peaks eluting before or after the major peak were observed. In the corresponding LS profile, another observable peak

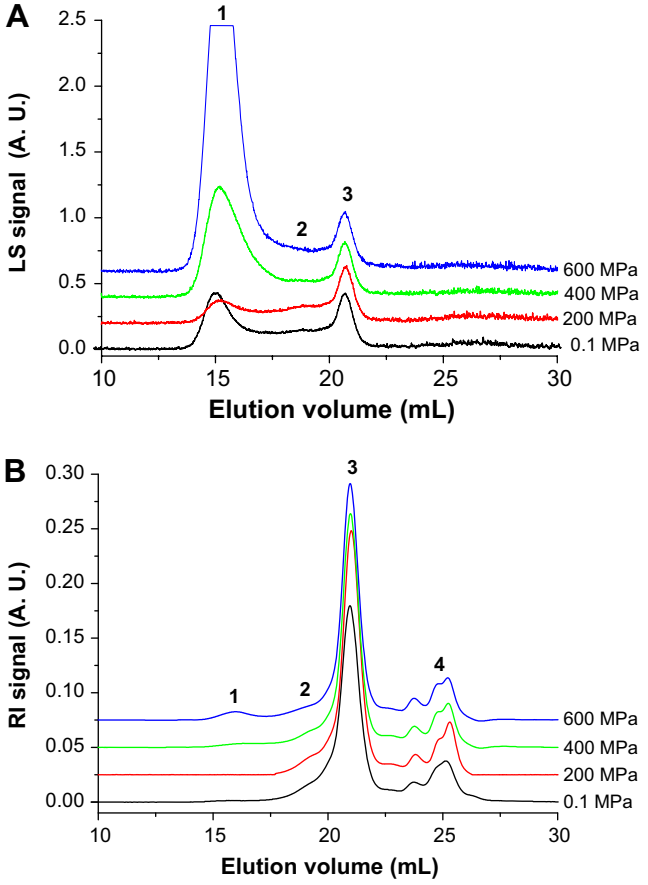

Fig. 1. Superimposed SEC–MALLS–RI elution profiles of untreated and HP-treated (200–600 MPa) KPI samples: (A) MALLS detection (at 90°) and (B) RI detection.

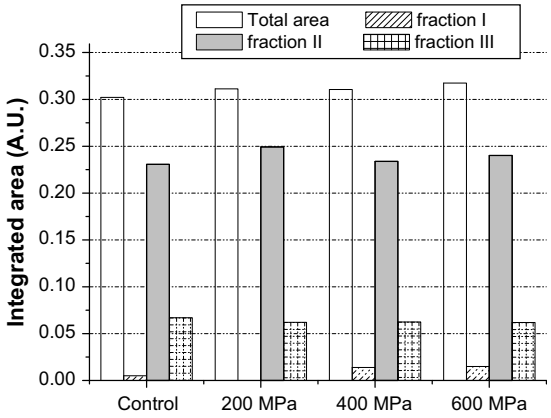

Fig. 2. Total or individual integrated area(s) of RI peaks for SEC profiles of untreated and HP (200–600 MPa)-treated KPI samples. Total area: total integrated RI peak area; Fraction I: <17.5 mL (peak 1); Fraction II: 17.5–23.0 mL (peaks 2 and 3); Fraction III: >23.0 mL (peak 4 and others).

at about 15.0 mL (peak 1) and a shoulder peak (peak 2) appeared, indicating the presence of large molecular weight (M_w) aggregates, but the amount was very low, since the RI signal of peak 2 was nearly invisible. The M_w of peak 3, calculated from the combined LS and RI signals, was about 161 kDa, in agreement with the M_w of vicilin in the trimeric form. The M_w of the shoulder peak was calculated to be about 6.2×10^5 Da (with a standard deviation of 4%), suggesting that it was mainly composed of tetramers of vicilin. From the RI profile, the vicilin (peak 3 plus peak 2) constituted about 78% of total protein in KPI. The data suggest that this protein isolate was rich in vicilin component. Furthermore, peak 4 or other peaks eluting at similar volumes may correspond to albumin or other components with small M_w in KPI.

