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Physicochemical and Antioxidant Properties of Buckwheat Protein Isolates with Different Polyphenolic Content Modified by Limited Hydrolysis with Trypsin

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Summary

Effects of limited hydrolysis with trypsin on the physicochemical and antioxidant properties of buckwheat protein isolates (BPIs) obtained with untreated and 2-propanol-extracted meal have been investigated and compared. The dephenolization treatment significantly improved the hydrolysis of BPI, which resulted in the gradual decrease in total and protein-bound polyphenolic content, but an increase in the free polyphenolic content. The hydrolysis of globulins was much easier than that of the albumins. The removal of polyphenols improved the hydrolysis of the albumin fraction. The modified BPIs with high polyphenolic content exhibited much higher DPPH radical scavenging activity and reducing power, but poorer ferrous ion chelating ability than those with low polyphenolic content. These results suggest that the limited hydrolysis is suitable for modification of the properties of buckwheat proteins.

Key words: buckwheat protein isolate (BPI), limited hydrolysis, dephenolization, antioxidant activity, physicochemical properties

Introduction

The storage proteins in buckwheat seeds, including common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum*), have been known as a valuable source of protein, with well-balanced amino acid composition (*1*) and many potential health effects (*2*,*3*).

In the buckwheat seeds, the protein content ranges from 8.51 to 18.87 %, depending on the variety (*4*). The buckwheat proteins include albumin, globulin, prolamin and glutelin, but the relative content of these individual protein fractions varies in a wide range also depending on the variety. Notwithstanding, it is generally recognized that albumin and globulin, namely seed storage proteins, are the major proteins in buckwheat seeds, and the content of prolamin and glutelin is very low. The storage proteins from common buckwheat seeds, including 8S and 13S globulins, and 2S albumins have been characterized (*4–7*). Salt-soluble 13S globulin is the main seed storage protein, and has a hexameric structure with disulphide-bonded subunits composed of acidic and basic polypeptides with relative molecular mass (M_r) of 43–68, 57–58 and 26–36 kDa (*6*). 2S albumins are composed of polypeptides in the M_r range of 8–16 kDa (*5*). The buckwheat protein isolate (BPI), obtained using conventional alkali solubilization and acid precipitation technique, is mainly composed of the globulin and albumin fractions.

To utilize this kind of valuable proteins as ingredients in food formulations, their physicochemical and other properties need to be recognized. To date, some works ad-

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dressing the functional properties of these protein products are available (*8–12*). However, these findings are inconsistent, which may be attributed to the differences in processing techniques, chemical compositions and physicochemical and conformational properties of buckwheat proteins or individual fractions. Thus, it is a prerequisite to investigate the physicochemical properties of these proteins.

Trypsin (EC 3.4.21.4) is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine. The limited hydrolysis using trypsin has been extensively applied to improve the properties of food proteins. The physicochemical and antioxidant properties of the hydrolysates obtained from BPI with Alcalase® have recently been reported (*13*). These hydrolysates exhibited excellent antioxidant activities, including DPPH radical scavenging ability, reducing power and inhibition of linoleic acid peroxidation, and these activities were closely related to their polyphenolic content. The influence of the removal of polyphenols on the physicochemical properties and hydrolysis pattern of BPI, as well as the antioxidant properties of its protease-modified products is still unknown. Furthermore, the influence of the removal of polyphenols from the buckwheat meal with various aqueous organic solvents (95 % ethanol, 70 % 2-propanol and 80 % methanol, by volume) influenced to a great extent the physicochemical and conformational properties of BPI (*14*).

The main objective of the present work is to characterize the influence of limited hydrolysis with trypsin (with much lower degree of hydrolysis than that with Alcalase®) on the physicochemical and antioxidant properties of BPI products with different polyphenolic content. The BPI with low polyphenolic content was obtained from the 2-propanol-extracted buckwheat meal.

Materials and Methods

Materials

The common buckwheat seeds (*Fagopyrum esculentum* Moench; a single cultivar), cultivated in Ganshou Province of China, were purchased in a retail outlet in Guangzhou (PR China). Prior to analysis, the seeds were milled to pass through a 60-mesh screen to produce fine whole meal, and then kept in refrigerator at 4 °C until used. Protein M_r marker kit (97–24 kDa) was purchased from Dingguo Biological Co. (Beijing, PR China). Butylated hydroxytoluene (BHT), 1-anilino-8-naphtalenesulfonate (ANS), linoleic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals were of analytical or higher grade.

Preparation of BPI products

The 2-propanol-extracted buckwheat meal was prepared according to the method of González-Pérez *et al.* (*15*) as follows: the untreated meal was extracted with cold (4 °C) mixture of 2-propanol and water (7:3, by volume) at a solid-to-solvent ratio of 1:10 (m/V) by stirring the suspension for 4 h, and then centrifuged at 8000×*g* for 20 min at 4 °C; the extraction was repeated until the supernatant no longer developed a yellow colour upon the addition of NaOH; finally, the obtained meal was dried at room temperature overnight to produce the extracted meal.

