Food Chemistry 115 (2009) 672–678

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Physicochemical and antioxidant properties of buckwheat (*Fagopyrum esculentum* Moench) protein hydrolysates

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ARTICLE INFO

Article history:
Received 15 July 2008
Received in revised form 6 December 2008
Accepted 22 December 2008

Keywords:
Buckwheat protein isolate
Hydrolysate
Antioxidant property
Enzymatic hydrolysis
Alcalase
Polyphenol

ABSTRACT

The enzymatic hydrolysis of common buckwheat (*Fagopyrum esculentum* Moench) protein isolate (BPI) by Alcalase and some physiochemical and antioxidant properties of the resulting hydrolysates were characterised. The hydrolysis resulted in remarkable decrease in the globulins or protein aggregates and concomitant increase in peptide fragments. The surface hydrophobicity of the hydrolysates decreased with increasing degree of hydrolysis (DH) and reached a minimum at DH 15%, but increased at further hydrolysis, whereas their amino acid compositions were unchanged. The polyphenol content of the hydrolysates gradually decreased with DH increasing from 0% to 15%, while it on the contrary increased upon further hydrolysis. The hydrolysates exhibited excellent antioxidant activities, including DPPH radical scavenging ability, reducing power and ability to inhibit linoleic acid peroxidation. The antioxidant activities of these hydrolysates were closely related to their polyphenol contents. The results indicated that polyphenol-rich buckwheat proteins are unique protein materials for the production of the hydrolysates with good nutritional and antioxidant properties.

1. Introduction

The proteins in buckwheat seeds, including common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum*) have recently attracted much interest, due to their well-balanced amino acid composition (Pomeranz & Robbins, 1972) and potential health effects, e.g., cholesterol-lowering and enhanced faecal steroid excretion effects (Krkošková & Mrázová, 2005; Li & Zhang, 2001). The prevalent hypothesis for this lipid-lowering mechanism of buckwheat proteins is based on its relatively insoluble nature and lipid-binding potential (Metzger, Barnes, & Reed, 2007; Tomotake, Shimaoka, Kayashita, Nakajoh, & Kato, 2002).

Protein content of buckwheat flour is ranging from 8.51% to 18.87% depending on variety (Krkošková & Mrázová, 2005). The proteins in buckwheat consist of albumin, globulin, prolamin and glutelin, with their relative contents varying with the variety. In defatted tartary buckwheat (cultivated in Liang Shan region of Sichuan province, China) flour, the contents of albumin, globulin, prolamin and glutelin are 43.8%, 7.8%, 10.5% and 14.6%, respectively (Guo & Yao, 2006). The relative contents of these protein fractions in common buckwheat flour are similar (our unpublished data). In general, only the albumin and globulin fractions of buckwheat protein can be extracted at alkali conditions (e.g., pH 8.0–8.5), and thus these two fractions constitute the major part of buckwheat protein isolate (BPI).

Buckwheat protein has been confirmed to be latent source of peptides with angiotensin I-converting enzyme (ACE) inhibiting activity (Li, Matsui, Matsumoto, Yamasaki, & Kawasaki, 2002). Nevertheless, to the authors' knowledge, no information is available about antioxidant properties of peptides from buckwheat protein, although the antioxidant activities of enzymatic hydrolysates from other plant proteins, including soy proteins (Chen, Muramoto, & Yamauchi, 1995), wheat protein (Zhu, Zhou, & Qian, 2006), chickpea protein (Li, Jiang, Zhang, Mu, & Liu, 2008), have been widely investigated using many *in vitro* antioxidant evaluation systems (water-soluble and oil-soluble). The antioxidant properties of the hydrolysates, largely depending on protease specificity, degree of hydrolysis (DH) and nature of released peptides (e.g., molecular weight and amino acid composition), have been attributed to cooperative or combined effects of a number of properties, including their ability to scavenge free radicals, to act as chelating agents of metal ions, or act as hydrogen donor.

The objective of the present work was to investigate the enzymatic hydrolysis of BPI by Alcalase, and characterise the physiochemical and antioxidant properties of the resulting hydrolysates, including DPPH radical scavenging ability, reducing power and ability to inhibit linoleic acid peroxidation. Considering that buckwheat seeds are rich in polyphenols (e.g., rutin), the polyphenol contents in BPI and its hydrolysates were also evaluated.

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doi:10.1016/j.foodchem.2008.12.068

2. Materials and methods

2.1. Materials

Common buckwheat seeds were purchased from a local supermarket in Guangzhou (China), which were cultivated in Ganshou Province of China. Buckwheat protein isolate (BPI) was prepared from the buckwheat flour according to the process as described by Tang (2007), with slight modifications. Briefly, the flour (without pretreatment) was fully dispersed in 10-fold volume of de-ionised water for 1 h at room temperature, and the pH of the dispersion adjusted to about 8.5 with 1 N NaOH. The dispersion was centrifuged at 7000*g* for 20 min, and the resultant supernatant was adjusted to pH 4.0 using 1.0 N HCl to precipitate the proteins. The precipitate was obtained by centrifugation at 4000*g* for 15 min, and re-dispersed in de-ionised water. Finally, the dispersion was adjusted to pH 7.0 and freeze-dried to obtain BPI.

