

Effects of High Pressure and Heat Treatments on Physicochemical and Gelation Properties of Rapeseed Protein Isolate

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Abstract High pressure (HP, 200, 400, and 600 MPa)- and heat (60, 80, and 100 °C)-induced gelation, aggregation, and structural conformations of rapeseed protein isolate (RPI) were characterized using gel permeation–size-exclusion chromatography, differential scanning calorimetry, and circular dichroism (CD) techniques. HP treatments significantly ($p<0.05$) increased the content of soluble protein aggregates and surface hydrophobicity of RPI. In contrast, heat treatments at 80 and 100 °C led to significant ($p<0.05$) decreases in the amount of soluble protein aggregates. At pressure treatment of 200 MPa, there was a significant ($p<0.05$) increase in free sulfhydryl group content of RPI, whereas 400- and 600-MPa treatments as well as temperature treatments (60–100 °C) caused significant decreases. Protein denaturation temperature was increased by about 6 °C by HP and heat treatments. The far-UV CD spectra revealed increases in α -helix content of RPI after HP treatments with 400 MPa producing the most increase. Near-UV data showed that HP and heat treatments of RPI led to increasing interactions among the aromatic amino acids (evidence of protein aggregation), and between aromatic amino acids and the hydrophilic environment, which indicates protein unfolding. Least gelation concentration of RPI was significantly ($p<0.05$) reduced by HP and heat treatments, but HP-treated RPI produced gels with

better textural properties (hardness increased from ~7.7 to 81.1 N, while springiness increased from ~0.37 to 0.99). Overall, pressure treatments (200–600 MPa) were better than heat treatments (60–100 °C) to modify the structure and improve gelation properties of RPI.

Keywords Rapeseed protein isolate · High pressure treatment · Heat treatment · Gelation · Differential scanning calorimetry · Circular dichroism

Introduction

Structural and functional properties are important determinants of protein quality; therefore, appropriate processing treatments that lead to structure modification can improve functional properties and enhance utilization of proteins as ingredients in the food industry. It is well known that heat treatment can induce thermal denaturation through covalent bond disruption, which can lead to increased exposure of hydrophobic and sulfhydryl (SH) groups with substantial effects on foaming, emulsion, and gelation properties of food proteins (Molina and Ledward 2003; Van der Plancken et al. 2007; Raikos 2010). In contrast to thermal processing, high pressure (HP) is a promising technology that has minor influence on small molecule nutrients such as amino acids, vitamins, and flavor compounds but can induce extensive changes in the structure of macromolecular components (e.g., proteins and carbohydrates) to produce changes in quality of food products (Barba et al. 2012; Devi et al. 2013). HP treatment has received tremendous research interest as a means of improving ingredient functionality and especially to influence protein structural changes that can enhance the formulation of food products with desirable qualities. HP processing can lead to protein denaturation and different states of aggregation or gelation depending on the protein system, the treatment temperature,

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the protein solution conditions, and the magnitude and duration of the applied pressure. Generally, the quaternary structure dissociates at moderate pressure (150–200 MPa). Pressure treatment at above 150 MPa induces unfolding of proteins and reassociation of subunits from dissociated oligomers. The tertiary structure is also significantly affected at above 200 MPa. Protein secondary structure changes take place at very high pressure (300–700 MPa), leading to nonreversible denaturation (Ahmed et al. 2010). Most covalent bonds participating in the protein primary structure are pressure insensitive, at least up to 1,000–1,500 MPa (Balny 2004). For example, some ordered structures of α -helix and β -sheet structures of soy glycinin were destroyed and converted to random coil after treatment with 500 MPa (Zhang et al. 2003). HP treatment at 300 MPa was shown to change soy protein flexibility in the tertiary or quaternary conformations and these changes were related to improvements in thixotropic and textural properties (Li et al. 2011). In addition, the underlying mechanism of these modifications may be largely attributed to changes in protein intra- and intermolecular interactions. It is accepted that hydrogen bonds are destabilized by heating, while they are stabilized by HP. Similarly, electrostatic interactions are disrupted by HP, while hydrophobic interactions are favored by moderate heating. Controversial statements exist about the stabilizing or destabilizing effects of HP on hydrophobic interactions (Boonyaratanakornkit et al. 2002; Ahmed et al. 2010). SH groups and SS bonds also undergo changes that accompany dissociation and refolding of proteins during HP treatments. The SH content of walnut protein was increased in the range 300–400 MPa, whereas a significant decrease was found in the range 400–500 MPa (Qin et al. 2012). Establishment of hydrophobic interactions and disulfide bonds under pressure (300–600 MPa) allowed the formation of soluble high molecular mass aggregates from the different polypeptides of both β -conglycinin and glycinin (Speroni et al. 2009).

Rapeseed protein isolate (RPI) is normally produced from rapeseed meal and is considered a suitable source of dietary protein when compared to other legume seeds due to the high bioavailability and excellent balance of essential amino acid composition such as high levels of lysine, cysteine, and methionine (Tan et al. 2011; Fleddermann et al. 2012). Several reports also confirm that RPI has some good functional properties, especially water holding capacity, oil binding capacity, and emulsifying ability. Krause and Schwenke (2001) separated globulin (12S) and albumin (2S) from RPI by an aqueous extraction, centrifugation, and dialysis, and believed that globulin possessed lower emulsifying or surface activity than that of albumin. A previous work also reported differences in the water- and oil-binding capacities of rapeseed protein concentrates prepared from different seed varieties (Yoshie-Stark et al. 2006). Additional studies confirmed that ultrafiltered 2S protein isolate had better emulsification capacity and solubility

than that of precipitated 12S protein isolate (Yoshie-Stark et al. 2008). In order to enhance functional properties, RPI has been treated with acid, heat, and proteinases. Heat treatment of RPI at alkaline pH resulted in significant lowering of emulsification capacity, but water binding capacity and emulsion stability were improved (Barbin et al. 2011). Limited enzymatic RPI hydrolysate with the lowest degree of hydrolysis was shown to have the best foam and emulsion stability properties (Vioque et al. 2000), while gelation of canola protein isolate can be improved by increasing the amounts of protein and transglutaminase and by keeping the treatment temperature close to 40 °C (Pinterits and Arntfield 2008). Rapeseed proteins have also been shown to be suitable functional ingredients for the production of edible protein films, as evident in the ability of RPI films to maintain the quality of Seolhyang strawberries (Jang et al. 2011a), and as a component of composite edible films for use in food packaging (Jang et al. 2011b).