Buckwheat protein isolate (control; BPI-U) and polyphenol-free BPI (BPI-P) were prepared from untreated and 2-propanol-extracted buckwheat meal according to the process described by Tang (*11*), with slight modifications. Briefly, the meal samples were fully dispersed in 10-fold volume of deionised water for 1 h at room temperature, and the pH of the dispersion adjusted to about 8.0 with 1 M NaOH. The dispersions were centrifuged at 7000×*g* for 20 min, and the resultant supernatants adjusted to pH=4.5 using 1.0 M HCl to precipitate the proteins. The precipitates were obtained by centrifugation at 4000×*g* for 15 min, and redispersed in deionised water. Finally, the dispersions were adjusted to pH=7.0 and freeze-dried to obtain individual BPI products. The protein content of these BPI products was measured by the Kjeldahl method (with nitrogen conversion factor of 6.25). The lipid content of the seed meal and the corresponding protein isolates was measured using standard AOAC method (*16*).

Enzymatic hydrolysis of BPI-U, BPI-P and the preparation of trypsin-modified BPI products

BPI-U or BPI-P was dispersed in deionised water at room temperature (2 %, m/V). The dispersions were pre-incubated at 55 °C, prior to adjusting the pH of the dispersions to 8.0. The mixture of protein and trypsin at a protein-to-enzyme ratio of 100:2 (m/V) was incubated in a temperature-controlled water bath at 55 °C. The pH of the mixture was kept constant during hydrolysis by the addition of 0.5 M NaOH. The change in the degree of hydrolysis (DH) during the enzymatic hydrolysis was monitored by pH-stat method (*17*). The DH was calculated according to the following equation:

$$\mathrm{DH}=\frac{B\cdot M_b}{a\cdot m_p\cdot N_{tot}}\times 100 \qquad /1/$$

where B, M_b, m_p and N_{tot} are the base consumption (mL), molarity of the base, mass of hydrolysed protein (g), and the total number of peptide bonds in the protein substrates (miliequivalent per gram of protein), respectively. The N_{tot} was calculated from the amino acid composition of BPI, according to the procedure described by Adler-Nissen (*17*). In the present study, the N_{tot} of BPI was calculated to be 8.14 mmol per g of protein. The $1/\alpha$ is the calibration factor for pH-stat, and also the reciprocal of the degree of dissociation of the α-NH_2 groups. The α was calculated as follows:

$$\alpha=\frac{10^{(\mathrm{pH}-\mathrm{p}K)}}{1+10^{(\mathrm{pH}-\mathrm{p}K)}} \qquad /2/$$

where pK is the average dissociation value for the α-amino groups, calculated according to the Gibbs-Helmholz equation (*17*). At 55 °C (in the present study), the average dissociation value pK was calculated to be 7.1.

The trypsin-modified BPI-U or BPI-P products were prepared as follows: at specific periods of hydrolysis time (1, 2, 3 and 4 h), aliquots of the digested mixtures were taken out, and heated at 90 °C for 10 min, then cooled immediately in ice water to room temperature. The re-

sultant digests (adjusted to pH=7.0) were centrifuged at 4000×*g* for 20 min to remove insoluble residues and further freeze-dried to produce the modified BPI products, which were stored at –20 °C before further analysis.

Determination of total, free and protein-bound polyphenolic content

The content of total, free and protein-bound polyphenols was determined according to the method of Carbonaro *et al.* (*18*), with slight modifications. Total polyphenolic content was determined after extraction in 0.1 M NaOH (0.02–0.15 g of sample per mL) and centrifugation at 20 000×*g* for 15 min. The concentration of polyphenols was calculated from the absorbance of the supernatant at 328 nm, using the standard curve of rutin in 0.1 M NaOH (determined at the same wavelength). The value obtained was reduced by the contribution of the absorbance at 328 nm of proteins of the sample in 0.1 M NaOH. The latter was estimated using a bovine serum albumin (BSA) solution at the same protein concentration as the control. The free polyphenolic concentration was determined from the absorbance of the supernatant obtained after protein precipitation with 5 % (*m*/*V*) trichloroacetic acid (TCA) and centrifugation. Protein-bound polyphenols were calculated from the difference between total and free polyphenols. The content of polyphenols was expressed as g of rutin equivalent per 100 g of the sample.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE experiment was performed on a discontinuous buffered system according to the method of Laemmli (*19*) using 12 % separating gel and 4 % stacking gel. The protein samples were solubilized in 0.125 M Tris-HCl buffer (pH=6.8), containing 1 % (*m*/*V*) SDS, 2 % (by volume) 2-mercaptoethanol (2-ME), 5 % (by volume) glycerol and 0.025 % (*m*/*V*) Bromophenol Blue, and heated for 5 min in boiling water before electrophoresis. A volume of 10 μL of each sample was applied to each lane. After the electrophoresis, the gel was stained in 0.25 % (*m*/*V*) Coomassie Brilliant Blue (R-250) in 50 % (*m*/*V*) TCA and then destained in 7 % (by volume) acetic acid (methanol/acetic acid/water 227:37:236, by volume).