Alcalase 2.4 L FG (2.4 Au/g) was kindly supplied by Guangzhou Office (China) of Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). Linoleic acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT), 1-anilino-8-naphtalene-sulphonate (ANS), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, USA). All the chemicals were of analytical or better grade.

2.2. Enzymatic hydrolysis of BPI and preparation of the hydrolysates

Four grams of BPI were dispersed in 200 mL of de-ionised water at room temperature. The dispersions were pre-incubated at 55 °C, prior to adjusting pH of the dispersion to 8.0. The mixture of protein and enzyme (Alcalase) at various enzyme-to-substrate (*E*/*S*) ratios of 1:100, 2:100 and 4:100 (v/w) was incubated in a temperature-controlled water bath at 55 °C. The pH of the mixture was kept constant during hydrolysis, by addition of 0.5 N NaOH. The change in degree of hydrolysis (DH) during the enzymatic hydrolysis was followed by pH-stat method (Adler-Nissen, 1986).

The percent DH was calculated according to the following equation:

$$DH(\%) = \frac{B \cdot N_b}{\alpha \cdot M_p \cdot h_{tot}} \times 100,$$

where B, N_b, M_p and h_{tot} are the base consumption in mL, the normality of the base, the mass of protein being hydrolysed (g), and the total number of peptide bonds in the protein substrate (meqv/g protein), respectively. The h_{tot} was calculated from the amino acid composition of BPI, according to the procedure described by Adler-Nissen (1986). In the present study, the h_{tot} of BPI was calculated to be 8.14 mmol/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 the following equation:

$$\alpha = \frac{10^{(\mathrm{pH}-pK)}}{1+10^{(\mathrm{pH}-pK)}},$$

where pK is the average dissociation value for the α-amino groups, calculated according to the Gibbs-Helmholz equation (Adler-Nissen, 1986). At 55 °C (in the present study), the average dissociation value pK can be calculated to be 7.1.

The hydrolysates were prepared using Alcalase at an *E*/*S* ratio of 2:100 (v/w). At specific periods of hydrolysis time, aliquots of the digested mixture were taken out, and heated at 90 °C for 10 min, and then cooled immediately in ice water to room temperature. The resulting digests were centrifuged at 4000*g* for 20 min to remove insoluble residues. The supernatants were then adjusted to pH 7.0, and lyophilised to produce the hydrolysate samples, which were stored at −20 °C before further analysis. The hydrolysates with DH values of 5%, 10%, 15%, 20% and 25% were obtained at hydrolysis times of 15, 40, 95, 210, 540 min, respectively (further denoted as DH-5, DH-10, DH-15, DH-20 and DH-25, respectively).

2.3. Chemical analysis

The chemical compositions of buckwheat flour and its protein products were determined according to AOAC procedures (AOAC, 1984). For amino acid analysis, the hydrolysis of the samples was performed in the presence of 6 N HCl for 24 h at 110 °C in a sealed tube. The amino acid composition of the samples was determined by an automatic amino acid analyser (Waters, Division of Millipore, Milford, MA, USA), using PICO.TAG column. The determination was carried out at 38 °C, with the detection wavelength 254 nm and flow rate 1.0 mL per minute. The amino acid tryptophan was not determined.

2.4. High performance size exclusion chromatography (HPSEC)

The HPSEC experiment was performed using a Waters HPLC 1525 system (Waters, Division of Millipore, Milford, MA, USA) fitted with a TSK-GELG2000SWXL column (0.78 × 30 cm, Tokyo, Japan) proceeded by a guard column Protein–Pak™ 125 (0.6 × 4 cm, Tokyo, Japan). Each sample (containing about 0.5% protein, w/v) in the 50 mM phosphate buffer (PBS; pH 7.0) containing 0.1 M NaCl was centrifuged at 15,000*g* for 10 min, and then the obtained supernatant was filtered with Millipore membrane (0.22 μm). The following chromatographic conditions were used: (1) injection volume, 20 μL; (2) eluting rate, 1.0 mL/min; (3) elution solvent: 50 mM phosphate buffer (PBS; pH 7.0) containing 0.1 M NaCl. The absorbance was recorded at 215 nm. All data were collected and analyzed by Breeze software (Waters, Division of Millipore, Milford, MA, USA).

2.5. Surface hydrophobicity (H_o)

Surface hydrophobicity (H_o) was determined with the fluorescence probe ANS according to the method of Haskard and Li-Chan (1998). Serial dilutions in 0.01 M PBS (pH 7.0) were prepared with the hydrolysates (stock solutions; 0.5%, w/w) to a final concentration of 0.005–0.2% (w/w). ANS solution (8.0 mM) was also prepared in the same phosphate buffer. Twenty microlitres of ANS solution was added to 4 mL of each dilution and fluorescence intensity (FI) of the mixture was measured at 365 nm (excitation) and 484 nm (emission) using F4500 fluorescence-spectrophotometer (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_o.