Though some processing technologies have been used to improve the quality of rapeseed proteins, there is scanty information on the improvement of RPI functional properties by the HP technology, especially evaluation of the relationships between the protein structure and gel-forming properties. Therefore, the objectives of this study are to determine the effects of high temperature or HP processing on protein gelation with relationships to protein structural characteristics such as molecular weight, free sulfhydryl content, denaturation enthalpy, surface hydrophobicity, and molecular conformation (secondary and tertiary structures).

Materials and Methods

Materials

The defatted rapeseed (*Brassica napus* L.) meal (DRM) was supplied by COFCO East Ocean Oils & Grains Industries Co., Ltd. (Zhang Jiagang, China). DRM refers to the residual of rapeseeds with lipids extracted out, and it is from the named species that is low in glucosinolates content (Fahey et al. 2001), which has minor toxic effect not advisable for large or frequent dosage since this may bring about unfavorable problem to human digestion. However, other varieties of rapeseeds include *B. rapa* L., which is often called turnip rape; *B. juncea* Coss (brown mustard); and *Sinapis alba* L. (yellow mustard). The meal was grounded to pass through a 15-mm screen sieve before used. 5,5-Dithiobis(2-nitrobenzoic acid) DTNB, glycine, ethylenediaminetetraacetic acid (EDTA), 1-anilino-8-naphthalene-sulphonate (ANS), and tris (hydroxymethyl)aminomethane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

Preparation of RPI

RPI was prepared from DRM by precipitation according to the method described by Yoshie-Stark et al. (2008). Briefly, DRM was dispersed in deionized water (1:15, w/v), adjusted to pH 10.0 with 1 M NaOH and then mixed at 45 °C for 2 h. The slurry was centrifuged at 10,000g for 30 min, the supernatant recovered, adjusted to pH 4.5 with 1 M HCl, and centrifuged. The precipitated proteins were recovered and redispersed in deionized water, adjusted to pH 7.0 with 1 M NaOH and freeze-dried to produce RPI powder. Protein content of RPI determined by the modified Lowry method (Markwell et al. 1978) was 85.25 % and was composed of three main fractions with estimated molecular weights (MW) of 212.8, 45.5, and 22.5 kDa from gel permeation–size-exclusion chromatography (GP–SEC). In addition, the RPI also contained 0.51 % phytic acid and 3.52 % fiber, as determined, respectively, by the methods of Wheeler and Ferrel (1971) and AOAC (1990).

HP and Heat Processing

Prior to pressure and heat treatments, 1 % (w/v) RPI slurry was prepared in 50 mM Tris–HCl buffer (pH 7.5) with stirring at 4 °C for 12 h. For HP treatment, the RPI slurry was sealed in a polyethylene bag and then subjected to a 4-l HP reactor unit equipped with temperature and pressure regulation as transmitting medium of water (High Pressure Systems, Bao Tou KeFa High Pressure Technology Co., Ltd., Baotou, China). HP was operated at 200, 400, and 600 MPa for 15 min each while the temperature was kept at 25 °C. Similarly, the RPI slurry was heated in a water bath at 60, 80, and 100 °C for 15 min each followed by freeze-drying and storage at –20 °C until needed for further analysis.

Molecular Weight Distribution

According to GP–SEC protocols, the MW distribution of RPI was analyzed using a fast protein liquid chromatography system (ÄKTA Purifier 10, GE HealthSciences, Montreal, PQ, Canada). The system was connected to a Superdex 75 10/300 GL column (10×300 mm, 1,000–300,000 fractionation range) and a UV detector ($\lambda=214$ nm). A 100- μ l aliquot of RPI solution (5 mg/ml) in phosphate buffer (50 mM, 0.15 M NaCl, pH 7.0) was loaded onto the column and eluted with the above phosphate buffer at a flow rate of 0.5 ml/min. Column was calibrated with bovine serum albumin (66 kDa), cytochrome c (12 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.8 kDa).

Free Sulfhydryl Content

Free SH content was determined according to the method of Beveridge et al. (1974). Ellman's reagent was prepared by

dissolving 4 mg of DTNB in 1 ml of Tris–glycine buffer (0.086 M tris, 0.09 M glycine, and 0.004 M EDTA, pH 8.0). A 1-ml aliquot of RPI solution (1 mg/ml, prepared in Tris–glycine buffer that contain 8-M urea) was mixed with 50- μ l Ellman's reagent and incubated for 30 min at room temperature; samples were then centrifuged for 20 min at 8,000g. Absorbance was measured at 412 nm in a spectrophotometer using the buffer as blank. The SH contents were obtained from dividing the absorbance value by the molar extinction coefficient value of 13,600 mol l⁻¹ cm⁻¹.