High performance size exclusion chromatography (HPSEC)

The HPSEC experiment was performed using a Waters HPLC 1525 system (Waters, Milford, MA, USA) fitted with a TSK-GEL G2000SWXL column (0.78×30 cm, Tosoh Bioscience Co., Tokyo, Japan) and a guard precolumn Protein-Pak™ 125 (0.6×4 cm, Tosoh Bioscience Co.). Each sample (containing about 0.5 % protein, by mass per volume) in the 50 mM phosphate buffer (PB; pH=7.2) containing 50 mM NaCl was centrifuged at 10 000×*g* for 10 min, and the obtained supernatant was filtered through a Millipore membrane (0.2 μm). The following chromatographic conditions were used: injection volume 20 μL, elution rate 0.8 mL/min, and elution solvent 50 mM PB (pH=7.2) containing 50 mM NaCl. The absorbance was recorded at 280 and 345 nm. All data were collected and analyzed by Breeze software (Waters).

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method described by Shimada *et al.* (*20*), with slight modifications. A volume of 2 mL of the sample solution with various concentrations (0–1.0 mg/mL) was fully mixed with 2 mL of $2 \cdot 10^{-4}$ M DPPH alcohol solution (freshly prepared). The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance value indicated a high DPPH radical scavenging activity. Methanol was used as the blank. The DPPH radical inhibition was calculated as follows:

$$[1\text{-}(\text{test sample absorbance}/\text{blank sample absorbance})] \times 100 \qquad /3/$$

Reducing power

The reducing power of untreated or modified BPI products was evaluated by the method developed by Oyaizu (*21*), with slight modifications. The sample solution (10 mL) at a concentration range of 0–5 mg/mL was mixed with 2.5 mL of 0.2 M PB (pH=6.6) and 2.5 mL of 1 % (*m*/*V*) potassium ferric cyanide solution. The mixture was then kept in a water bath at 50 °C for 20 min. The resulting solution was cooled rapidly, and then mixed with 2.5 mL of 10 % (*m*/*V*) TCA, and centrifuged at 3000×*g* for 10 min. Finally, the supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % (*m*/*V*) ferric chloride solution. The absorbance of the resulting mixture was measured at 700 nm after the reaction time of 10 min. A high absorbance indicated strong reducing power.

Ferrous ion chelating activity

Ferrous ion chelating activity of the samples was evaluated using the method of Decker and Welch (*22*), with a slight modification. A volume of 4 mL of individual sample solutions at different solid concentrations (0–2.0 mg/mL) was spiked with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. After reaction for 20 min, the absorbance of the resulting solutions was recorded at 562 nm. A low absorbance in the test samples indicated high ferrous ion chelating activity. The ferrous ion chelating ability was calculated using Eq. 3.

Statistical analysis

An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) with a confidence interval of 95 % was used to compare the mean values.

Results and Discussion

Characterization of untreated BPI products

Two BPI products (BPI-U and BPI-P), obtained from the untreated and 2-propanol-extracted buckwheat meal, respectively, were used in the present study. The protein content of BPI-P (88.7 %) was much higher than that of BPI-U (80.9 %), although the protein yield (49.6 %) of the latter was slightly lower than that of the former (52.3 %). The lipid content of BPI-U (about 3 %, wet basis) was

similar to that of the untreated meal. The extraction treatment with 2-propanol considerably decreased the lipid content of the meal (the lipid content of the extracted meal was less than 1 %). As expected, the lipid content (about 1 %) of BPI-P was much lower than that of BPI-U.

In the untreated meal, total polyphenolic content was about 0.74 g of rutin equivalent per 100 g of the sample, of which about 45 % of polyphenols were present in the free form. The dephenolization treatment with 2-propanol considerably decreased the total polyphenolic content (including free and protein-bound polyphenols) to about 0.11 % rutin equivalent. Interestingly, the polyphenolic content (total or protein-bound) of BPI-U and BPI-P was 2.7 and 0.62 %, respectively, which is 3- to 4-fold higher than that of their corresponding meal (Fig. 1). Furthermore, more than half of the polyphenols were in the protein-bound form, about 85 and 56 % for BPI-U