2.6. Quantification of total, free and protein-bound polyphenols

The contents of total, free and protein-bound polyphenols were determined according to the method of Carbonaro, Grant, Cappelloni, and Pusztai (2000), with slight modifications. Total polyphenol content was determined after extraction in 0.1 N NaOH (0.02–0.15 g of the samples/mL) and centrifugation at 20,000*g* for 15 min. The concentration of polyphenols was calculated from the absorption at 328 nm of the supernatant, using the standard curve of rutin in 0.1 N NaOH (determined at the same wavelength). The value obtained was reduced by the contribution of the absorption at 328 nm of proteins of the sample in 0.1 NaOH. The latter was estimated using a bovine serum albumin solution at the same protein concentration as the sample. The free polyphenol concentration was determined from the absorption of the supernatant obtained after protein precipitation with 5% trichloroacetic acid (TCA)

and centrifugation. Protein-bound polyphenols were obtained by the difference. The contents of the polyphenols were expressed in the unit of gram of rutin equivalent per 100 grams of the sample.

2.7. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method described by Shimada, Fujikawa, Yahara, and Nakamura (1992), with slight modifications. Two millilitres of the sample solution with various solid concentrations (0–1.0 mg/mL) were fully mixed with 2 mL of 2.0×10^{-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 at 517 nm indicates a high DPPH radical scavenging activity. The methanol was used as the blank. The DPPH radical inhibition as a percentage is calculated by [1-(test sample absorbance/blank sample absorbance)] × 100. The IC_{50} value (mg/mL; meaning the concentration that causes a decrease in initial DPPH concentration by 50%) was determined from the linear regression equation of the DPPH radical inhibition against the concentration.

2.8. Reducing power

The reducing power of the hydrolysates was evaluated by the method developed by Oyaizu (1986), with slight modifications. The sample solution (10 mL) with a concentration in the range of 0–5 mg/mL was mixed with 2.5 mL of 0.2 M PBS (pH 6.6) and 2.5 mL of 1% potassium ferric cyanide solution. The mixture was then kept in a 50 °C water bath for 20 min. The resulting solution was cooled rapidly, and then mixed with 2.5 mL of 10% TCA (w/v), and centrifuged at 3000*g* for 10 min. Last, the supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL 0.1% ferric chloride solution. The absorbance at 700 nm of the resulting mixture was measured after reaction for 10 min. A high absorbance indicates strong reducing power.

2.9. Antioxidant activity in a linoleic acid system

The inhibition of linoleic acid peroxidation of the hydrolysates or antioxidants (Trolox and BHT) was evaluated by the thiocyanate method (Dong & Yao, 2008; Yen, Chang, & Su, 2003). Linoleic acid emulsion was prepared with linoleic acid (0.2804 g) and Tween 20 (0.2804 g) in 50 mL of 0.2 M PBS (pH 7.0). A reaction solution, containing different concentrations of the samples (0.5 mL), linoleic acid emulsion (2.5 mL) and 0.2 M PBS (pH 7.0; 2 mL) were mixed with a ULTRA-TURRAX® T25 digital homogenizer (IKA Co., Germany). The reaction mixture was incubated at 37 °C in dark to accelerate the oxidation process. At specific periods of time (e.g., 24 h), aliquots (0.1 mL) of the mixture were taken out for evaluation of the inhibition of the oxidative rancidity. To 9.7 mL of ethanol (75%), 0.1 mL of ammonium thiocyanate solution (30%, w/v), 0.1 mL of sample solution and 0.1 mL of ferrous chloride solution (20 mM in hydrochloric acid) were added in sequence. Last, the resultant mixture was well mixed for 3 min, and its absorbance at 500 nm determined as the peroxide value. The oxidation process of linoleic acid in the absence of the hydrolysates or antioxidants was performed as the same process above, and used as the blank. All the tests were performed in duplicate.

2.10. 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% was used to compare the means.

3. Results and discussion

3.1. Chemical composition of BPI

The chemical composition of the buckwheat flour as the material to produce BPI was as follows (wet basis): protein 11%, lipid 3.1%, moisture 12.0% and ash (2.9%). From this flour, about 74% of total proteins were extracted at pH 8.5, at a solid-to-water ratio of 1:10. The chemical composition of the obtained BPI was as follows (wet basis): protein 69.7%, lipid 2.7%, moisture 5.7% and ash 2.4%. The protein content data of BPI is lower than that reported by Tomotake et al. (2002) and Tang (2007), but similar to that by Metzger et al. (2007).

3.2. Enzymatic hydrolysis

The DH changes during enzymatic hydrolysis of BPI using Alcalase with *E*/*S* ratios of 1:100, 2:100 and 4:100 (v/w), respectively, were monitored by pH-stat method for up to 360 min, as shown in Fig. 1. As expected, the DH increased with hydrolysis time, indicating gradual release of peptide fragments during the hydrolysis. The rate of the hydrolysis or the release of peptide fragments was fast during initial hydrolysis (e.g., <30 min) and gradually decreased with the hydrolysis time increasing. Upon further hydrolysis, e.g., >180 min, the rate of hydrolysis was nearly unchanged. The change of DH with hydrolysis time is similar to that of defatted wheat germ protein isolate treated by Alcalase (Zhu et al., 2006).