Surface Hydrophobicity (*So*)

So of RPI was measured using an aromatic fluorescence probe (ANS) according to the method described by Wu et al. (1998). RPI sample stock solutions were serially diluted to final concentrations of 0.005–0.025 % (w/v) using 0.01 M phosphate buffer (pH 7.0). A 20- μ l aliquot of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 4 ml of each diluted RPI solution and fluorescence intensity (FI) of the mixture was measured with a spectrofluorimeter (JASCO FP-6300, Tokyo, Japan) at 390 nm (excitation) and 470 nm (emission). The initial slope of FI versus RPI concentration (percent) plot (calculated by linear regression analysis) was used as an index of *So*.

Determination of Denaturation Enthalpy

Thermal properties of RPI were examined with a differential scanning calorimeter (DSC) Q200 (TA Instruments, New Castle, DE, USA). In a typical experiment, approximately 1 mg of protein sample and 10 mg distilled water were weighed into an aluminum pan and hermetically sealed. The pans were heated from 30 to 120 °C at a rate of 10 °C/min with a sealed empty pan as reference. Denaturation temperature (T_d) and enthalpy of denaturation (ΔH) were calculated using computer software (Universal Analysis 2000, Version 4.5). All experiments were conducted in triplicate. In all cases, the sealed pans containing protein isolate samples and buffers were equilibrated at 25 °C for 2 h prior to measuring thermal properties.

Circular Dichroism (CD)

Far- and near-UV CD spectra were measured using a JASCO J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) under constant nitrogen flush according to the method of Omoni and Aluko (2006). Near-UV spectrum was recorded from 250 to 320 nm with 4 mg/ml protein solutions (dissolved 0.1 M phosphate buffer, pH 7). Far-UV CD spectra were recorded from 180 to 240 nm using 2 mg/ml protein solutions in different buffers (0.1 M phosphate buffer, pH 7.0). Stock protein solutions were first centrifuged at 10,000g followed by

protein content determination and then dilution with the phosphate buffer to obtain desired protein concentration. Though there is the potential for native protein structure disruption at high centrifugal force, the variability due to high speed centrifuge is beyond the current scope of investigation and was not determined. A 1-mm path length quartz cell was used for near-UV while 0.5 mm was used for far-UV measurements, and the recorded spectra were each the average of three scans after automatic subtraction of the buffer spectrum. Deconvolution of far-UV spectra to calculate secondary structure fractions was performed using the CDSSTR secondary structure determination algorithm (Lobley et al. 2002; Whitmore and Wallace 2004, 2008) accessed via the DichroWeb website (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

Gel Preparation and Textural Measurement

RPI gels were prepared according to the method of Aluko et al. (2009) with slight modification. Protein samples were suspended in distilled water at concentrations of 5–20 % (w/v) and stored in a cylindrical glass vial with an inner diameter of 28 mm and height of 65 mm. All solutions were mixed uniformly and heated at 95 °C in water bath for 30 min and then cooled rapidly to room temperature in an ice bath followed by storage at 4 °C for 24 h. The sample concentration at which the gel did not slip when the tube was inverted was taken as the least gelation concentration (LGC). The formed gels were analyzed for textural properties, as described by Keim and Hinrichs (2004), using a Texture Analyzer (Stable Micro System, Ltd., Surrey, UK). Gels were penetrated to 30 % deformation, with a 10-mm diameter probe at a constant rate of 0.2 mm s⁻¹. Gels were analyzed directly within the cylindrical vial in order to get information about weak gels, and the following four parameters were used to evaluate textural properties of gels. Hardness (peak force in the first compression cycle), adhesiveness (maximum negative force generated during upstroke of probe), springiness (height that the food recovers during the time elapsed between the end of the first bite and the start of the second), and cohesiveness (ratio of positive area during the second to that of the first compression cycle, downward strokes only). Chewiness was then calculated as hardness × cohesiveness × springiness.

Statistical Analysis

All assays were conducted in triplicate and analyzed by one-way analysis of variance (ANOVA). The means were compared using Duncan's multiple range test and significant differences accepted at $p < 0.05$.

Results and Discussion

Molecular Weight Distribution

The molecular weight distribution of native, HP-, and heat-treated RPI is shown in Fig. 1a. The native (untreated) RPI was mainly composed of three components labeled as ii, iii, and iv with estimated MW values of 212.8, 45.5, and 22.5 kDa, respectively. Fraction iv was the most abundant of the three protein groups and accounted for 57.97 ± 0.77 % of the total proteins in RPI (Table 1). The ii, iii, and iv fractions probably contain the three main rapeseed proteins named cruciferin (12S globulin), lipid transfer protein (LTP), and napin (2S albumin), respectively, as previously reported by Bérot et al. (2005). The HP treatment at different levels (200, 400, and 600 MPa) significantly increased