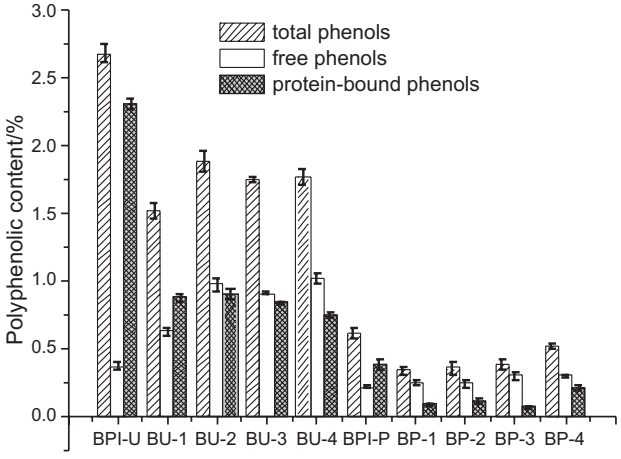

Fig. 1. Total, free and protein-bound polyphenolic content of untreated and trypsin-modified buckwheat protein isolates (BPI-U and BPI-P). BPI-U and BPI-P: buckwheat protein isolates obtained from the untreated and 2-propanol-extracted meal, respectively; BU-1, BU-2, BU-3 and BU-4: BPI-U products modified with trypsin for 1, 2, 3 and 4 h, respectively; BP-1, BP-2, BP-3 and BP-4: BPI-P products modified with trypsin for 1, 2, 3 and 4 h, respectively

and BPI-P, respectively. The data indicate that the polyphenols in the buckwheat meal preferably interacted with their proteins during the preparation of the protein isolates using alkali solubilization and acid precipitation technique. Similar results were obtained in our previous work (*13*).

Enzymatic hydrolysis

Limited hydrolysis with trypsin was applied to modify the properties of BPI products. Fig. 2 shows the curves of BPI-U and BPI-P after hydrolysis up to 5 h, using DH change as an indicator. In both cases, the DH progressively increased with the hydrolysis time, but after 30 min the rate of increase gradually slowed down. At specific periods of hydrolysis time (0.5–5 h), the DH of BPI-P was significantly higher than that of BPI-U, indicating that the proteins in BPI-P were more easily digested by trypsin than those in BPI-U. In general, trypsin splits preferentially those peptide linkages that contain

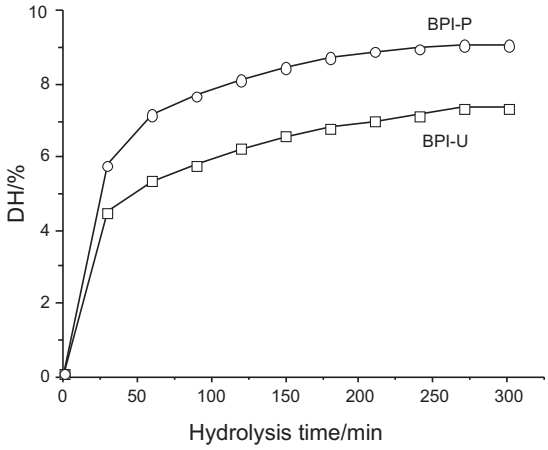

Fig. 2. DH changes in BPI-U and BPI-P during hydrolysis by trypsin. BPI-U and BPI-P are the same as in caption of Fig. 1. Each data is the mean of duplicate measurements

either lysine or arginine as amino acid side chains (*23*). It is also well recognized that phenolic substances may react with ε-amino groups of lysine side chains and tryptophan (*24*). Thus, the improvement of hydrolysis by the dephenolization treatment may be mainly due to reduced interactions between the proteins and the polyphenols, as a consequence of significant decrease in polyphenolic content (Fig. 1). A similar result was obtained in a previous work, where it was shown that the extraction of sunflower meal with aqueous organic solvents significantly increased the rate of hydrolysis of its proteins by trypsin (*25*).

Compared to Alcalase® 2.4 L FG (*13*), the DH of BPI-U and BPI-P hydrolyzed by trypsin was much lower. At the end of hydrolysis (5 h), the DH values reached about 7.3 and 9.1 % for BPI-U and BPI-P, respectively (Fig. 2), much less than that by Alcalase® (18.4 %) even at 1 % (by volume per mass) (*13*). The difference may be due to the difference in susceptibility of the proteins to these two proteases.

Polyphenolic content

According to the hydrolysis curves (Fig. 2), various trypsin-modified BPI-U or BPI-P products were prepared at hydrolysis times of 1, 2, 3 and 4 h. At these specific periods of hydrolysis, the DH values were about 5.4, 6.2, 6.8 and 7.2 % for modified BPI-U products (further denoted as BU-1, BU-2, BU-3 and BU-4), and 7.1, 8.1, 8.8 and 9.0 % for modified BPI-P products (further denoted as BP-1, BP-2, BP-3 and BP-4), respectively. The polyphenolic content (total, free and protein-bound) of these protein products is also included in Fig. 1.