The change pattern of DH for the hydrolysis of BPI was closely dependent upon the applied enzyme concentration, namely the *E*/*S* ratio. With the *E*/*S* ratio increasing (from 1:100 to 4:100, v/w), the rate of DH increase during initial hydrolysis process (e.g., at <15 min) increased. However, the time needed to keep the rate of DH increase constant seemed to correspondingly become short (Fig. 1). Interestingly, during final process (e.g., at a hydrolysis time >270 min), the rate of DH increase at different *E*/*S* ratios became similar. The observed hydrolysis pattern of BPI by Alcalase was attributed to sequential hydrolysis of the two kinds of proteins in buckwheat (i.e., globulins and albumins). The increase in DH during initial hydrolysis seems to be mainly attributed by the hydrolysis of the globulins in BPI, while during final hydrolysis, the hydrolysis of the albumins may mainly account for the DH increase.

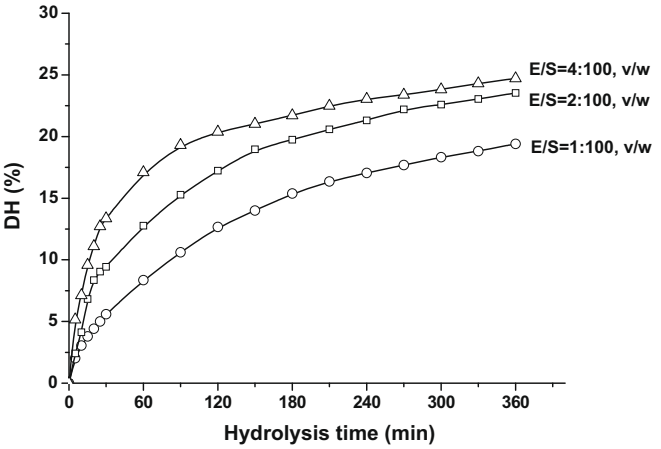

Fig. 1. DH changes of BPI during hydrolysis by Alcalase at various *E*/*S* ratios of 1:100, 2:100 and 4:100 (v/w), respectively. Each data was the mean of duplicate measurements.

3.3. Characterisation of the hydrolysates

3.3.1. SEC analysis

The molecular weight (MW) distribution of BPI (control) and its hydrolysates with DH of 5–25% was evaluated by HPSEC. Fig. 2 shows typical SEC elution profiles of BPI and its hydrolysates, as detected by UV absorbance at 215 nm. In order to better reflect the differences in SEC chromatograms, we divided the peaks on the chromatograms into four groups. Groups I–IV corresponded to the constituents of the protein or the hydrolysates eluting at <8 min, 8–12 min, 12–14 min and >14 min, respectively. The area percentages of individual groups of BPI and its hydrolysates are listed in Table 1.

In the SEC profile of BPI, there were two major peaks eluting at about 6.2 and 11.5 min, respectively (Fig. 2). The former peak is clearly attributed to the protein constituents with high MW in BPI, while the latter may mainly correspond to the 2S albumins (Radovic, Maksimovic, Brkljacic, Gasic, & Savic, 1999). The 2S albumin is the major protein fraction in buckwheat storage proteins (Guo & Yao, 2006; our unpublished data). Thus, it could be suggested that besides the 8S and 13S globulins (and its aggregates), the high MW peak also included some aggregates of the albumins. The remarkable increase in integrated peak areas of the elution profiles of the hydrolysates (relative to BPI; Data not shown) confirmed the presence of the aggregates (especially those insoluble aggregates).

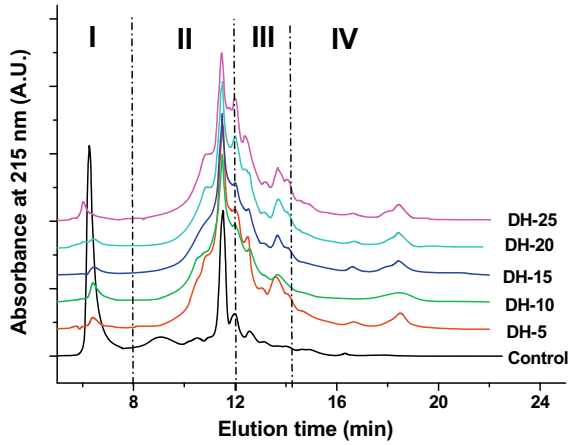

Fig. 2. HPSEC elution profiles of BPI and its hydrolysates with various DH values. Control: BPI; DH-5, DH-10, DH-15, DH-20 and DH-25 are the hydrolysates with DH values of 5%, 10%, 15%, 20% and 25%, respectively. The numbers (I–IV) indicate Groups I–IV of the protein constituents eluting at <8, 8–12, 12–14 and >14 min, respectively.

