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Functional Properties of Buckwheat (*Fagopyrum esculentum* Moench) Seed Protein Isolate: Effects of Limited Enzymatic Hydrolysis with Trypsin

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ABSTRACT

The effects of limited enzymatic hydrolysis with trypsin on the physicochemical and functional properties of buckwheat protein isolate (BPI) were investigated. The protein constituents, free sulfhydryl (SH) contents and surface hydrophobicity (H_o), as well as some selected functional properties, such as protein solubility (PS), emulsifying (including emulsifying activity index, EAI and emulsion stability index, ESI) and foaming (including foaming capacity, FC and foam stability, FS) activities of BPI hydrolysates (freeze-dried) were evaluated. The influence of heat treatment prior to freeze-drying on these properties was also evaluated. The limited hydrolysis led to transformation of insoluble aggregates into soluble peptide fragments. Progressive decreases in total SH content (or exposed free SH content) and H_o were observed for both untreated and heated hydrolysates with the increase in degree of hydrolysis (DH). In the unheated case, the hydrolysis treatment resulted in gradual increases in PS, EAI and ESI, and decreases in FC and FS of BPI. The heat pretreatment remarkably decreased the EAI and ESI of the hydrolysates, but significantly increased the FC for the hydrolysates with DH less than 8.3%. The results indicated that the limited hydrolysis with trypsin could be applied to modify the physiochemical and functional properties of buckwheat proteins.

Keywords: buckwheat seeds, functionality, modification, storage proteins

INTRODUCTION

The storage proteins from buckwheat (*Fagopyrum esculentum* Moench) seeds have attracted increasing interest due to their balanced essential amino acid composition (Pomeranz and Robbins 1972), as well as many potential health effects, e.g. hypocholesterolemic activity in rats, suppression in body fat, constipation, mammary carcinogenesis and colon carcinogenesis and in the formation of cholesterol gallstones in hamsters (Kayashita *et al.* 1996, 1997; Tomotake *et al.* 2000; Liu *et al.* 2001; Tomotake *et al.* 2001).

Protein content of buckwheat flour ranges from 8.51 to 18.87% depending on the variety (Krkošková and Mrázová 2005). The proteins in buckwheat seeds consist of albumin, globulin, prolamin and glutelin, and their relative contents vary 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 estimated to be about 43.8, 7.8, 10.5 and 14.6%, respectively (Guo and 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 proteins 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 proteins (especially BPI) have also been shown to have some superior functional properties, including higher nitrogen solubility index and higher water holding, emulsifying and foaming capacities, as compared to soy protein isolate (SPI) (Zheng *et al.* 1998; Bejosano and Corke 1999; Tomotake *et al.* 2002). In our recent study, it was shown that the functional properties of BPI were highly dependent upon their lipid content, as affected by the method of extraction, as well as defatting treatment (Tang 2007). The protein fractions in buckwheat seeds seem to be preferably associated with the lipid fraction, and as a result, the lipids are usually extracted together with the proteins during the production of BPI from undefatted buckwheat flour (Tomotake *et al.* 2002; Tang 2007). The BPI obtained from untreated flour exhibited poor functional properties, especially protein solubility at neutral condition (Tang 2007). Due to the economic and practical consideration, it is preferable to prepare the BPI from untreated flour. Thus, modification should be conducted to improve the functional properties of BPI, contributing to its applications in food formulations.

Relative to other physical and chemical treatments, enzymatic hydrolysis has been considered to be the most potential to improve the functional properties of food proteins, due to its mild process conditions required, and easier control of the reaction and minimal formation of by-products (Mannheim and Cheryan 1992). To date, limited enzymatic proteolysis has been used to modify the functional and physicochemical properties of soy protein (Jung *et al.* 2005), sunflower protein (Martineza *et al.* 2005), rapeseed protein isolates (Vioque *et al.* 2000), whey protein (Chobert *et al.* 1988), oat bran protein concentrate (Guan *et al.* 2007), legumin from faba bean (*Vicia faba*) (Dudek *et al.* 1996) and hemp proteins (Yin *et al.* 2008).

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 or lysine (Kishimura *et al.* 2007). In our previous study about *in vitro* pepsin plus trypsin digestibility, it was indicated that the proteins in BPI are much more easily digested by trypsin, after initial pepsin digestion, compared to SPI (Tang 2007). Thus, it would be a good choice to apply the trypsin to modify the functional properties, through limited hydrolysis. The main objective

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of the present work was to investigate the influence of limited enzymatic hydrolysis with trypsin on the functional properties of BPI, obtained from the untreated buckwheat flour.