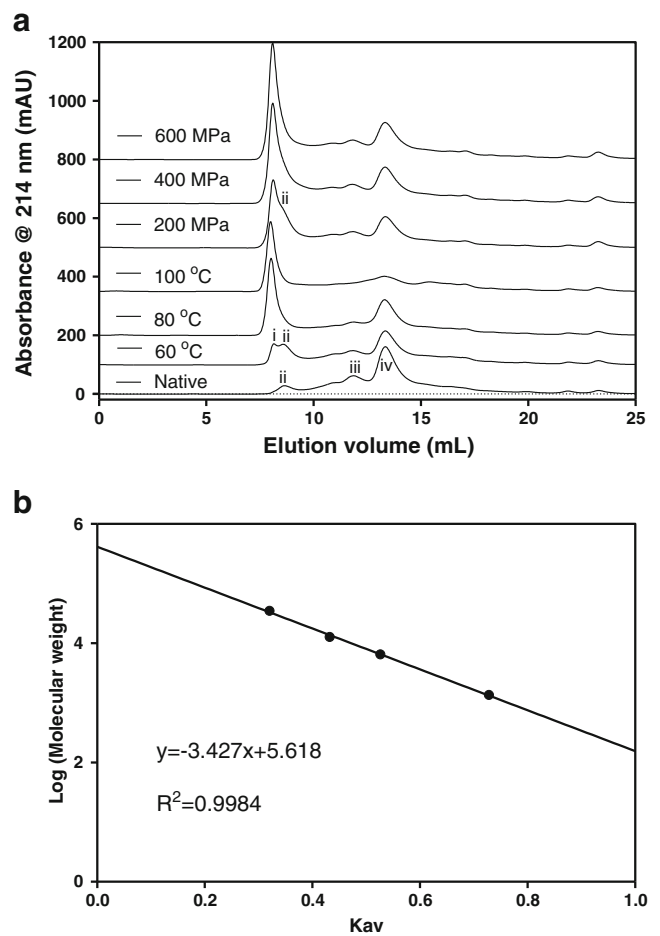


Fig. 1 a Gel permeation–size-exclusion chromatography of the native, high pressure-, and heat-treated rapeseed protein isolate after passage through a Superdex 75 10/300 GL column. Molecular weights (MW) of labeled peaks in the graph are: *i* (284.8 kDa), *ii* (212.8 kDa), *iii* (45.5 kDa), and *iv* (22.5 kDa). b MW calibration curve using protein standards: serum albumin (66 kDa), cytochrome c (12 kDa), aprotinin (6.5 kDa), vitamin B12 (1.86 kDa). $K_{av} = (V_e - V_o) / (V_t - V_o)$, where V_e is the elution volume of protein sample, V_o is the column void volume, and V_t is the column total volume

RPI aggregation, which is evident in a new distinct peak with MW of 284.8 kDa (i), as shown in Fig. 1a. Level of protein aggregation was directly proportional to HP treatment, as shown by the increased ratio of fraction i from 32 % at 200 MPa to 49 % at 600 MPa (Table 1). There were also simultaneous decreases in the ratios of fractions iii and iv as level of fraction i increased. Thus, it is possible that most of the proteins present in fraction i are HP-induced aggregates of the iii and iv fraction proteins. The behavior of fraction ii proteins was slightly different because there was an initial increase when treated with 200 MPa, but treatments with 400 and 600 MPa led to complete conversion into higher aggregates that were probably detected in fraction i. Thus, the results suggest that the 400- and 600-MPa treatments were more effective than the 200-MPa treatment in producing high MW RPI aggregates. Tang and Ma (2009) and Cecilia Condes et al. (2012) have reported similar aggregation patterns during HP treatment of soy protein isolate (SPI) and amaranth protein isolate (API), respectively. In addition, heat treatment, especially at 80 and 100 °C, also resulted in the aggregation of RPI (Fig. 1). The 80 and 100 °C treatments presented similar aggregation of RPI as HP treatment at 400 and 600 MPa, but resulted in the reduction of total soluble RPI, as evident in the least total areas of 541.18±12.98 and 421.15±6.36 % AU/ml in their chromatogram, which agrees with the work of Barbin et al. (2011). Lower temperature treatment (60 °C) led to a slight denaturation of the RPI, as shown by the total area of 788.44±7.07 % AU/ml and the smaller i peak when compared to higher temperatures. Since high weight protein aggregates have been shown to enhance the formation of protein gels (Wang and Damodaran 1990), it means that the HP- and heat-induced aggregation of RPI could contribute to better gelation properties. Overall, cruciferin (fraction ii) was the most susceptible to pressure- and heat-induced aggregation, as evident in the lack of detection after 400–600 MPa and 80–100 °C treatments (Table 1). The napin protein subunit (fraction iv) was the second most susceptible to pressure-induced aggregation, as shown by ~52 % decrease in ratio after application of 600 MPa, while LTP

(fraction iii) was the least susceptible with 33 % decrease (Table 1). However, for the temperature treatment, fraction iii was the second most susceptible at 81 % decrease in ratio and fraction iv was the least susceptible with 68 % decrease after 100 °C treatment. The high susceptibility of cruciferin to heat and high pressure treatments suggests minimal stabilization of the native protein structure by disulfide bonds when compared to napin and LTP. This is supported by the fact that the two polypeptide chains that make up napin are linked and stabilized by a disulfide bond, whereas cruciferin has several polypeptide chains that are not linked by disulfide bonds (Wu and Muir 2008). The cruciferin polypeptides that are not linked by disulfide bonds will more readily undergo aggregation when compared to the disulfide-linked napin polypeptides.

Free Sulfhydryls

The contents of free SH groups in the RPI, as shown in Fig. 2, suggest that moderate HP treatment (200 MPa) led to a significant ($p<0.05$) increase with the value of 27.10±0.05 μM/g protein, which probably reflects pressure-induced exposure of inaccessible thiol groups buried within the hydrophobic interior. In contrast, at 400- and 600-MPa treatments, there was a progressive decrease in free SH groups, which may be due to formation of disulfide bonds as pressure-induced protein–protein interactions intensified. The newly formed disulfide bonds will contribute to protein aggregation, which enhances formation of the high MW proteins (fraction i) generated under the high pressure treatment of RPI. The HP-induced protein unfolding and subsequent aggregations were also observed in the 200- and 600-MPa pressure-treated soy proteins (Wang et al. 2008). However, results from this work differ slightly from those reported for a HP-treated amaranth protein, where free SH content increased by 12, 52, and 78 % for 200-, 400-, and 600-MPa treatments, respectively (Cecilia Condes et al. 2012). Therefore, it is possible that the differences in structural properties of RPI and amaranth protein are reflected in their behaviors towards HP treatments. However, heat