Table 1
Area percentages of individual groups and surface hydrophobicity of buckwheat protein isolate and its hydrolysates.

Samples	Area percentages of individual groups[a]				Surface hydrophobicity
	Group I	Group II	Group III	Group IV	
Control (BPI)	46.6	34.0	16.3	3.1	678
DH-5	7.3	55.9	26.2	10.6	202
DH-10	4.6	56.1	27.4	11.8	134
DH-15	1.7	55.8	30.2	12.4	66
DH-20	3.2	47.8	35.0	14.1	79
DH-25	3.2	47.3	34.4	15.2	105

[a] Groups I-IV indicate the protein constituents of BPI and its hydrolysates, eluting at <8, 8–12, 12–14 and >14 min, respectively. Values represent the mean of duplicate measurements.

The hydrolysis led to remarkable decreases in the high MW peak (at 6.2 min) and concomitant increases in the peaks (at about 11.5 min) (Fig. 2), indicating the breakdown of high MW proteins or aggregates and the formation of low MW protein fragments. By comparing the changes in area percentages of individual groups (Table 1), it was shown that the DH increase (up to about 15%) was mainly contributed by the decrease in the high MW protein components in the BPI, and those low MW protein components (eluting at about 11.5 min) was hydrolysed only after the former components were almost completely digested. This phenomenon is consistent with the DH data (Fig. 1), further suggesting the sequential hydrolysis of the globulins and albumins in BPI.

During the whole hydrolysis process, the major peak (at about 11.5 min) mainly referred to the native albumin seems to be unchanged (Fig. 2). On the other hand, it should be noted that the buckwheat proteins (especially those obtained by alkali-solubilisation and acid-precipitation) have low solubility in aqueous solution (Metzger et al., 2007; Tomotake et al., 2002). Thus, it is reasonably assumed that the DH increase during final hydrolysis was mainly caused by the hydrolysis of the aggregated albumin (especially those insoluble), not that of the native albumin.

3.3.2. Surface aromatic hydrophobicity (H_o)

The H_o values of BPI and its hydrolysates are also included in Table 1. In general, the influence of enzymatic hydrolysis on the surface aromatic hydrophobicity of food proteins is dependent upon the type or nature of these proteins. For example, Mahmoud, Malone, and Cordle (1992) indicated that the enzymatic hydrolysis decreased the hydrophobicity of casein, while in another study concerning about soy protein isolates, the hydrolysis resulted in significant increases in the surface hydrophobicity (Wu, Hettiarachchy, & Qi, 1998). As shown in Table 1, the enzymatic hydrolysis led to gradual and significant decreases in H_o (from 678 to 66) with DH increasing up to 15%, while further hydrolysis (with DH > 15%) on the contrary gradually increased the H_o. The data seem to be consistent with the result of SEC analysis (Table 1), reflecting that the H_o change for BPI hydrolysates was closely related to the hydrolysis pattern of BPI.

The gradual decreases in H_o by the hydrolysis (relative to BPI) can be attributed to two main reasons. First, enzymatic cleavage of hydrophobic clusters at the surface of the globulins (or the aggregates) might partially result in decline in H_o. Second, the heat inactivation treatment of the enzyme also might remove some hydrophobic peptides or protein fragments that are released during the hydrolysis. The increases in H_o during extensive hydrolysis (Table 1) may be attributed to release of more stable hydrophobic peptides (especially those with low MW) from the globulins or the aggregates (e.g., the albumin aggregates).

3.3.3. Amino acid composition

Table 2 shows the amino acid composition of BPI and its hydrolysates, as well as FAO/WHO suggested essential amino acid contents for children and adult. The amino acid composition of BPI is consistent with that previously reported by other researchers (Pomeranz & Robbins, 1972; Tomotake et al., 2002). It is more similar to that of the albumin fraction as compared to that of globulin fraction of tartary buckwheat flour (Guo & Yao, 2006), further confirming that the albumin fraction was the major component in the protein isolate from buckwheat. All the essential amino acids of this protein meet up with the FAO/WHO suggested requirements for 2–5 years old children, except the content of sulphur-containing amino acids is slightly lower (Table 2). The amino acid composition of BPI was nearly unaffected by the hydrolysis, except sulphur-containing amino acids slightly decreased (Table 2). The data suggest that the protein products (including the hydrolysates) have well-balanced amino acid com-

Table 2
Amino acid composition of BPI and its hydrolysates (g/100 g protein), as well as FAO/WHO suggested essential amino acid contents for children and adult.