MATERIALS AND METHODS

Materials

Common buckwheat seeds were purchased from a local supermarket in Guangzhou (China), which were cultivated in Ganshou Province of China. Trypsin powder (from porcine pancreas; catalog no. T4799, 1,000-5,000 BAEE units mg^{-1} solid) and Folin and Ciocalteu's Phenol Reagent (F-9252**)** were from Sigma-Aldrich Inc. (St. Louis, MO, USA). 5, 5'-dithio-bis 2-nitrobenzoic acid (DTNB), 2, 4, 6-trinitrophene sulphonic acid (TNBS) and 1, 8-anilinonaphthalenesulfonate (ANS) reagents were also purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other chemicals used in the present study were of analytical or better grade.

Preparation of BPI

BPI was prepared from 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-ionized water for 1 h at room temperature, and the pH of the dispersion adjusted to about 8.0 with 1 N NaOH. The dispersion was centrifuged at 8,000 × *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 4,000 × *g* for 15 min, and re-dispersed in de-ionized water. Finally, the dispersion (about 5%, w/v) was neutralized to pH 7.0, and freeze-dried to produce raw BPI sample.

Enzymatic hydrolysis of BPI and preparation of the hydrolysates

Four grams of BPI were dispersed in 200 mL of de-ionized water at room temperature. The dispersions were pre-incubated at 37°C, prior to adjusting pH of the dispersion to 8.0. The mixture of protein and enzyme (trypsin) at an enzyme-to-substrate ratio of 1:200 (w/w) was incubated in a temperature-controlled water bath at 37°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 the pH-stat method (Adler-Nissen 1986).

Unheated and heated hydrolysates were prepared as follows. At specific periods of time (e.g. corresponding to DH of 0, 4.5, 8.3 and 12.8%, respectively), aliquots of the digested mixture were taken out, and heated at 95°C for 10 min, and then cooled immediately in ice water to room temperature. The resulting digests were centrifuged at 4,000 × *g* for 20 min to remove insoluble residues. The supernatants were then adjusted to pH 7.0, and lyophilized to produce the heated hydrolysate samples, which were stored at -20°C before further analysis. The unheated hydrolysates were prepared according to the same above-mentioned process, but without the heat treatment. The obtained hydrolysates with DH values of 0, 4.5, 8.3 and 12.8% were further denoted as control, DH-4.5, DH-8.3 and DH-12.8, respectively.

Determination of DH

DH is defined as the ratio of the number of peptide bonds cleaved (number of free amino groups formed during proteolysis) expressed as hydrolysis equivalents (*h*), in relation to the total number of peptide bonds before hydrolysis (h_{tot}). The h_{tot} is equivalent to the amino acid composition of the protein, calculated from amino acid analysis by summing the mmoles of each individual amino acid per gram of protein (Adler-Nissen 1986). The DH for enzymatic hydrolysis was measured by the 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 is the base consumption in mL, N_b the normality of the base, M_p the mass of protein being hydrolyzed (g), and h_{tot} the total number of peptide bonds in the protein substrate (meqv g^{-1} protein). The h_{tot} of BPI was about 8.14 meqv g^{-1} protein (Tang *et al.* 2009).

The calibration factor α, or the reciprocal of degree of dissociation of the α-NH_2 groups, was calculated as follows:

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

where pK is the average dissociation value for the α-amino groups, calculated according to the Gibbs-Helmholz equation (Adler-Nissen 1986). At 37°C as in the present study, the average dissociation value (pK) can be calculated to be 7.4.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (1970), using a 12% separating gel (pH 8.6) and a 4% stacking gel (pH 6.7). The protein samples were solubilized in 0.125 M Tris-HCl buffer, containing 1% (w/v) SDS, 5% (v/v) glycerol and 0.025% (w/v) bromophenol blue (with or without 2% (v/v) 2-mercaptoethanol, 2-ME), and heated for 5 min in boiling water before electrophoresis. After the electrophoresis, the gel was dyed in 0.25% Coomassie blue (R-250) in 50% trichloroacetic acid and then destained in methanol solution containing acetic acid [methanol: acetic acid: water, 227: 37: 236 (v/v/v)].

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^{TM} 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^{-1}; 3) elution solvent: 50 mM 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).

Free sulfhydryl group (SH) content

Free SH contents of BPI and its hydrolysates, including total and exposed SH contents, were determined according to the same process described by Yin *et al.* (2008). The SH contents were expressed as μmol/g of protein.

Surface aromatic hydrophobicity (H_o)

H_o was determined with the fluorescence probe ANS according to the method described by Haskard and Li-Chan (1998). Serial dilutions in 0.01 M PBS (pH 7.0) were prepared with the hydrolysates to a final concentration of 0.005-0.2% (w/w). ANS solution (8.0 mM) was also prepared in the same buffer. Twenty microliters 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^{-1}) plot (calculated by linear regression analysis) was used as an index of H_o.