The limited hydrolysis treatment resulted in considerable decreases in total and protein-bound polyphenolic content, and concomitant increases in their free polyphenolic content of BPI products, but the extent of the decrease or increase depended on the type of BPI products and the period of hydrolysis time (Fig. 1). In the BPI-U case, the total polyphenolic content of BU-1 was the lowest among all tested products. Further hydrolysis for 2–4 h, on the contrary, increased the total polyphenolic content relative to that obtained after 1 h. The free polyphenolic content of BPI-U gradually and significant-

ly increased from 0.38 to 0.97 % rutin equivalent with the increase of hydrolysis time from 0 to 2 h, and did not change upon further hydrolysis (Fig. 1). In the BPI-P case, maximal decreases in total and protein-bound polyphenolic content were observed in the products treated for 1–3 h. The hydrolysis for 4 h, on the contrary, significantly increased these polyphenolic values relative to those obtained after 1–3 h. In this case, the free polyphenolic content slightly but gradually increased from 0.22 to 0.30 % rutin equivalent. The data indicated that the hydrolysis changed the pattern of polyphenol-protein interactions to a great extent, and some of the protein-bound polyphenols were transformed into the polyphenols in the free form. Similar results had been observed in our previous work, where it was clearly indicated that the total and protein-bound polyphenolic content progressively decreased with the increase of DH from 0 to 15 %, and further hydrolysis with 20 or 25 % DH considerably increased the polyphenolic content relative to that at 15 % DH (*13*). By comparison, the changes in polyphenolic content were much more distinct in the BPI-U than those in the case of BPI-P.

SDS-PAGE and SEC analyses

The protein or polypeptide compositions of untreated and trypsin-modified BPI-U and BPI-P products were investigated using SDS-PAGE and SEC techniques (Figs. 3 and 4). In the SDS-PAGE profiles of untreated BPI-U or BPI-P, only the polypeptides or subunits of the globulin fraction (including 8S and 13S globulins) with M_r of about 45–60 kDa (in the absence of reducing agents) were observed (and the 2S albumins with M_r less than 16 kDa were not observed in the gels). Interestingly, the hydrolysis with trypsin led to degradation of these polypeptides or subunits to a homogenous polypeptide with M_r of about 30 kDa (Fig. 3). There was no distinct difference between the hydrolysis pattern of the BPI-U and BPI-P globulins. This is an indirect evidence that the polyphenols may be mainly associated with the albumins, not the globulins.

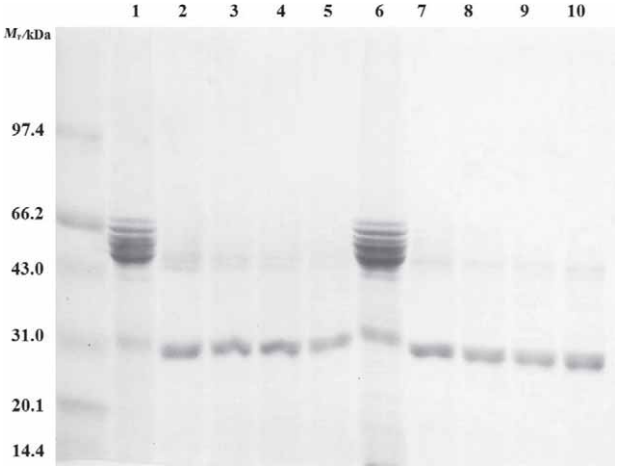

Fig. 3. SDS-PAGE profiles of untreated and trypsin-modified BPI-U and BPI-P products. Lanes 1–5 represent BPI-U samples treated with trypsin for 0, 1, 2, 3 and 4 h, respectively. Lanes 6–10 represent BPI-P samples treated with trypsin for 0, 1, 2, 3 and 4 h, respectively. M_r indicates the standard protein markers