Amino acids	Amino acid content						FAO/WHO suggested requirements		
	BPI	DH-5	DH-10	DH-15	DH-20	DH-25	2–5 years old	10–12 years old	Adult
Asp+Asn	8.3	8.7	8.8	9.2	9.0	8.9			
Glu+Gln	16.8	17.6	17.6	18.6	17.7	17.0			
Ser	4.5	4.4	4.4	4.6	4.4	4.5			
Gly	4.0	4.3	4.0	4.3	4.3	6.8			
His[a]	3.9	3.1	3.5	2.6	3.1	2.6	1.9	1.9	1.6
Arg	10.6	10.5	10.5	11.4	11.2	11.2			
Thr[a]	3.3	3.2	3.1	2.8	3.3	2.8	3.4	2.8	0.9
Ala	3.5	3.3	3.2	3.7	3.5	3.7			
Pro	3.6	3.5	3.6	3.1	3.1	3.0			
Tyr	3.4	3.5	3.4	3.3	3.3	3.3			
Val[a]	5.1	5.0	4.9	4.7	4.8	4.7	3.5	2.5	1.3
Met	1.4	1.4	1.2	1.0	1.1	0.9			
Cys	0.5	0.5	0.5	0.2	0.3	0.4			
Ile[a]	3.7	3.8	3.7	3.4	3.5	3.4	2.8	2.8	1.3
Leu[a]	6.3	6.0	5.9	5.9	6.1	5.9	6.6	4.4	1.9
Phe	5.3	5.3	5.3	5.2	5.2	5.2			
Lys[a]	5.6	6.1	6.2	5.9	6.1	6.0	5.8	4.4	1.6
SAA[b]	1.9	1.9	1.7	1.2	1.4	1.3	2.5	2.2	1.7
ARM[c]	8.7	8.8	8.7	8.5	8.5	8.5	6.3	2.2	1.9
Hydrophobic amino acids [d]	28.9	28.3	27.8	27.0	27.2	26.8			

[a] Essential amino acids.
[b] SAA: sulphur-containing amino acids Met and Cys.
[c] ARM: aromatic amino acids Phe and Tyr.
[d] Hydrophobic amino acids: Ala, Pro, Val, Met, Ile, Leu and Phe.

position, and are suitable for human consumption as a source of protein nutrition.

3.3.4. Polyphenol content

It is well-known that buckwheat seeds are rich in flavonoids and flavones, especially rutin. These polyphenols might interact with the proteins during the preparation of BPI, and as a consequence, would be present in the resulting BPI. The presence of the polyphenols may account for the low solubility of buckwheat proteins (Metzger et al., 2007; Tomotake et al., 2002). The polyphenol contents (including those in free or protein-bound form) of the buckwheat flour, BPI and its hydrolysates were analyzed, as shown in Fig. 3. In the seed flour, total polyphenol content was about 0.28 g of rutin equivalent /100 g of sample, less than of that (total flavonoid content; about 0.37–0.40 g/100 g) reported by Oomah and Mazza (1996). The difference may be attributed to the differences in variety and/or cultivation condition, as well as choice of the standard polyphenol compound. All the polyphenols in the flour were in the protein-bound form (Fig. 3). Additionally, total polyphenol content of BPI was surprisingly more than 10-fold higher than that of the flour (Fig. 3). The data indicated that there was high affinity or strong interaction between proteins and the polyphenols. Thus, the low solubility of buckwheat proteins (Metzger et al., 2007; Tomotake et al., 2002) may largely be related to the presence of high level of polyphenols associated with proteins.

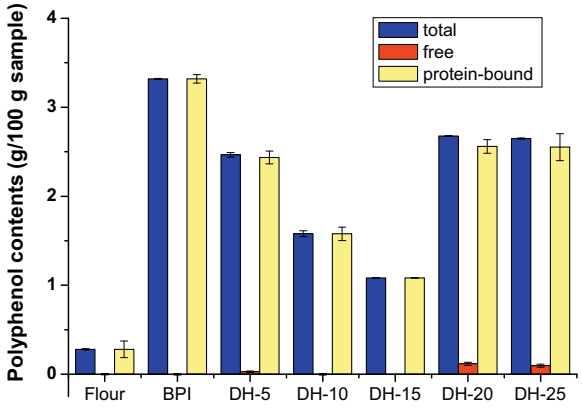

Fig. 3. The contents of total, free and protein-bound polyphenols in BPI and its hydrolysates. Each data point is the mean of three determinations, and error bars represent standard deviation. The polyphenol contents were expressed as grams of rutin equivalent per 100 grams of the sample. The 'flour' in the figure indicates buckwheat seed flour. For labels (DH-5, DH-10, DH-15, DH-20 and DH-25), refer to Fig. 2.

Taking the DH and SEC data (Figs. 1 and 2) together, it is reasonable to suggest that the polyphenols would preferentially interact with the albumins to form high MW aggregates, making these aggregates more susceptible to protease hydrolysis (since the globulins were easily digested, while the native albumins were nearly unaffected during the hydrolysis). The improvement of the susceptibility may be attributed to polyphenol-induced exposure of catalytic sites initially buried within the molecules of native albumins. This assumption has been interestingly evidenced by a recent study about pepsin digestibility of buckwheat protein, where it was found that the presence of rutin significantly improved the pepsin digestibility of its albumin fraction, although it was not indicated which level of rutin was used for the experiment (Guo, Yao, & Chen, 2007).