Protein solubility (PS)

An aqueous solution (1%, w/v) of protein samples was stirred magnetically for 30 min, and then with either 0.5 N HCl or 0.5 N NaOH, the pH of the solutions was adjusted to the required values.

After 30 min of stirring, the pH was readjusted if necessary. Then it was centrifuged at 8,000 × *g* for 20 min at 20°C in a CR22G centrifuge (Hitachi Co., Japan). After appropriate dilution, the protein content of the supernatant was determined by the Lowry method (Lowry *et al.* 1951) using bovine serum albumin monomer (BSA; Sigma-Aldrich Co., USA) as the standard. The PS was expressed as grams of soluble protein per 100 g of protein. All determinations were conducted in duplicate.

Emulsifying activities

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined according to the method of Pearce and Kinsella (1978), with minor modifications. For emulsion formation, 15 mL of 0.1% (w/v) dispersions of BPI and its hydrolysates in 50 mM phosphate buffer (pH 7.0) and 5 mL of corn oil were homogenized in ULTRA-TURRAX® T25 digital homogenizer (IKA Co., Germany) at 24,000 turn/min for 1 min. Fifty microliters of emulsion were taken from the bottom of the homogenized emulsion, immediately (0 min) or 10 min after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) SDS solution. After shaking in a vortex mixer for about 5 sec, the absorbance of diluted emulsions was read at 500 nm in a spectrophotometer (Lengguang Technol. Co., Shanghai). EAI and ESI values were calculated by equations as follows:

$$\text{EAI } (m^2/g) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \phi \times (1-\theta) \times 10000},$$

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

where DF is the dilution factor (100), c the initial concentration of protein (g mL^{-1}), ϕ the optical path (0.01 m), θ the fraction of oil used to form the emulsion (0.25), and A_0 and A_{10} the absorbance of the diluted emulsions at 0 and 10 min, respectively. Measurements were performed in more than triplicate.

Foaming properties

Foaming properties including foaming capacity (FC) and foam stability (FS) were determined by the method of Fernandez and Macarulla (1997) with minor modifications. Aliquots (10 mL) of sample solutions (1%, w/v) at pH 7.0 in measuring cylinder (25 mL) were homogenized with an ULTRA-TURRAX® T25 digital homogenizer at 10,000 turns/min for 2 min. FC was calculated as the percent increase in volume of the protein dispersion upon mixing, while FS was estimated as the percentage of foam remaining after 30 min.

Statistics

An analysis of variance (ANOVA) of the data was performed using SPSS 13.0 for windows software and a least significant difference (LSD) or Tamhane's with a confidence interval of 95 or 99% was used to compare the means.

RESULTS AND DISCUSSION

Enzymatic hydrolysis

The chemical composition of the buckwheat flour used to prepare 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-water ratio of 1: 10. The chemical composition of 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 (about 80%) of Tomotake *et al.* (2002) and Tang (2007), but similar to that (about 64%) of Metzger *et al.* (2007).

The enzymatic hydrolysis of BPI by trypsin was monitored by pH-stat method, and the DH change with hydrolysis time is shown in **Fig. 1**. As expected, the DH progressively increased with hydrolysis time. At the end of hydrolysis (540 min), the DH reached about 24%. According to the pattern of DH increase, the hydrolysis process of BPI by trypsin could be appropriately divided into three phases: during initial incubation with trypsin (up to about 258 min), the rate of DH increase gradually decreased; then, it gradually increased till about 446 min, but at a higher rate of DH increase; last, it gradually decreased again. The hydrolysis pattern is similar to that of legumin-rich hemp protein isolate by trypsin (Yin *et al.* 2008), where it was attributed to sequential hydrolysis of soluble proteins and insoluble aggregates.

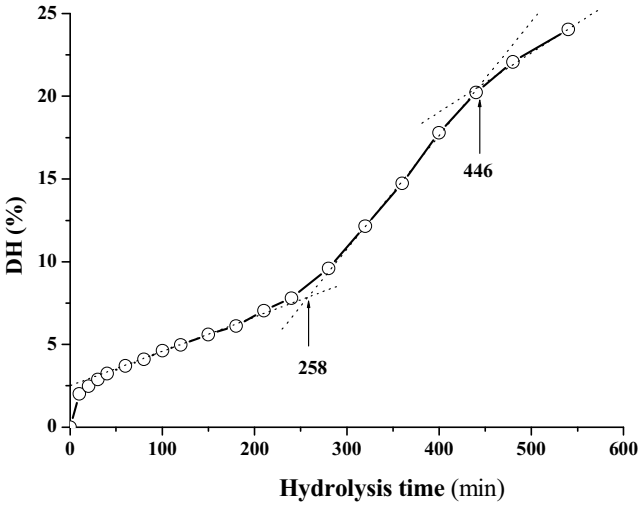

Fig. 1 Trypsin-induced enzymatic hydrolysis of BPI, as analyzed by DH change.