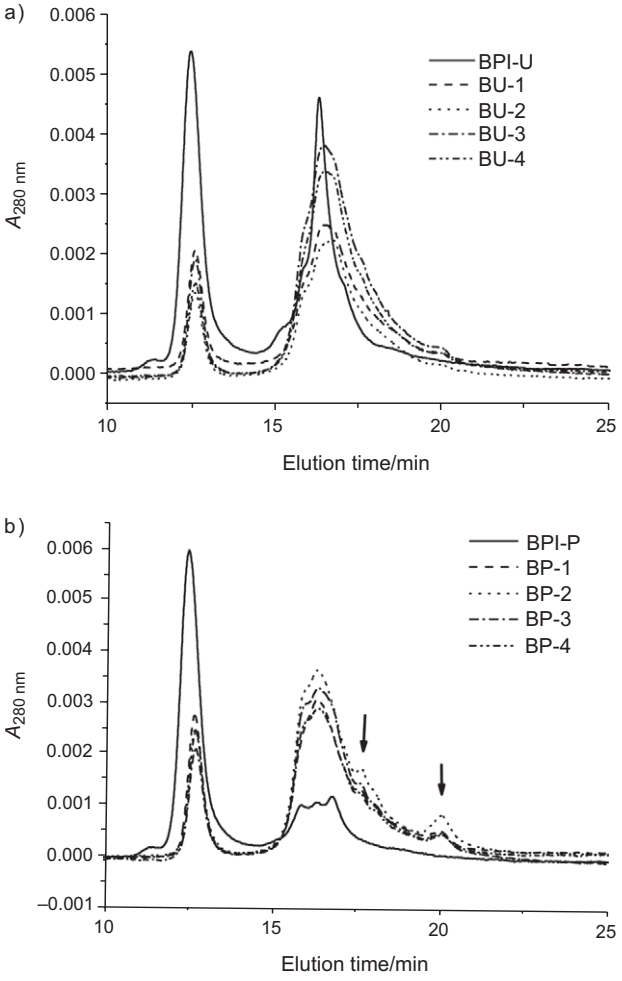

Fig. 4. SEC elution profiles of: a) BPI-U and b) BPI-P samples, with UV detection at 280 nm. BPI-U and BPI-P refer to the caption of Fig. 1. BU-1, BU-2, BU-3 and BU-4 represent BPI-U samples treated with trypsin for 1, 2, 3 and 4 h, respectively. BP-1, BP-2, BP-3 and BP-4 represent BPI-P samples treated with trypsin for 1, 2, 3 and 4 h, respectively. The arrows within the figures indicate the new peaks

To further confirm that the polyphenols might preferably interact with the albumins, we carried out the SEC analyses of untreated and trypsin-modified BPI-U or BPI-P products, solubilized in neutral phosphate buffer containing 50 mM NaCl. The difference in SEC elution profiles (detected by UV detector) between BPI-U and BPI-P can reflect to some extent the interactions between protein and polyphenols. There were two major elution peaks at about 12.5 and 15.8 to 16.9 min attributed to the globulin and 2S albumin fractions respectively (as evidenced by the SEC profiles of purified globulin and albumin fractions in our preliminary experiments; unpublished data). The globulin peaks of BPI-U and BPI-P were similar, while the intensity of the albumin peaks of BPI-U was much stronger than that of BPI-P (Fig. 4). In combination with the polyphenolic content data (Fig. 1), this observation clearly confirms that the polyphenols preferably interact with the 2S albumin fraction.

The hydrolysis treatment led to considerable decrease in the intensity of the globulin peak, and the extent of the decrease seems to be independent of the type of

protein isolates and the period of hydrolysis time (Fig. 4). This is consistent with the SDS-PAGE analyses (Fig. 3), confirming similar limited degradation of the polypeptides or subunits of globulins by trypsin. In contrast, the albumin peaks changed in a more complex way. For BPI-U, the hydrolysis for 1 h also resulted in the decrease in the intensity of the major albumin peak, but the intensity and width of the peak (eluted at the albumin position) gradually increased as the hydrolysis time increased from 1 to 3 or 4 h (Fig. 4a). In this case, the changes in the albumin elution peak in SEC profiles are consistent with the data of total polyphenolic content (Fig. 1), indicating that the polyphenols contained in the albumin fraction were to a greater extent affected by the hydrolysis than those in the globulin fraction. However, in the BPI-P case, the intensity of the peaks eluted at the albumin position remarkably increased, and the difference in the intensity among various modified BPI-P products was slight, suggesting that in this case, the increase in albumin elution peak intensity was mainly due to the contribution of the released degraded fragments or polypeptides from the globulin fraction. Interestingly, in the SEC profiles of modified BPI-P products, new elution peaks appeared at 17.6 and 20.0 min, while these two peaks were much less distinct in the case of BPI-U (Fig. 4b). These two new peaks may be the degraded products of the albumin fraction. This observation, together with the DH data (Fig. 2), suggests that the removal of polyphenols might improve the hydrolysis of albumins to a great extent.