Table 1 Analysis of the molecular weight distribution of native, heat-, and high pressure-treated rapeseed protein isolate

| Treatment | Percentage of total area (%) | | | | Total area (AU ml) |
|-----------|------------------------------|-------------------------|--------------------------|-------------------------|-----------------------------|
| | i | ii | iii | iv | |
| Native | Not detectable | 6.05±0.15 ^c | 16.21±0.66 ^a | 57.97±0.77 ^a | 781.11±8.82 ^d |
| 200 MPa | 32.68±0.79 ^d | 11.38±0.61 | 12.53±0.59 ^{bc} | 30.61±0.28 ^d | 1,063.18±6.36 ^c |
| 400 MPa | 42.93±0.82 ^c | ND | 11.73±0.27 ^{cd} | 30.52±0.12 ^d | 1,226.23±13.44 ^b |
| 600 MPa | 49.37±0.85 ^b | ND | 10.87±0.49 ^d | 27.71±0.39 ^c | 1,278.12±7.78 ^a |
| 60 °C | 11.21±0.15 ^e | 13.21±0.25 ^a | 13.42±0.19 ^b | 44.34±1.41 ^b | 788.44±7.07 ^d |
| 80 °C | 48.55±0.16 ^b | ND | 6.58±0.42 ^c | 40.33±0.36 ^c | 541.18±12.98 ^e |
| 100 °C | 55.41±0.84 ^a | ND | 3.04±0.04 ^f | 18.61±1.96 ^f | 421.15±6.36 ^f |

Results are presented as mean±standard deviation ($n=3$). For each column, mean values that contain different alphabets are significantly different at $p<0.05$. ND not detected

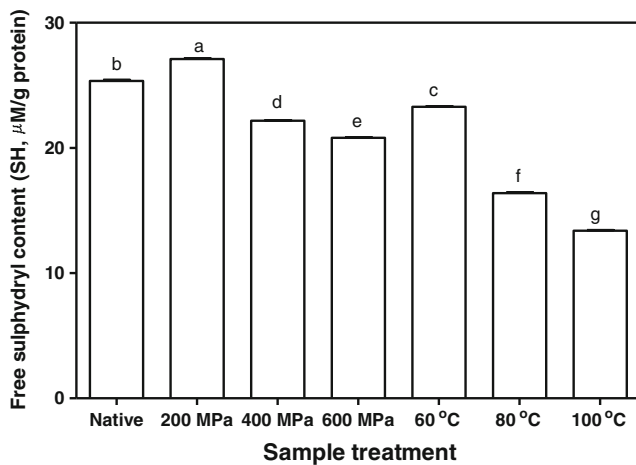


Fig. 2 Free sulphhydryl content of the native, high pressure-, and heat-treated rapeseed protein isolate. Bars (mean±standard deviation, $n=3$) with different letters have mean values that are significantly different ($p<0.05$)

treatment resulted in progressive decreases in free SH content with the least value obtained for the 100 °C treatment (Fig. 2). The results suggest that the 80 and 100 °C treatments were significantly ($p<0.05$) more effective in reducing the SH content of RPI when compared to HP treatments. The lower SH contents of 80 and 100 °C-treated RPI suggest high insoluble protein aggregation effect, which is supported by the least total area of soluble proteins for these treatments, as shown in Table 1.

Surface Hydrophobicity

Figure 3 shows that S_o of RPI was significantly ($p<0.05$) increased following HP and thermal treatments, which suggests that the native protein had a higher degree of globular (folded) structure when compared to the treated samples.

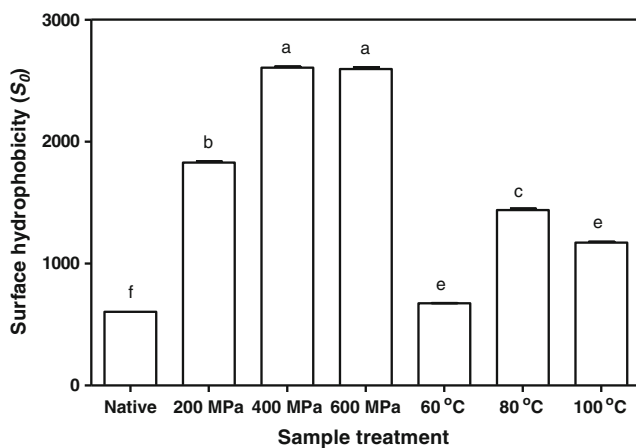


Fig. 3 Surface hydrophobicity of the native, high pressure-, and heat-treated rapeseed protein isolate. Bars (mean±standard deviation, $n=3$) with different letters have mean values that are significantly different ($p<0.05$)

Although HP and heat treatment improved the S_o of RPI, the effect was significantly ($p<0.05$) higher for the HP-treated proteins than the thermally treated samples. Additionally, the increase in S_o was dependent on HP level with the 400- and 600-MPa treatments, leading to higher S_o than the 200-MPa treatment. The higher protein unfolding efficiency of HP treatment may be due to the effective ability to disrupt hydrogen bonds that hold proteins in a folded state (Hayakawa et al. 1996). Increases in S_o following HP treatments are consistent with previous reports for food proteins such as kidney bean (Yin et al. 2008) and soybean (Li et al. 2011, 2012). However, in contrast to results obtained in this work, Li et al. (2012) showed a significant increase in S_o of soybean protein treated at 300 MPa, but higher pressure treatments (400 and 500 MPa) led to the decreased S_o . Differences in the HP and heat treatment effects on S_o of RPI may be attributed to the differences in level of protein aggregation, as shown by the presence of less amounts of soluble aggregates (least total area in Table 1) for the heat-treated samples. Thus, it is possible that heat treatments led to intense protein–protein interactions with formation of complex molecular aggregates, which reduced exposure of hydrophobic groups. The lower S_o values for heat-treated samples when compared to the HP-treated samples are consistent with results presented in Fig. 2, showing least exposure of free SH groups for RPI treated with 80 and 100 °C, and more soluble protein observed in Fig. 1a. Therefore, increased formation of disulfide bonds at high temperatures would have enhanced protein aggregate-dependent formation of soluble RPI, which reduced exposure of hydrophobic groups and, hence, decreased S_o values when compared to the HP-treated samples.