SDS-PAGE and SEC analyses

The protein constituents of BPI and its hydrolysates (DH-4.5, DH-8.3 and DH-12.8) were analyzed using SDS-PAGE and SEC techniques, as shown in **Fig. 2**. In the presence of 2-ME, raw BPI exhibited typical bands corresponding to subunits of 8S (minor) and 13S (major) globulins, while the 2S albumins with MW from 8 to 16 kDa could not be reflected in this gel electrophoresis (**Fig. 2A**, lane 1). The hydrolysis resulted in disappearance of subunits of 8S globulin and acidic subunits (AS) of 13S globulin, while basic subunits (BS) of 13 S globulin also decreased remarkably, but the extent of decrease seemed to be similar for the hydrolysates with DH in the range 4.5-12.8% (**Fig. 2A**, lanes 2-4). The data suggest that the subunits of 8S globulin and AS of 13S globulins are much more susceptible to trypsin hydrolysis than BS of 13 S globulins. The susceptibility difference between AS and BS of 13S globulins may be attributed to the accessibility difference of both subunits to the catalytic sites of trypsin, since the former is usually located at the exterior of the protein molecules, while the latter is usually buried in the interior (Plietz *et al.* 1984). Similar results have been obtained in many plant 11S globulins (Kamata and Shibasaki 1978; Plumb and Lambert 1990; Dudek *et al.* 1996; Schwenke 2001; Yin *et al.* 2008).

In the absence of 2-ME, BPI displayed the bands with MW in the range 55-66 kDa (**Fig. 2A**, lane 5), confirming that AS and BS of 13S globulins in raw BPI were mainly in the AB form, via disulfide bond. In this sample, some acidic subunits of 13S globulins were also observed, reflecting that these subunits could be released from native structure of 13S globulin, during the preparation of BPI. The hydrolysis led to disruption of the major bands (55-66 kDa) into the bands with MW of about 30 kDa (**Fig. 2A**, lanes 6-8). The latter bands are clearly attributed to partially digested AB subunits with more intact basic subunits (as indicated by SDS-PAGE profile in the presence of 2-ME; **Fig. 2A**, lanes 2-4).