The effects of HP (200–600 MPa) treatment prior to freeze-drying on SEC elution profiles (LS and RI signals) are also presented in Fig. 1. In the RI profile, HP-induced changes in total or individual fraction integrated area(s) are shown in Fig. 2. HP treatment resulted in a slight but gradual increase of total integrated area, suggesting that the HP treatment might improve protein solubility of KPI in the applied buffer (pH 7.4). However, the changes of relative integrated area for various elution fractions were different. HP treatment at 200 MPa resulted in dissociation of aggregate (peak 1) into peaks 2 or 3 (Figs. 1B and 2). Concomitantly, LS signal of peak 1 of untreated KPI was considerately higher than that of HP (200 MPa)-treated KPI (Fig. 1A). However, HP at a pressure above 200 MPa markedly increased the LS and RI signals of the aggregate peak (peak 1) (Figs. 1 and 2). Interestingly, from Fig. 2, it was observed that the integrated areas for peaks 2, 3 or 4 were nearly not affected by HP treatment. Thus, the increase in total integrated area by HP treatment at a pressure above 200 MPa can be considered to be due to the increase in integrated area of peak 1 (aggregate). This is an indication that in the present case, HP at pressures of 400 MPa or higher led to transformation of insoluble precipitate to soluble aggregate.

3.2. Emission fluorescence spectroscopy analysis and surface hydrophobicity (H_0)

The emission fluorescence spectroscopic technique was used to characterize the conformational changes of KPI, induced by HP treatment, as shown in Fig. 3. The fluorescence spectrum is determined chiefly by the polarity of the environment of the tryptophan and tyrosine residues and by their specific interactions and provides a sensitive means of characterizing proteins and their conformation, since the fluorescence emission maximum suffers a red shift when chromophores become more exposed to solvent, and the quantum yield of fluorescence decreases when the chromophores interact with quenching agents either in a solvent or in the protein itself (Pallarès, Vendrell, Avilès, & Ventura, 2004). The intrinsic fluorescence spectrum of untreated KPI shows an emission maximum at around 332 nm (Fig. 3A). This is a characteristic fluorescence profile of tryptophan residues in a relatively hydrophobic environment, such as the interior of the globulin (Dufour, Hoa, & Haertlé, 1994). This maximum wavelength was nearly unaffected by HP treatment at a pressure of 200 and 400 MPa, while it red-shifted to about 335 nm when HP treatment at 600 MPa was applied. On the other hand, the fluorescence intensity of untreated KPI was increased by HP treatment at pressures of 400 MPa or higher. The increase in fluorescence intensity after HP treatment was also observed for β-lactoglobulin (Yang, Powers, Clark, Dunker, & Swanson, 2002). These observations indicated that HP treatment at higher pressure levels (especially at 600 MPa) resulted in an increased exposure of the tryptophan residues of the protein to the solvent.

The fluorescence emission spectra of ANS (a polarity-sensitive fluorescent probe) upon binding to untreated and HP-treated KPI samples were also analyzed. At a constant pH (e.g. pH 7.0), ANS binds to exposed hydrophobic surfaces in partially unfolded proteins with much higher affinity than to native or completely unfolded proteins, resulting in an increase in fluorescence emission compared with the emission of free ANS in aqueous solution (Pallarès et al., 2004). As expected, HP treatment at pressures of 200 and 400 MPa led to similar increases in fluorescence emission intensity, while further increase in intensity was observed at a pressure of 600 MPa (Fig. 3B), indicating the gradual exposure of hydrophobic clusters initially buried in the untreated proteins.