The hydrolysis resulted in reduction in total polyphenol contents to a variable extent, depending upon the DH (Fig. 3). With DH increasing from 5% to 15%, total polyphenol content of the hydrolysates gradually decreased from about 2.4 to 1.1 g of rutin equivalent per 100 g of the sample, and it on the contrary distinctly increased upon further hydrolysis (with DH of 20–25%). The change pattern of polyphenol content is consistent with the pattern of protein hydrolysis and the change pattern of surface hydrophobicity (Table 1). The data can be presumably explained in the view of thermal stability of the protein–polyphenol complexes. From this view of point, the decrease in total polyphenol content upon hydrolysis (with DH increasing from 0% to 15%) reflected the decline in the thermal stability of the complexes. On the other hand, extensive hydrolysis might lead to complete disruption of the complexes, and concomitant release of more low MW polypeptide or peptide fragments associated with the polyphenols (relative to the hydrolysis with low DH), and the latter would be relatively

thermally stable, and still kept in the hydrolysates, as evidenced by increased polyphenol contents during further hydrolysis (with 20–25% DH) (Table 1). This point is also manifested by the distinct increases in free polyphenol contents in the DH-20 and DH-25 (Table 1).

3.4. Antioxidant activities

3.4.1. DPPH radical scavenging ability

Fig. 4A shows DPPH radical inhibition of the hydrolysates of BPI, at various concentrations (0–1.0 mg/mL). In the present study, the data for BPI was not provided, due to its low solubility. The relatively 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 (Jao & Ko, 2002; Shimada et al., 1992). All the hydrolysates showed dose-dependent DPPH radical scavenging activities to varying extents (Fig. 4A). At any tested concentration, the DPPH radical inhibition for all the hydrolysates was considerably lower than that of Trolox (control). At concentrations >0.05 mg/mL (and <1.0 mg/mL), the DPPH radical inhibition of Trolox was independent of concentration. Thus, it is possible that if the concentration is high enough, the BPI hydrolysates might exhibit a similar DPPH radical scavenging activity to that of Trolox.

The IC_{50} value is applied as an indication to evaluate the scavenging activity. The lower the IC_{50} value, the higher the free radical scavenging ability. The IC_{50} values for DH-5, DH-10, DH-15, DH-20 and DH-25 were calculated from the regression equation to be 0.56, 0.78, 0.94, 0.67 and 0.65 mg/mL, respectively. The IC_{50} values (0.56–0.94 mg/mL) of the hydrolysates are much less than the lowest value (1.3 mg/mL) of wheat germ protein hydrolysate prepared with Alcalase (Zhu et al., 2006). The DPPH radical scavenging inhibition for DH-5 or DH-25 at a concentration of 1.0 mg/mL is close to the highest value of chickpea protein hydrolysate obtained by Alcalase digestion (Li et al., 2008).

Among all tested DH values, lowest DPPH radical scavenging ability for the BPI hydrolysates was found at DH 15%. This DH-dependence of DPPH radical scavenging ability is different from that reported for porcine collagen hydrolysate (Li, Chen, Wang, Ji, & Wu, 2007), where high DPPH radical scavenging ability was obtained for the hydrolysates with high DH. By comparison with the protein constituents, amino acid composition and polyphenol content, it can be suggested that at a specific concentration, the DPPH radical scavenging ability of the hydrolysates is closely related to their polyphenol contents (Fig. 3). Some previous studies pointed out that high DPPH or other radical scavenging activities for the protein hydrolysates or peptides are usually associated with high hydrophobic amino acid or hydrophobicity (Li et al., 2008; Rajapakse, Mendis, Byun, & Kim, 2005). This view seems to be consistent with the data of DPPH radical scavenging ability between DH-5 and DH-25 (Fig. 4A), since DH-5 had higher surface hydrophobicity relative to DH-25, although the latter contained higher polyphenol content.

3.4.2. Reducing power

The reducing capacity of a compound can be used to evaluate its potential antioxidant activity (Huang, Chen, How, Lin, & Lin, 2004). The reducing power is denoted as absorbance at 700 nm to monitor the Fe^{2+} concentration (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). BPI and its hydrolysates also exhibited dose-dependent reducing power (Fig. 4B). Interestingly, the dependence of the reducing power of the hydrolysates on concentration was similar to ascorbic acid (a well-known antioxidant), even though the latter exhibited better reducing power. At the highest experimental concentration (5 mg/mL), the reducing power of the BPI hydrolysates was in the range of 0.77–1.24, which is higher than 0.08–0.25 reported for similar concentration of haemoglobin hydrolysates (Chang, Wu, & Chiang, 2007), but comparable to that of purified fractions of chickpea protein hydrolysate (Li et al., 2008).