The hydrolysis of globulins was reflected by SDS-

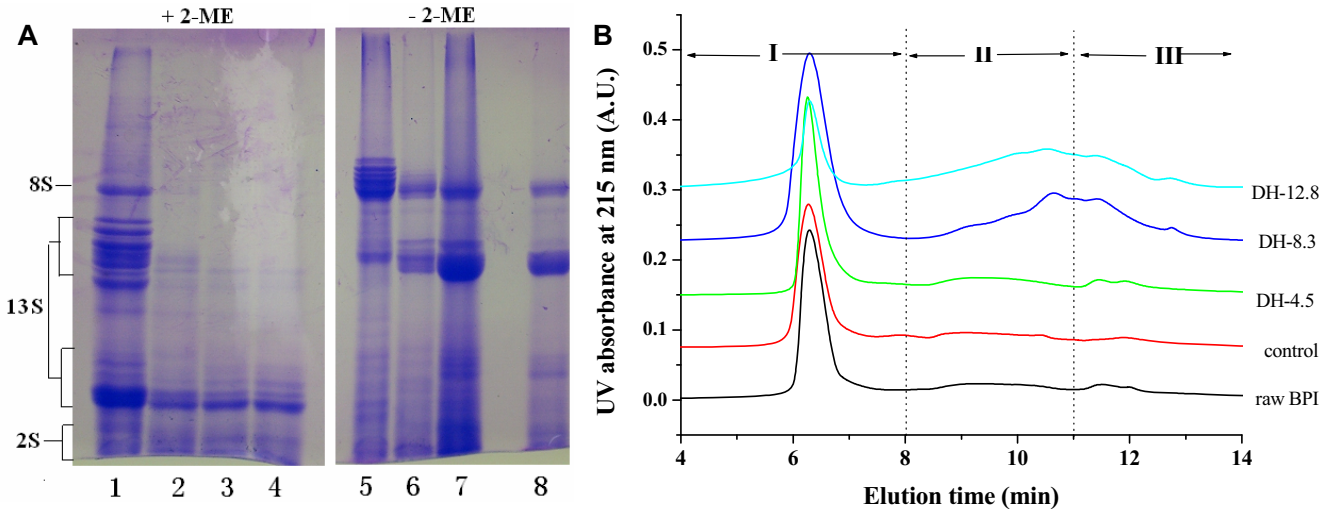

Fig. 2 SDS-PAGE (A) and SEC (B) analyses of raw BPI and its hydrolysates with DH values of 4.5, 8.3 and 12.8%, respectively. Panel A: Lanes 1-4 and 5-6 represent BPI (control), DH-4.5, DH-8.3 and DH-12.8, in the presence and absence of 2-ME, respectively; 8S, 13S and 2S indicate the 8S globulins, 13S globulins and 2S albumins, respectively. Panel B: The numbers (I-III) represent the groups corresponding to the constituents of the samples eluting at < 8 min, 8-11 min and > 11 min, respectively.

Table 1 Area percentages of individual groups of BPI and its hydrolysates (in the SEC chromatograms).

Samples	Unheated				Heated			
	Total integrated area (%)[a]	Group I[b]	Group II	Group III	Total integrated area (%)	Group I	Group II	Group III
Raw BPI	100.0	68.9	15.1	16.0				
Control	95.0	60.4	21.2	13.4	114.3	74.9	18.5	20.9
DH-4.5	157.2	66.2	43.3	47.7	126.1	25.6	63.3	37.2
DH-8.3	179.7	64.6	40.8	74.3	134.8	20.0	71.4	43.4
DH-12.8	170.0	58.6	37.2	74.2	140.2	22.3	69.2	48.7

[a] The total integrated area of peaks in SEC profile of raw BPI was defined as 100 %, and that of others calculated relative to this value. [b] Groups I, II and III correspond to the protein constituents eluting at < 8 min, 8-11 min, and > 14 min, respectively.

PAGE, but the hydrolysis of 2S albumins accounting for the major components of BPI could not. Therefore, we also analyzed unheated BPI and its hydrolysates using SEC technique, as shown in **Fig. 2B**. In order to better reflect the differences in SEC chromatograms, we divided the peaks on the chromatograms into three groups. Groups I-III corresponded to the constituents of the protein (or the hydrolysates) eluting at < 8 min, 8-11 min and > 11 min, respectively. The area percentages of individual groups of BPI and its hydrolysates are listed in **Table 1**. In the SEC profile of raw BPI, a major peak eluting at near void volume was observed, accounting for about 68.9% of total integrated peak area (**Fig. 2B** and **Table 1**). This data indicates the presence of high level of protein aggregates in the BPI, also confirming insoluble nature of this kind of protein (Tomotake *et al.* 2002). The additional freeze-drying treatment (control) slightly decreased total integrated peak area of BPI, mainly at the expense of its Group I, suggesting decrease in protein solubility due to transformation of soluble to insoluble aggregate. Meanwhile, the hydrolysis (with DH 4.5-12.8%) considerably increased total integrated peak area (**Table 1**), indicating improvement of protein solubility by limited enzymatic hydrolysis. The increase in total peak area was mainly contributed by the increases in the contents of Groups II and III, while that of Group I on the contrary slightly decreased upon hydrolysis (relative to raw BPI; **Table 1**). The data suggest that the improvement of protein solubility was mainly due to transformation of insoluble aggregates into soluble peptide fragments.

The influence of heat pretreatment (95°C, 10 min) used to inactivate the enzyme on the SEC profiles was also investigated. The heat pretreatment prior to freeze-drying significantly increased total integrated peak area of raw BPI (**Table 1**). The data suggest that the pretreatment led to partial disruption of insoluble aggregates (or precipitates) in raw BPI into soluble components. However, the pretreatment remarkably decreased the total integrated peak area of the hydrolysates (with DH 4.5-12.8%) (**Table 1**). For a given heated hydrolysate, Groups I and III remarkably decreased with concomitant increase in Group II, relative to its unheated counterpart (**Table 1**). The results clearly indicated heat-induced aggregation of hydrophobic peptide fragments (e.g. those included in Group III) released during trypsin digestion, or formation of insoluble macroaggregates from soluble aggregates (e.g. Group I).