DSC Properties

Native RPI was found to have denaturation temperature (T_d) of 98.45 °C and denaturation enthalpies (ΔH) of 10.25 J/g, which is probably due to the thermal denaturation of 12S globulin (Fig. 4a). Since hydrophobic interactions contribute to high T_d values, while electrostatic interactions and hydrogen bonds contribute low T_d values (Ahmed et al. 2010), the increased T_d (from 98.45 to 104.14 °C) after HP treatment indicates increased protein–protein interactions as a result of aggregation of unfolded polypeptide chains that led to enhanced thermal stability of the RPI (Fig. 4a). The sharpness of the thermal denaturation temperature peak disappeared gradually as pressure was increased from 200 to 600 MPa, which indicates loss in RPI native structure and is consistent with formation of complex protein aggregates with lack of a well-defined conformational structure. This is consistent with the fact that the total enthalpy changes (ΔH , joules per gram), which represent the proportion of proteins with defined structural conformation, was reduced (from 10.25 to 3.72 J/g) as pressure level was increased (Fig. 4). Thus, the enthalpy data

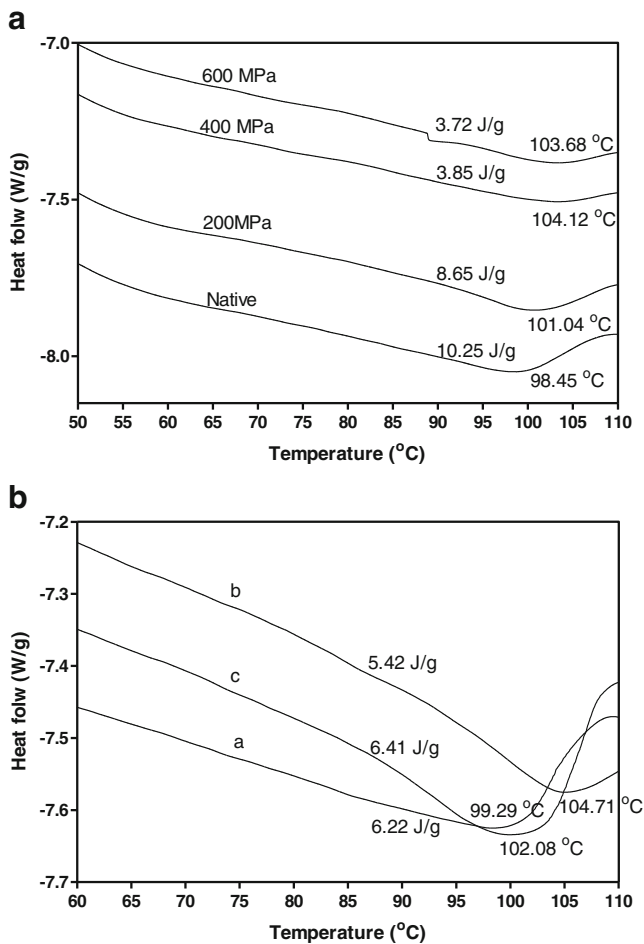


Fig. 4 DSC curves for: **a** high pressure- and **b** heat (*a* 60 $^{\circ}\text{C}$, *b* 80 $^{\circ}\text{C}$, *c* 100 $^{\circ}\text{C}$)-treated rapeseed protein isolate. Insets show the denaturation temperature, T_d ($^{\circ}\text{C}$) and enthalpy, ΔH (J/g) values

also confirms protein denaturation and unfolding as pressure increased to 600 MPa, leading to more disordered structures. These results are similar to those reported for HP-treated soybean protein, where T_d value of 11S glycinin increased from 94.7 to 98.0 $^{\circ}\text{C}$ and ΔH values decrease from 7.8 to 0.6 J/g as pressure was increased from 200 to 600 MPa (Wang et al. 2008).

As a comparison, the endothermic peaks of heat-treated RPI were much more distinctive than those obtained for the HP-treated RPI, and T_d values were also higher than that of the native protein (Fig. 4b). Thus, the heat treatments also led to a higher degree of protein–protein interactions that tended to enhance thermal stability (higher T_d values). The enthalpy values of 6.22, 5.42, and 6.41 J/g, respectively, for 60, 80, and 100 $^{\circ}\text{C}$ treatments (Fig. 4b) are not only higher than those obtained for 400- and 600-MPa treatments but also indicate a lower degree of defined structural conformation when compared to the native protein (10.25 J/g). The enthalpy data suggest that the heat-treated soluble aggregates had less structural conformation than the 200-MPa-treated sample but had

more defined structure than the 400- and 600-MPa-treated samples.