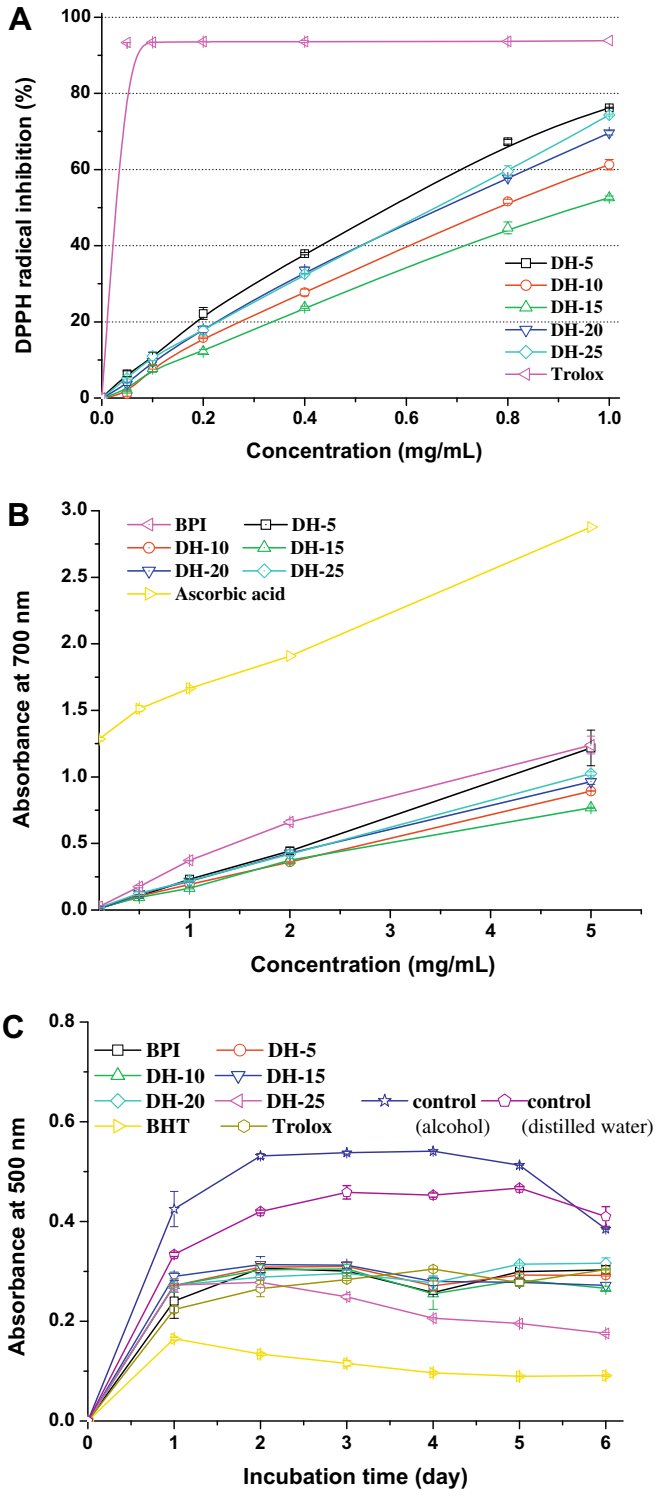

Fig. 4. Antioxidant activities, including DPPH radical scavenging ability (A), reducing power (B) and linoleic acid autoxidation inhibition (C) of BPI and its hydrolysates. Each data point is the mean of duplicate or more measurements, and error bars represent standard deviation. For legends, refer to Fig. 2.

3.4.3. Inhibition of linoleic acid peroxidation

The inhibition of linoleic acid peroxidation by BPI and its hydrolysates, as shown in Fig. 4C. In the present experiment, the concentration of the tested samples was 1.2 mg/mL, and the

time-course plots were determined by the thiocyanate method (using the absorbance at 500 nm as an indication). The autoxidation of linoleic acid (in alcohol or distilled water) was inhibited to varying extents by the BPI hydrolysates (Fig. 4C). The inhibition activity of all the hydrolysates was similar to that of Trolox (in alcohol), except that after two days, the inhibition activity of DH-25 was distinctly better than that of Trolox. The underlying mechanism for higher activity of DH-25 when compared with the other hydrolysates is still not clear, but it may partially be related to changes in the state of polyphenols resulting from protein–polyphenol interactions in the extensively hydrolysed system. However, this antioxidative activity of all the hydrolysates was much lower than BHT (Fig. 4C), in agreement with other reports on wheat germ hydrolysate (Zhu et al., 2006) and chickpea protein hydrolysate (Li et al., 2008).

4. Conclusions

The enzymatic hydrolysis by Alcalase resulted in effective breakdown of the globulins or the protein aggregates in BPI, and the resultant hydrolysates exhibited considerable decrease in surface hydrophobicity, but amino acid compostion was similar to BPI. The hydrolysis also decreased the polyphenol content, to a variable extent, depending on the DH. The obtained hydrolysates showed excellent antioxidant activities, including DPPH radical scavenging ability, reducing power and the ability to inhibit linoleic acid peroxidation. The antioxidant activities of these hydrolysates were closely related to their polyphenol contents. Further studies should be carried out to investigate the mechanism concerning the influence of polyphenol on the antioxidant properties of buckwheat protein hydrolysates.

Acknowledgments

This work is part of the research project from National Natural Science Foundation of China (Serial Number: 20876057). The authors especially thank for valuable and critical comments by anonymous reviewers, which greatly improve the quality of the manuscript.

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