Sulfhydryl (SH) contents

Table 2 shows total SH [combined disulfide bond (SS) and free SH] and exposed free SH contents of unheated and heated BPI and its hydrolysates. In the raw BPI, total SH content and exposed SH contents were 27.3 and 7.8 umol SH g^{-1}, respectively, indicating that most of sulfhydryl groups were in the SS form), or in the form of buried state. This value of total SH content is even much higher than that of soy protein isolate (18.4 μmol SH g^{-1}) (Tang 2008). This is consistent with the fact that the albumin is the major protein fraction of common buckwheat, which had very high level of methionine residue (9.2%) (Radovic *et al.* 1999; Guo and Yao 2006). The total SH content slightly but significantly ($p < 0.05$) decreased after another additional freeze-drying treatment, while the exposed free SH content was unchanged (**Table 2**). The decrease in total SH content seems to be consistent with the decrease in total integrated peak area in SEC analysis (**Table 1**), suggesting that the decrease in protein solubility in 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl might be due to formation of aggregates, mainly from the albumins.

In the unheated hydrolysates, considerable and significant decrease in total SH content was observed (relative to unheated control) as the DH increased from 4.5 to 8.3% (**Table 2**). Similar decreases occurred for the exposed free

SH content, as DH increased from 0 to 12.8%, though there was no significant difference between DH-4.5 and DH-8.3. The data suggest that the hydrolysis progressively led to formation of insoluble aggregates, via disulfide bonds. In other words, the aggregates formed during the hydrolysis were mainly contributed by the albumins, since the latter contained high content of methionine residue.

The heat pretreatment led to slight but significant increases in total SH content and exposed free SH content of the hydrolysates, except that the total SH content of DH-4.5 was on the contrary decreased by the pretreatment (**Table 2**). The increases in SH contents could be explained by transformation of insoluble aggregates (containing SH-rich proteins or peptides) into the hydrolysates.

H_0

The H_0 values of unheated and heated BPI and its hydrolysates are also included in **Table 2**. The treatment of additional freeze-drying (control) did not affect the H_0 of unheated BPI. However, the hydrolysis led to the hydrolysates with considerably and significantly decreased H_0 relative to raw BPI or untreated control (**Table 2**). Contrasting results have been observed in soy protein isolates modified by enzymatic hydrolysis, where the hydrolysis resulted in significant increases in surface hydrophobicity (Hettiarachchy and Kalapathy 1997; Wu *et al.* 1998). The difference may be attributed to the difference of hydrolysis pattern for different proteins in the different forms of state. In combination with the SEC analysis (**Figs. 1, 2B, Table 1**), it can be suggested that the hydrolysis led to release of hydrophilic peptide fragments, mainly from insoluble albumin aggregate. Together with the data of total SH content (**Table 2**), the data also suggested that the insoluble albumin aggregates were associated mainly due to hydrophobic interactions and disulfide bonds.

The heat pretreatment significantly ($p < 0.05$) increased the H_0 value of raw BPI (**Table 2**). This is clearly due to heat-induced unfolding of protein structure and subsequent exposure of hydrophobic clusters initially buried within the molecules. The pretreatment also led to significant increases in H_0 value of the obtained hydrolysates, as compared to their individual counterparts (**Table 2**). This is consistent with the SH data (**Table 2**), further suggesting that the pretreatment resulted in disruption of insoluble aggregates and subsequent formation of soluble hydrophobic peptide fragments.

Functional properties

PS. The PS of BPI and its hydrolysates (untreated and heated) was determined at pH 7.0, as shown in **Table 3**. The PS of raw BPI was just about 54.4%, which is consistent with the view that the proteins from buckwheat are insoluble in nature (Tomotake *et al.* 2002). The additional freeze-drying treatment didn not affect the PS of raw BPI. The untreated hydrolysates (with DH 4.5-12.8%) exhibited significantly higher PS than raw BPI or control (**Table 3**). Interestingly, the heat pretreatment slightly but insignificantly ($p > 0.05$) increased the PS of the hydrolysates (obtained at DH of 0-12.8%). This result seems to be different from the SEC analysis (**Table 1**) that the pretreatment led to significantly decreased total integrated peak area. The difference may be attributed to the differences of pH and ionic strength in applied buffers. In the SEC experiments, the pH of the buffer was pH 7.2, while it also contained additional 0.1 M NaCl. The presence of 0.1 M NaCl might increase the PS of the protein aggregates, associated with weak interactive forces, like in the case of unheated hydrolysates, while in the heated cases, the heat pretreatment would lead to formation of the insoluble aggregates, with strong hydrophobic interactions. In general, the improvement of PS by hydrolysis for BPI seems to be not as prominent as in other cases, such as hemp protein isolate (Yin *et al.* 2008). The reasonable explanation for this phenomenon is that the hydrolysis of the albumins and the release of the peptide fragments may be different from that of the 11 S globulins in hemp proteins.