Secondary and Tertiary Structure Conformations

HP and heat treatments produced differences in the secondary structure of soluble proteins as measured using the absorbance of polarized light in the 180–240 nm far-UV range (Fig. 5a). The native RPI showed a positive band near 190 nm with a zero crossing at 200 nm and a negative band near 208 nm. After pressure treatment, the zero-crossing points shifted to near 200 nm and, simultaneously, the ellipticity decreased at 208 nm, which indicated an increase of α -helical structure and a slight decrease in β -sheet structure. The 400-MPa-treated RPI had greater ellipticity at 208 nm, which indicates more α -helical structure when compared to the 200- and 600-MPa treatments (Fig. 5a). This is reflected by the higher α -helix content for the 400-MPa-treated RPI, as shown in Table 2. The results are similar to a previous report for 300-MPa-treated SPI, where the α -helix and β -sheet contents increased by 195 % and decreased by 472 %, respectively, when compared to the

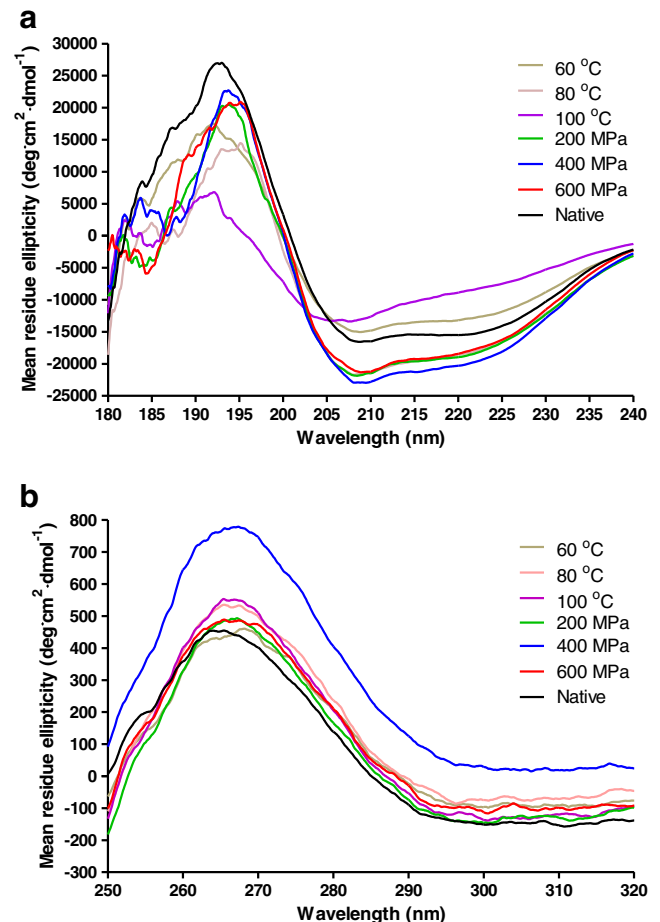


Fig. 5 Far-UV (**a**) and near-UV (**b**) circular dichroism of the native, high pressure-, and heat-treated rapeseed protein isolate

Table 2 Secondary structure composition of the native, high pressure-, and heat-treated rapeseed protein isolate

| Treatment | α helix | β strand | β turn | Unordered |
|-----------|----------------|----------------|----------------|----------------|
| Native | 43.6 \pm 0.3 | 10.3 \pm 0.1 | 15.5 \pm 0.2 | 22.6 \pm 0.2 |
| 200 MPa | 44.5 \pm 0.3 | 10.8 \pm 0.1 | 16.6 \pm 0.2 | 17.5 \pm 0.2 |
| 400 MPa | 49.0 \pm 0.3 | 8.8 \pm 0.1 | 16.2 \pm 0.2 | 15.7 \pm 0.2 |
| 600 MPa | 45.5 \pm 0.3 | 10.1 \pm 0.1 | 16.3 \pm 0.2 | 17.9 \pm 0.2 |
| 60 °C | 35.6 \pm 0.3 | 16.2 \pm 0.2 | 17.3 \pm 0.2 | 25.2 \pm 0.2 |
| 80 °C | 41.3 \pm 0.3 | 13.1 \pm 0.2 | 17.3 \pm 0.2 | 18.1 \pm 0.2 |
| 100 °C | 19.0 \pm 0.3 | 33.2 \pm 0.3 | 18.9 \pm 0.3 | 28.3 \pm 0.3 |

Results are presented as mean \pm standard deviation ($n=3$)

native protein (Li et al. 2012). On the other hand, the zero-crossing point of 100 °C-treated RPI shifted to 196 nm and its ellipticity values at 208 and 218 nm are higher than those of native RPI, which indicates a decrease in α -helix but an increase of β -sheet structure (Fig. 5a). The changes in ellipticity are reflected as increased β -sheet content and decreased α -helix content for the 100 °C-treated RPI (Table 2). The heat-treated samples also showed higher contents of unordered protein structure when compared to the HP-treated samples (Table 2). The unordered secondary structure results are consistent with data presented in Table 1 that shows stronger effects of heat treatment on protein structure (reflected as decreased total area) when compared to HP treatment.

The tertiary conformation of rapeseed protein was analyzed by near-UV CD spectra in the region of 250–320 nm to delineate contributions of the three aromatic amino acid residues, phenylalanine (255, 262, and 268 nm) tyrosine (275 and 283 nm), and tryptophan (280, 290, and 298 nm) (Kelly et al. 2005). As shown in Fig. 5b, the near-UV CD spectra of native, HP-, and heat-treated RPI consisted of a prominent positive dichroic band in the 255–285 nm range, which indicates changes in the microenvironment of the three aromatic amino acids. There was no peak observed around 290–300 nm, probably because rapeseed proteins contain few tryptophan

(Chabanon et al. 2007) but also suggests that the tryptophan residues are located in the vicinity of tyrosine. In addition, when compared to the native RPI, the near-UV CD spectra peak at 262 nm underwent a red shift of 3–5 nm after HP and heat treatments, which suggests protein denaturation (or partial unfolding) that increased interaction of the aromatic residues with a more polar environment. Besides the observed shifts of peak wavelength, the band magnitudes and the intensities were higher for the HP- and heat-treated RPI when compared to the native RPI, which is an indication of structural changes that increased interactions of the aromatic amino acid residues (Schmid 1989). The ellipticity changes were more pronounced for the 400-MPa-treated RPI, which suggests even greater interactions between the aromatic amino acid residues. The observed increases in ellipticity are typical of increased protein–protein interactions that arise from HP and heat treatments. The work of Li et al. (2011) also showed that the 300-MPa-treated SPI possessed a higher magnitude of phenylalanine ellipticity.