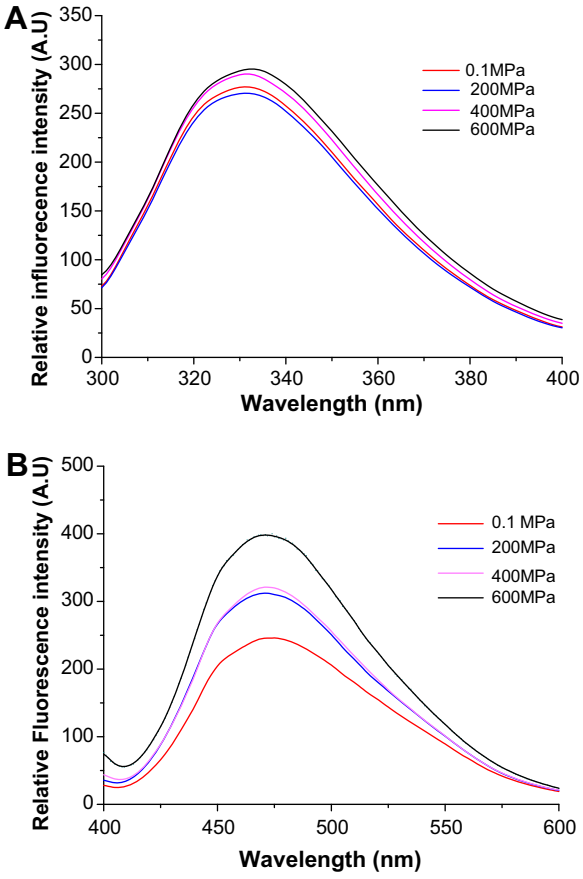

Fig. 3. Conformational changes in KPI monitored by intrinsic and ANS fluorescence emission at various pressure levels. Panel A: Intrinsic fluorescence spectra of untreated and HP-treated (200–600 MPa) KPI samples, in 10 mM phosphate buffer (pH 7.0). Panel B: Fluorescence emission spectra of ANS upon binding to untreated and HP-treated KPI samples (0.15 mg/mL).

Fig. 4 shows surface hydrophobicity (H_0) values of untreated and HP-treated KPIs, as determined from ANS emission fluorescence spectra. HP treatment at pressures of 200 and 400 MPa significantly increased the H_0, however, there was no significant difference of H_0 between 200 and 400 MPa. The HP treatment at 600 MPa further significantly increased H_0, relative to that at 400 MPa. These results are consistent with the data of maximum emission fluorescence intensity (Fig. 3B), indicating gradual exposure of hydrophobic groups initially buried in the interior of the molecules. Molina et al. (2001) used a different method based on SDS binding and Puppo et al. (2004) used the same method as in the present to determine H_0, and also observed a pressure-induced increase in H_0 of SPI and/ or its 7S and 11S fractions in the range of 200–600 MPa (though Puppo et al. performed the study at pHs 3 and 8).

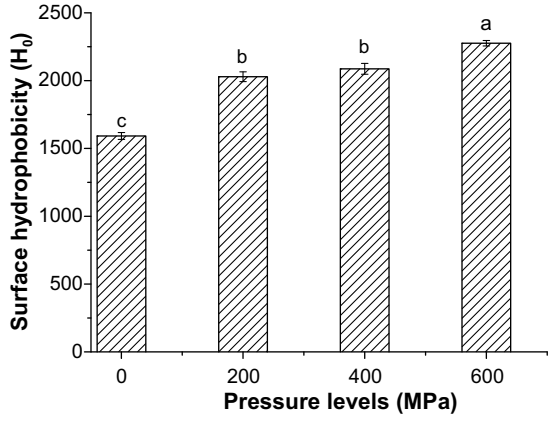

Fig. 4. Surface hydrophobicity (H_0) of untreated (0.1 MPa) and HP-treated (200–600 MPa) KPI samples in 10 mM phosphate buffer (pH 7.0). Different characters (a–d) on the top of columns indicate significant ($P < 0.05$) difference between different pressure levels.

3.3. SH and SS contents

Fig. 5 shows free SH (including total SH and exposed SH) and SS contents of untreated and HP-treated KPI samples. With pressure increasing from 0.1 to 600 MPa, total free SH content of SPI significantly and gradually decreased with a concomitant increase in SS content (Fig. 5). The data clearly indicate that HP treatment resulted in formation of SS bonds among protein components, in a pressure level dependent manner. Similar results of exposed SH content change have been observed in egg white proteins (Van der Plancken, Van Loey, & Hendrickx, 2005), and soy protein (Puppo et al., 2004). It is reasonably supposed that the formation of new SS bonds is involved in the formation of soluble aggregate by HP treatment.