Emulsifying activities. **Table 3** also shows emulsifying activity index (EAI) and emulsion stability index (ESI) of unheated and heated BPI and its hydrolysates, as determined at pH 7.0. The EAI (32.2 m^2 g^{-1}) of raw BPI was lower than that (about 50 m^2 g^{-1}) reported in our previous paper (Tang 2007). The difference in pH of the applied buffer clearly accounts for this difference, since the EAI at high pH value (e.g. pH 7.5 in this previous work) is usually higher than that at lower pH value (e.g. pH 7.0). The additional freeze-drying treatment (control) did not affect the EAI of raw BPI. Relative to raw BPI or the control, the unheated hydrolysates exhibited significantly increased EAI (**Table 3**). The increase in EAI is consistent with the increase in PS, however, the relationship between the EAI and H_o is clearly against the general view that there is a positive relationship between EAI and H_o. The data suggest that in the present case, the EAI of BPI was much more contributed by PS, than by H_o.

Various hydrolysates (including the control) obtained

Table 2 Total SH (SS and free SH) and exposed free SH contents, as well as surface hydrophobicity (H_o) of unheated and heated BPI and its hydrolysates (μmol SH g^{-1} sample).

Samples	Unheated			Heated		
	Total SH	Exposed free SH	H_o	Total SH	Exposed free SH	H_o
Raw BPI	27.3 ± 1.2a	7.8 ± 0.3a	687.8 ± 13.0 a			
Control	22.9 ± 0.6b,E	7.0 ± 0.6 a,E	696.2 ± 12.8 a,F	21.1 ± 0.4 a,F	6.8 ± 0.4 a,E	804.3 ± 10.0 a,E
DH-4.5	22.3 ± 1.1b,E	3.2 ± 0.2 b,F	276.2 ± 10.2 b,F	20.1 ± 1.0 a,F	5.1 ± 0.6 b,E	590.4 ± 14.3 b,E
DH-8.3	15.8 ± 0.4c,E	3.0 ± 0.2 b,F	254.1 ± 12.4 b,F	16.4 ± 0.8 b,E	3.7 ± 0.1 c,E	560.6 ± 6.2 c,E
DH-12.8	14.2 ± 0.2c,E	2.4 ± 0.1 c,F	202.07 ± 8.3 c,F	14.9 ± 0.6 b,E	3.0 ± 0.1 d,E	494.0 ± 10.2 d,E

Different letters (a-d) indicate significant difference at $p < 0.05$ level in the same column. Different letters (E-F) indicate significant difference at $p < 0.05$ level between unheated and heated samples.

Table 3 Protein solubility (at pH 7.0), emulsifying (EAI and ESI) and foaming (FC and FS) activities of unheated and heated BPI and its hydrolysates.

Samples	Unheated					Heated				
	PS (%)	EAI (m^2 g^{-1})	ESI (min)	FC (%)	FS (%)	PS (%)	EAI (m^2 g^{-1})	ESI (min)	FC (%)	FS (%)
Raw BPI	54.4 ± 1.9^{c}	32.2 ± 1.2^{c}	12.4 ± 0.1^{c}	79.2 ± 5.2^{a}	77.0 ± 5.0^{b}					
Control	50.3 ± 2.6^{c,E}	33.7 ± 1.4^{c,E}	12.2 ± 0.2^{c,F}	77.5 ± 0.4^{a,F}	86.9 ± 2.2^{a,E}	53.6 ± 5.7^{c,E}	20.5 ± 1.1^{c,F}	14.4 ± 0.8^{a,E}	117.7 ± 2.0^{a,E}	46.0 ± 0.0^{d,F}
DH-4.5	62.6 ± 4.0^{b,E}	44.2 ± 0.3^{b,E}	17.1 ± 0.4^{b,E}	53.9 ± 1.6^{b,F}	76.2 ± 4.6^{b,E}	63.4 ± 4.0^{b,E}	21.4 ± 2.2^{c,F}	14.3 ± 1.5^{a,F}	89.1 ± 2.2^{b,E}	58.3 ± 0.6^{c,F}
DH-8.3	64.9 ± 1.0^{b,E}	47.2 ± 1.4^{a,E}	18.0 ± 0.1^{a,E}	49.4 ± 1.7^{c,F}	56.3 ± 3.3^{c,F}	67.0 ± 0.9^{b,E}	26.1 ± 0.4^{b,F}	12.9 ± 0.4^{a,F}	55.1 ± 3.2^{c,E}	66.9 ± 2.8^{b,E}
DH-12.8	70.1 ± 0.8^{a,E}	43.8 ± 1.6^{b,E}	18.2 ± 0.4^{a,E}	33.5 ± 2.3^{d,E}	60.7 ± 3.4^{c,F}	70.6 ± 1.1^{a,E}	33.9 ± 2.3^{a,F}	13.0 ± 0.5^{a,F}	35.8 ± 1.5^{d,E}	85.4 ± 1.2^{a,E}