DPPH and hydroxyl radical scavenging activities

Fig. 5a shows DPPH radical inhibition of untreated and trypsin-modified BPI-U and BPI-P products, at various solid concentrations in the range of 0–1.0 mg/mL. The inhibition of BHT (butylated hydroxytoluene), a well known synthetic antioxidant, was evaluated. The stable DPPH radical in ethanol has been widely used to test the ability of some compounds to act as free radical scavengers or hydrogen donors (*20,26*). All the tested products showed dose-dependent DPPH radical inhibition, but the extent of inhibition diversely varied with the type of the protein isolates and the solid concentration applied. At low concentrations, *e.g.* 0.1 and 0.4 mg/mL, the DPPH radical inhibition of BPI-U was higher than that of BPI-P, while at a concentration of 1.0 mg/mL, the inhibition of these two products was similar (Fig. 5a). It is reasonably expected that the protein products with high polyphenolic content have higher DPPH radical scavenging ability than those with low polyphenolic content. However, in the present study, at the concentration of 1.0 mg/mL this situation does not seem to be the case. The difference in protein solubility may account for the difference of the DPPH radical inhibition, since the protein solubility of BPI-U was poorer than that of BPI-P, especially at high solid concentrations (*e.g.* 1.0 mg/mL).

The hydrolysis treatment resulted in the decrease in DPPH radical inhibition of BPI-U and BPI-P at solid concentrations of 0.1 and 0.4 mg/mL. Surprisingly, remarkable decreases were observed in the case of BPI-P when it was modified with trypsin (Fig. 5a). The underlying mechanism for this phenomenon is not yet known. The DPPH radical inhibition at the various tested concentrations slightly decreased with the hydrolysis time. In contrast, it was observed that the DPPH radical inhibition of trypsin-modified BPI-U products was much higher than that of modified BPI-P products. For example, at the solid concentration of 1.0 mg/mL, the DPPH radical inhibition was about 54–61 and 11–17 % for trypsin-modified BPI-U and BPI-P products, respectively. Together with the data about the polyphenolic content (Fig. 1), the results are in agreement with the proposed view in our previous work that the DPPH radical scavenging ability of BPI digests or hydrolysates was closely related to their polyphenolic content (*13*). The inhibition of all the tested products was much less than that (86 % at concentrations of 0.1–1.0 mg/mL) of BHT. However, it should be noted that the DPPH radical inhibition of trypsin-modified BPI-U products at solid concentration of 1.0 mg/mL was comparable to 62–71 % of BHT at a concentration of 0.1 mg/mL.

Reducing power

Fig. 5b shows the reducing power of untreated and trypsin-modified BPI-U and BPI-P products at various concentrations in the range from 0 to 1.0 mg/mL, as estimated by the Fe^{2+}-Fe^{3+} transformation in the presence of various products. The reducing power is denoted as absorbance at 700 nm to monitor the Fe^{2+} concentration (*27*). The reducing power of ascorbic acid (a well-known antioxidant) was also included in Fig. 5b. In all tested samples, the reducing power was linearly dependent on the concentration of the samples. The slope of the regression lines for absorbance *vs.* concentration can reflect the difference in reducing power. The protein products with high slope have high reducing power. The slopes were about 0.36 and 0.17 for BPI-U and BPI-P, respectively, suggesting that the reducing power of BPI-U was about twice that of BPI-P. The difference in their reducing power can also be attributed to the difference in their polyphenolic content.

In the BPI-U case, the hydrolysis for 1–4 h resulted in slight decrease in the slope from 0.36 to about 0.29–0.32, while in the case of BPI-P, it decreased from 0.17 to 0.07–0.10 (Fig. 5b). The difference in reducing power between modified BPI-U and BPI-P products can also be attributed to their polyphenolic content (Fig. 1). The slopes of untreated and modified BPI-U products were higher than that (0.15–0.21) of BPI hydrolysates obtained at DH of 10–25 % (*13*). This further confirms the decrease in the reducing power with the increase in DH for BPI digests or hydrolysates. A similar result has been obtained for porcine haemoglobin hydrolysates obtained by Alcalase® and/or Flavourzyme® (*28*).

At the highest experimental concentration (5 mg/mL), the reducing power (1.5–1.6) of modified BPI-U products was much higher than 0.08–0.25 reported for haemoglobin hydrolysates at similar concentrations (*28*) and 0.25–0.60 for purified fractions of chickpea protein hydrolysates (*29*). The results suggest that limited modified BPI-U products with high polyphenolic content have excellent reducing power, thus exhibiting good potential to be applied as food ingredients with the ability to inhibit Fe^{2+}-Fe^{3+} transformation.

Ferrous ion chelating ability

Fig. 5c shows the ferrous ion (Fe^{2+}) chelating ability of trypsin-modified BPI-U and BPI-P samples at various