The decreases in total SH content clearly corresponded with the increases observed in SS content (Fig. 5). This observation suggests that the total SH and SS content changes are primarily due to oxygen-catalyzed oxidation of the free SH groups. In the present study, although the protein solutions were packaged in a polyethylene

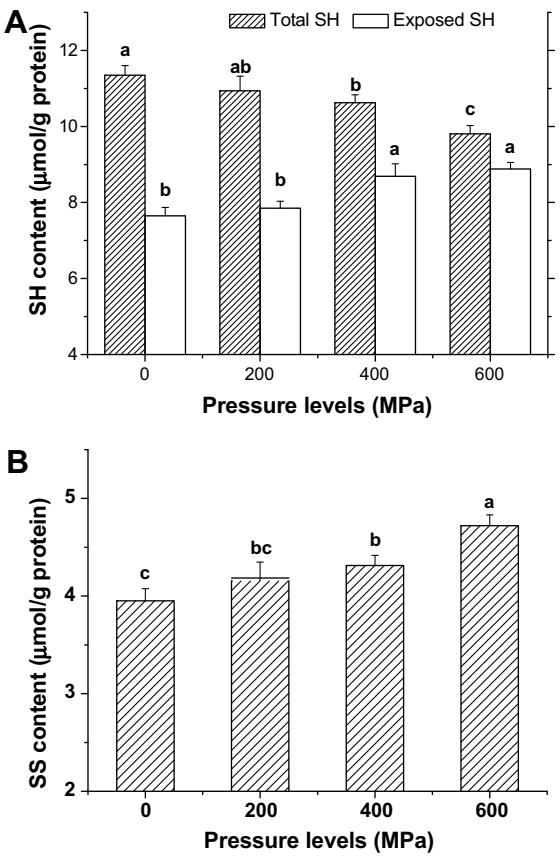

Fig. 5. SH (A) and SS (B) contents of untreated (0.1 MPa) and HP-treated (200–600 MPa) KPI samples. Different characters (a–c) on the columns represent significant difference at $P < 0.05$ level among various pressure levels. Within panel A, two kinds of SH are included: total and exposed.

bag under vacuum condition, prior to HP treatment, residual oxygen was still present. Cheftel, Hayashi, Heremans, and Masson (1992) had pointed out that, free sulfhydryl groups of protein could be oxidized under pressure above 300 MPa, when oxygen was present. On the other hand, only HP treatment at pressures of 400 and 600 MPa led to a significant ($P < 0.05$) increase in exposed free SH content, relative to the control (untreated) (Fig. 5A). This was a result of HP-induced protein unfolding and denaturation at high-pressure levels.

3.4. Functional properties

3.4.1. Protein solubility

As shown in Fig. 6, protein solubility (PS) of untreated KPI was about 77%, which is higher than that of amorphous protein isolates from white kidney bean (about 65.7%), and lower than that of the crystalline (85–90%) (Di Lollo et al., 1993). The PS was significantly improved by HP treatment at a pressure of 400 MPa or higher ($P < 0.05$). This result was consistent with SEC analysis using RI detection as shown in Figs. 1 and 3, indicating that the PS improvement at pressures above 200 MPa was due to the transformation of insoluble aggregates to soluble ones with lower molecular weight. The formation of stable soluble aggregate seems to be closely related to the newly formed SS bonds after HP treatment at higher pressure levels (above 200 MPa). A contrary result has been observed in lupin proteins, wherein PS decreased after HP treatment at a pressure above 200 MPa (Chapleau & De Lamballerie-Anton, 2003). The difference may be attributed to differences of protein type (11S or 7S globulins and/or albumin), nature and conformational stability between these two proteins.