Different letters (a-d) indicate significant difference at $p < 0.05$ level in the same column. Different letters (E-F) indicate significant difference at $p < 0.05$ level between unheated and heated samples.

after heat pretreatment had much less EAI values, than their unheated counterparts (**Table 3**). On the other hand, the PS of heated hydrolysates was similar to that of the untreated ones and the H_o was much higher (**Tables 2, 3**). Thus, it can be ascertained that the PS and H_o are not major factors contributing to the influence of heat treatment on the EAI of the hydrolysates of BPI. Here, the distinct decrease in EAI by the heat treatment may be attributed to more compact structural conformation of the proteins or peptide fragments in the hydrolysates, by means of strengthened hydrophobic interactions. Factually, this result is consistent with the general view that the proteins associated with stronger hydrophobic interactions usually exhibit higher thermal stability (Gorinstein *et al.* 1996).

In the unheated cases, the hydrolysates exhibited significantly higher ESI than in the raw BPI or in the control (**Table 3**). The increase in ESI may be associated with increases in PS and conformational flexibility of the protein. For the control, the heat treatment significantly ($p < 0.05$) increased the ESI, however, the ESI of the hydrolysates was on the contrary significantly decreased (**Table 3**). The increase in ESI is clearly associated with the increases in PS and H_o whereas the decrease in ESI of the hydrolysates may also be attributed to decline in molecular flexibility of the proteins or peptide fragments by the heat treatment. The progressive decrease in ESI of the hydrolysates with increase in DH (from 0 to 12.8%) also corroborates this point of view, since more peptide fragments released during the hydrolysis may contribute to the decrease in molecular flexibility by the heat treatment.

Foaming activities. Good foaming proteins must (i) rapidly adsorb during whipping and bubbling, (ii) have a rapid conformational change, rearranging at the air-water interface with reduction of surface tension and (iii) form a viscoelastic cohesive film through intermolecular interactions (Hettiarachchy and Ziegler 1994). The foaming capacity (FC) and foam stability (FS) of untreated and heated BPI and its hydrolysates are also included in **Table 3**. From **Table 3**, it was found that the additional freeze-drying did not affect the FC of raw BPI, but significantly increased the FS. The combined heat pretreatment and freeze-drying remarkably and significantly increased the FC, with concomitant decrease in FS (**Table 3**). The data confirm the diversity of the factors affecting the foaming activities of the proteins. The heat pretreatment seems to result in increase in adsorption of the proteins to air-water interface, possibly due to the increase in surface hydrophobicity, while the prominent decrease in FS by the pretreatment may be attributed to the decreases in ability of conformational changes and viscoelasticity of formed protein film on the surface of air bubbles.

The FC and FS of the untreated hydrolysates progressively and significantly ($p < 0.05$) decreased with increasing DH from 0 (control) to 12.8% (**Table 3**). Similar results of the hydrolysis on foaming activities have been observed for the trypsin-modified hemp proteins (Yin *et al.* 2008), where the decreases in FC and FS were attributed to decreases in chain length of peptides as a result of enzymatic hydrolysis. The explanation is consistent with the general point of view that the larger the molecular size for a protein, the higher foaming stability (Damodaran 1997). Factually, the decreases in FC and FS are consistent with the progressive decreases in surface hydrophobicity (**Table 2**), suggesting that the surface hydrophobicity is also a major factor affecting the foaming activities of BPI. In the heated hydrolysates, similar decreases in FC with increasing DH were also observed, but the increase in DH (e.g. 4.5-12.8%) on the contrary increased the FS (**Table 3**). The increase in FS with DH for the heated hydrolysates seems to be related with the gradual increase in PS (**Table 3**), but it is not yet confirmed.

CONCLUSIONS

The prospective studies on the flavonoid pathway give us The physicochemical and functional properties of BPI were modified by the limited hydrolysis with trypsin, to a variable extent depending on the DH. The properties of the obtained hydrolysates could be further affected by an additional heat pretreatment prior to freeze-drying. The hydrolysis resulted in distinct changes in total SH content, H_o, PS as well as those surface-related functional properties, including EAI, ESI, FC and FS of BPI. However, these changes were related with the DH and the heat pretreatment. The results confirmed that the limited hydrolysis could be applied to modify the physiochemical and functional properties of buckwheat proteins. However, further investigations should be carried out to optimize these modifications, by selecting the type of proteases and the conditions of the pretreatment.

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