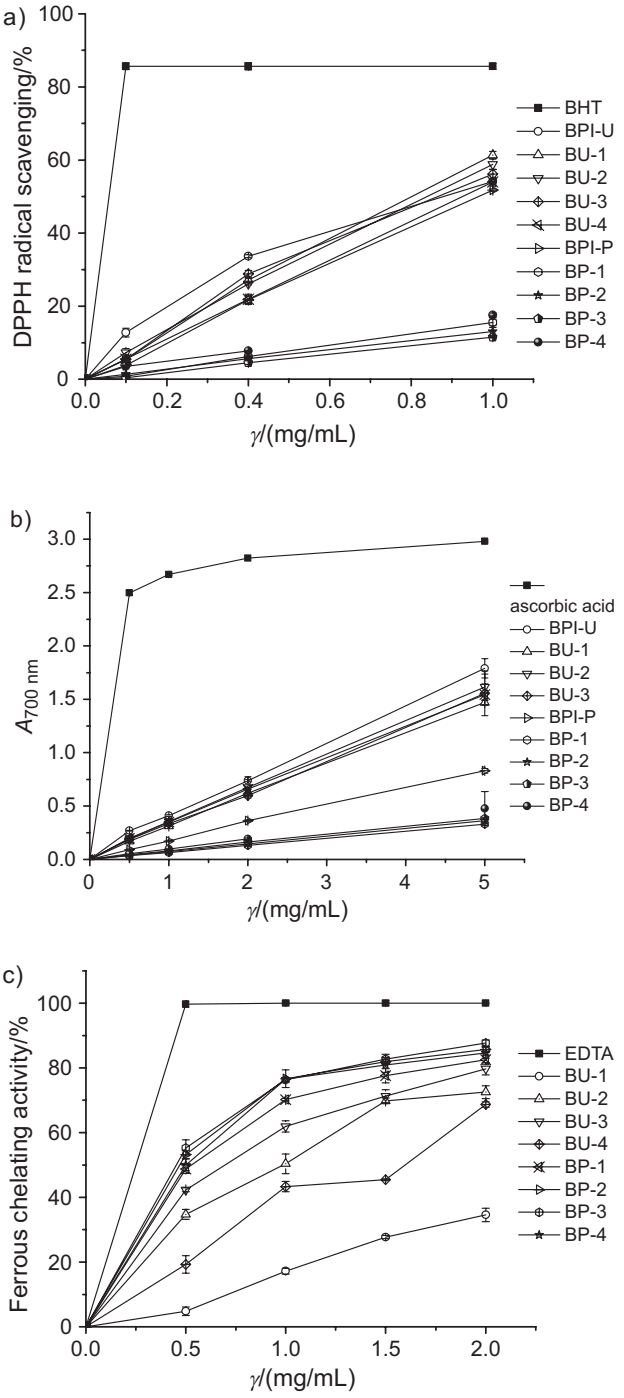

Fig. 5. Antioxidant activities, including: a) DPPH radical scavenging ability, b) reducing power, and c) Fe^{2+} chelating ability of untreated and trypsin-modified BPI-U and BPI-P products. For legends, refer to Fig. 4

concentrations in the range of 0–2.0 mg/mL. In this case, ethylene diamine tetraacetic acid (EDTA, a well-known metal ion chelating agent) was applied as the reference. The Fe^{2+} chelating ability of all the tested samples also progressively increased with the increase of the concentration of the samples. At specific concentrations, the Fe^{2+} chelating ability of modified BPI samples increased with the increase of the hydrolysis time from 1 to 3 h, while further hydrolysis (4 h) led to the decrease in this ability (relative to that at hydrolysis time of 2 or 3 h; Fig. 5c). In contrast, the Fe^{2+} chelating ability of all modified BPI-P samples at various concentrations was similar, and even higher than the maxima of modified BPI-U samples. The much higher Fe^{2+} chelating ability of modified BPI-P samples may be attributed to greater exposure of effective sites (especially those of the albumins) capable of chelating ferrous ion.

At a concentration of 0.5 mg/mL, the modified BPI-P samples exhibited 48–56 % chelating activity, while at the same concentration, EDTA still displayed almost 100 % chelating effect (Fig. 5c). However, the Fe^{2+} chelating ability of all modified samples was much stronger than that of haemoglobin hydrolysates (*28*) and hemp protein hydrolysates (*30*), and even comparable to that of wheat germ protein hydrolysate (except B-1 and B-4; *31*).

Conclusion

Two BPI products with different levels of polyphenols were prepared from untreated and 2-propanol-extracted buckwheat seed meal. The proteins in BPI with low polyphenolic content were more easily hydrolyzed with trypsin than those with high polyphenolic content. Besides the globulin fraction, the albumin fraction of BPI with low polyphenolic level was also digested to a lesser extent. The hydrolysis led to considerable decreases in total and protein-bound polyphenolic content, but concomitantly, the free polyphenolic content gradually increased with increasing hydrolysis time (in the range 1–4 h). The antioxidant properties of trypsin-modified BPI products depended to various extent on the polyphenolic level of the applied BPI material, the type of the antioxidant evaluation systems and the degree of hydrolysis. By comparison, the modified BPIs with high polyphenolic content exhibited much higher DPPH radical scavenging activity and reducing power, but poorer ferrous ion chelating ability than those with low polyphenolic content.

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