3.4.2. Emulsifying properties

Fig. 7 shows emulsifying activity index (EAI) and emulsion stability index (ESI) values of untreated and HP-treated KPIs at pH 7.0. HP treatment at 200 and 400 MPa resulted in a similar and significant increase in EAI, while a significant decrease was observed at 600 MPa, relative to untreated KPI. As for EAI, highest ESI was also obtained at 400 MPa. With pressure level increasing from 0.1 to 400 MPa, ESI gradually and significantly increased, while there was no significant difference of ESI between 600 MPa and the control (Fig. 7). The results suggest that moderate protein unfolding by HP treatment could improve emulsifying activities of KPI, and severe HP treatment on the contrary impaired its emulsifying activities. In the case of HP-treated soy 7S globulin, highest EAI was also obtained at 400 MPa, and the increase in EAI was also attributed to protein unfolding of its subunits (Molina et al., 2001).

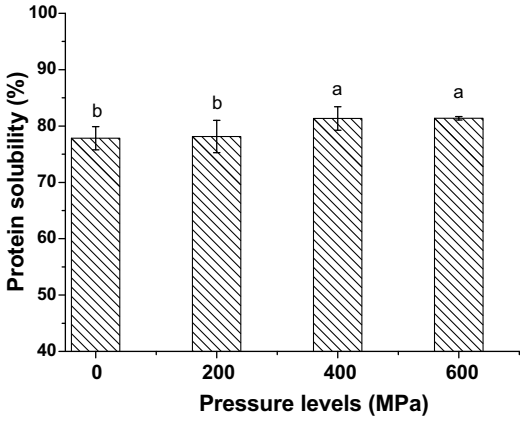

Fig. 6. Protein solubility of untreated (0.1 MPa) and HP-treated (200–600 MPa) KPI samples in de-ionized water (pH 7.0). Different characters (a–b) on the top of columns indicate significant difference ($P < 0.05$) among different pressure levels.

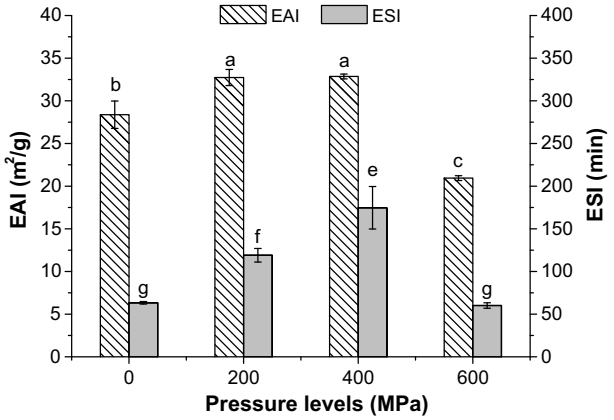

Fig. 7. Emulsifying activity index and emulsion stability index of untreated (0.1 - MPa) and HP-treated (200–600 MPa) KPI samples in de-ionized water (pH 7.0). Different characters (a–g) on the top of columns represent significant difference ($P < 0.05$) in EAI or ESI among different pressure levels.

3.4.3. DSC characteristics

The thermal properties of untreated and HP-treated SPI samples in 50 mM phosphate buffer (pH 7.0) were evaluated by DSC, and related DSC characteristics are summarized in Table 1. In the DSC profiles of all tested KPI samples, there was a prominent endothermic peak, clearly attributed to thermal denaturation of vicilin component (7S) (data not shown). The on-set temperature of denaturation (T_o) and thermal denaturation temperature (T_d) of the vicilin component were unaffected by HP treatment at 200–600 MPa (Table 1), suggesting that the structure of this protein component was very compact and not easily susceptible to HP treatment. Jivotovskaya et al. (1996) also pointed out that the vicilin (also called phaseolin) in KPI has unique structural peculiarities relative to other vicilins, since it was least susceptible to protease digestion.

However, HP treatment led to a gradual decrease in enthalpy change (ΔH) of the endothermic peak of vicilin (Table 1), indicating HP-induced unfolding of undenatured protein in KPI (Arntfield & Murray, 1981). On the other hand, the width at half peak height of the endotherm, related to cooperativity of transition from native to denatured state (Privalov, 1982), also slightly decreased with increase in pressure (Table 1). This situation is very different from the influence of HP on other 7S globulins. For example, Molina et al. (2001) indicated that HP treatment at a pressure of 400 MPa and higher led to remarkable decrease in ΔH, and the T_d was also significantly decreased by HP treatment at 600 MPa. In this work, the HP influence was attributed to the dissociation of 7S fraction (β-conglycinin) of SPI into partially or completely denatured monomers. The differences of structural characteristic and