Gelation and Protein Gel Properties

The gel properties of native, HP-, and heat-treated RPI are summarized in Table 3. LGC of RPI was significantly ($p < 0.05$) decreased from 15 to 6 % by HP treatment, while heat treatment decreased LGC from 15 to 10 %. These observed values are consistent with the fact that there were increases in surface hydrophobicity as a result of protein unfolding during HP and heat treatments. The unfolded proteins are then able to interact through hydrophobic bonding to increase strength of resultant gel networks and reduce amount of proteins required to form the gel, i.e., decreased LGC. Previous works with pea and soybean gels also showed that hydrophobic interaction is a key factor during formation of protein gel networks (O’Kane et al. 2005). As shown in Table 3, the observed rheological properties of HP- and heat-treated RPI gels are directly dependent on their LGCs

Table 3 Gelation properties of the native, high pressure-, and heat-treated rapeseed protein isolate

| Treatment | LGC (%) | Hardness (N) ^a | Adhesiveness ^a | Springiness ^a | Cohesiveness ^a | Chewiness ^a (mJ) |
|-----------|-----------------|---------------------------------|----------------------------------|--------------------------------|--------------------------------|-----------------------------|
| Native | 15 ^b | 7.733 \pm 0.611 ^g | -1.876 \pm 0.064 ^g | 0.371 \pm 0.004 ^h | 0.352 \pm 0.003 ^h | 1.010 |
| 200 MPa | 8 ^f | 23.467 \pm 0.451 ^d | -22.990 \pm 0.608 ^d | 0.803 \pm 0.004 ^d | 0.419 \pm 0.004 ^d | 7.896 |
| 400 MPa | 7 ^g | 42.433 \pm 1.211 ^c | -35.425 \pm 0.431 ^c | 0.869 \pm 0.004 ^c | 0.436 \pm 0.004 ^c | 16.077 |
| 600 MPa | 6 ^g | 81.133 \pm 0.709 ^b | -44.880 \pm 0.198 ^b | 0.992 \pm 0.004 ^b | 0.448 \pm 0.004 ^b | 36.057 |
| 60 °C | 12 ^c | 12.403 \pm 1.212 ^f | -5.240 \pm 0.113 ^f | 0.562 \pm 0.008 ^f | 0.392 \pm 0.004 ^f | 2.732 |
| 80 °C | 10 ^e | 19.567 \pm 1.484 ^e | -9.460 \pm 0.311 ^e | 0.669 \pm 0.005 ^e | 0.409 \pm 0.006 ^e | 5.354 |
| 100 °C | 11 ^d | 12.933 \pm 0.751 ^f | -4.900 \pm 0.255 ^f | 0.425 \pm 0.005 ^g | 0.378 \pm 0.006 ^g | 2.078 |

Results are presented as mean \pm standard deviation ($n=3$). For each column, mean values that contain different letters are significantly different at $p < 0.05$
LGC least gelation concentration

^a Tests were done at the 12 % protein concentration

at the tested concentration of 12 % protein content. The hardness, springiness, and cohesiveness of HP-treated RPI gels are significantly ($p < 0.05$) higher than those of native and heat-treated RPI, while the adhesiveness of HP-treated RPI gels are of lower magnitudes when compared to the native and thermally treated RPI. Gels prepared from HP-treated SPI have been shown to be relatively softer than gels obtained by heat-treated proteins (Ahmed et al. 2007; Speroni et al. 2009), which is in contrast to data obtained in the present study. The differences could be attributed to structural variations in protein structure coupled with differences in degree of protein susceptibility to HP or heat treatments. Interestingly, the HP-treated RPI had higher gel hardness than transglutaminase cross-linked canola protein isolate (Pinterits and Arntfield 2008) at the same protein concentration, suggesting superiority of the HP process when compared to the enzymatic modification. The formation of high MW proteins coupled with high level of hydrophobic interactions in HP-treated RPI could have contributed to the increased hardness, springiness, and cohesiveness of RPI gels. Wang and Damodaran (1990) had also suggested that the high MW globular proteins improved the strength of SPI gels, and the increased polypeptide chain length that accompany protein unfolding can enhance molecular entanglement within the gel structure. In addition, the proportion of hydrogen bonds, hydrophobic interactions, electrostatic interactions, and disulfide bonds are different in heat- and pressure-induced gels when compared to native proteins (Dumoulin et al. 1998). The observed higher hardness, springiness, and cohesiveness of RPI may also be attributed to increased hydrophobicity, reduced content of free SH groups, and a more disordered structural conformation after heat and HP treatments. The chewiness, which is expressed as the energy required to chew a solid prior to swallowing were, respectively, 16.077 and 36.057 mJ at 400 and 600 MPa; the results further confirm the ability of HP treatment to induce formation of stronger rapeseed protein gels.

Conclusions

In this study, HP- and heat-induced gelation, aggregation, and conformational changes of RPI have been successfully characterized using GP-SEC, DSC, and CD techniques combined with textural analysis of protein gels. Content of soluble protein aggregates was higher for HP-treated samples and, therefore, was less effective than heat treatment in enhancing protein-protein interactions. For all the treatments, the napin protein fraction was more resistant to HP- or heat-induced protein aggregation probably because of the presence of more protein cross-links in the native structure when compared to the more susceptible cruciferin and LTP fractions. The higher content of soluble protein aggregates in the HP-treated RPI is

probably responsible for the better gelling ability (reduced LGC values) and gel textural properties (increased hardness and springiness) when compared to heat-treated RPI.

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