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

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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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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)