Table 1
DSC characteristics of untreated (0.1 MPa) and HP (200–600 MPa)-treated KPI samples

	T_o (°C)	T_d (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)
Control	88.3 ± 0.08^a	93.9 ± 0.16^a	16.2 ± 0.50^a	5.47 ± 0.09^a
200 MPa	88.1 ± 0.40^a	93.8 ± 0.27^a	13.8 ± 0.10^b	5.37 ± 0.08^{ab}
400 MPa	88.7 ± 0.26^a	93.9 ± 0.22^a	12.4 ± 0.18^c	5.32 ± 0.05^{bc}
600 MPa	88.5 ± 0.05^a	93.7 ± 0.06^a	11.2 ± 0.84^c	5.19 ± 0.08^c

The scan rate was 5 °C per min. The KPI samples were dispersed at about 20% (w/v) in 50 mM phosphate buffer (pH 7.0).
Values were expressed as mean and standard deviations of triplicate measurements. T_o, on-set temperature of denaturation; T_d, thermal denaturation temperature; ΔH, enthalpy changes of the endotherm and $\Delta T_{1/2}$, width at half peak height of endothermic peak. Superscript letters (a–c) indicate significant ($P < 0.05$) difference within the same column.

Table 2
In vitro trypsin digestibility of untreated (0.1 MPa) and HP-treated (200–600 MPa) KPI samples

Samples	TCA-soluble nitrogen (%)	
	30 min	120 min
Control	5.73 ± 0.22[a]	15.19 ± 0.33[a]
200 MPa	5.61 ± 0.14[a]	14.99 ± 0.20[a]
400 MPa	5.52 ± 0.27[a]	13.33 ± 0.57[b]
600 MPa	5.82 ± 0.05[a]	13.60 ± 0.47[b]

Values were expressed as means and standard deviations of triplicate measurements. Different superscript characters (a–b) indicate significant difference ($P < 0.05$) within the same column due to the pressure level.

ability of refolding after unfolding may account for the differences of HP treatment on different vicilin samples.

3.5. *In vitro* trypsin digestibility

The *in vitro* trypsin digestibility of untreated and HP-treated KPI samples was evaluated by the release of TCA-soluble nitrogen, after incubation time of 30 and 120 min respectively, as shown in Table 2. HP treatment at 200–600 MPa did not affect *in vitro* trypsin digestibility at a digestion time of 30 min, however, HP treatment at a pressure of 400 MPa or higher significantly decreased the digestibility at a digestion time of 120 min (Table 2). Low susceptibility of this vicilin (phaseolin) to trypsin and pepsin hydrolysis has been also previously observed, and attributed to structure pecularity of this protein (Jivotovskaya et al., 1996; Romero & Ryan, 1978).

Susceptibility to trypsin hydrolysis has been taken as an index of structural integrity for many proteins, e.g. ovalbumin and egg lysozyme (Iametti et al., 1998; Van der Plancken, Van Remoortere, Van Loey, & Hendrickx, 2003). This seems to be not suitable in the present case, since HP treatment resulted in unfolding of vicilin component (as indicated by DSC and fluorescence spectrum analyses). Factually, the influence of HP treatment on the trypsin digestibility can be considered to be a consequence of protein unfolding or denaturation (positive) as well as protein aggregation (negative). The decreased digestibility at a digestion time of 120 min (after HP treatment at a pressure above 200 MPa) may reflect that the negative influence of protein aggregation on the digestibility was larger than the positive influence of protein denaturation.

4. Conclusions

HP treatment at 200–600 MPa could to some extent improve protein solubility of vicilin-rich KPI, via the formation of soluble aggregate from insoluble precipitate. Additionally, HP treatment could result in gradual unfolding of protein molecules, and formation of SS bonds from free sulfhydryl groups. These changes seemed to largely account for the influence of HP treatment on the functional properties of KPI, especially the emulsifying activities. Furthermore, the *in vitro* trypsin digestibility (at a digestion time of 120 min) was significantly decreased by HP at a pressure above 200 MPa.

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