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Physicochemical and Conformational Properties of Buckwheat Protein Isolates: Influence of Polyphenol Removal with Cold Organic Solvents from Buckwheat Seed Flours

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The effects of polyphenol removal from common buckwheat seed flours with cold aqueous organic solvents (including 95% ethanol, 70% 2-propanol, and 80% methanol, v/v) on the physicochemical and conformational properties of their protein isolates (BPI) were investigated. The extraction resulted in considerable reduction in its polyphenol content, especially protein-bound polyphenol content, and concomitant increase in its protein content. The efficiency of the removal of the polyphenols was much better in the 2-propanol case than in other two cases. The surface hydrophobicity of the proteins changed slightly, while the disulfide bond contents remarkably increased, partially at the expense of free sulfhydryl group contents. The protein solubility in the pH range of 7.0−11.0 and the proportion of undenatured globulins in BPI products were variably improved by the organic solvent extraction, and the extent of the improvements was highest in the 2-propanol case. Intrinsic emission fluorescence and far-UV and/or near-UV CD spectra showed that polyphenol removal resulted in significant changes in tertiary and/or secondary conformations of the proteins in BPI, and the changes were also related to the efficiency of the removal of the polyphenols. These results suggest that the physicochemical and conformational properties of BPI are closely related to its polyphenol level, and there is also a close relationship between its physicochemical properties and tertiary and/or secondary conformations.

KEYWORDS: Buckwheat protein isolate; physicochemical property; protein conformation; polyphenol; organic solvent extraction

INTRODUCTION

The proteins in buckwheat seeds, including common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum*) have attracted much interest worldwide because of their well-balanced amino acid composition (*1*) and a number of potential health effects, e.g., hydrocholesterolemic activity, suppression in body fat, constipation, mammary carcinogenesis and colon carcinogenesis, and in the formation of cholesterol gallstones as well as inhibition of mammary cancer cells in vitro (*2*−*9*). In the seeds, the protein content ranges from 8.51% to 18.87% depending on variety (*10*). The major storage proteins from common buckwheat seeds, including 8 and 13S globulins, and 2S albumins have been characterized (*11*−*14*).

Some functional properties of buckwheat protein products, especially buckwheat protein isolate (BPI), obtained with alkali solublization and acid precipitation technique have been recently investigated (*15*−*19*). However, the knowledge about the functional properties and protein solubility and surface-related properties in particular is inconsistent on the basis of different works. For example, Zheng et al. (*16*) indicated that the buckwheat protein concentrate spray-dried showed a higher nitrogen solubility index at neutral pH and higher water holding, emulsifying, and foaming capacities compared to those of a freeze-dried protein sample. Whereas in another study, the buckwheat protein concentrate, prepared by drying at 70 °C and then treating with petroleum ether, showed poor foaming capacity (*15*). Additionally, poor water holding capacity and emulsifying stability of spray-dried buckwheat protein products were reported recently (*17*). The differences may be mainly due to a lack of knowledge about the chemical compositions of buckwheat protein products and the physicochemical and conformational properties of their individual protein fractions. In our previous work, we had pointed out that the buckwheat proteins preferably interact with the lipids and that, thus, buckwheat protein products with different lipid contents (2.3−17.9%) might be obtained by selecting different processing, though the lipid content (about 3.0%) in the seed flour was low (*18*). The diversity in the lipid contents also resulted in changes in the thermal transition of buckwheat globulins, especially the cooperativity of the transition from native to the denatured state (*19*).

Besides the lipids, the buckwheat proteins also might interact with the polyphenols (e.g., flavonoids and rutin in particular) during the processing of the related protein isolate. In our

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previous work, it was found that BPI, obtained with undefatted common buckwheat flour with polyphenol content of about 0.28 g rutin equivalent/100 g, had a polyphenol content of 3.3 g rutin equivalent/100 g, of which most of the polyphenols were in the protein-bound form (*20*). This observation clearly confirms the formation of protein−polyphenol complexes during the preparation of BPI. Factually, the interactions of the polyphenols or other phenolic compounds (e.g., chlorogenic and caffeic acids) with food proteins, such as soy proteins and whey proteins, have been well characterized, and the covalent interactions that are favored at alkali conditions would result in changes in their physicochemical and structural properties (*21*−*23*). Thus, it can be expected that the physicochemical and structural properties of BPI would be to a great extent related to its polyphenol levels.

The polyphenol level in the protein isolate can be modulated using the extraction treatment of the flour with various organic solvents. To date, several works in literature have been available, addressing the influence of the removal of the polyphenols from sunflower flour with organic solvents on the physicochemical and/or conformational properties of the proteins in the flours or its protein products (*24*−*28*). The extraction treatment of sunflower flour with organic solvents (e.g., 40% aqueous acetone, 40% aqueous ethanol, and 40% aqueous methanol) resulted in a decrease in nitrogen solubility of the flours in the pH range 2−8 (*24*). The decrease in nitrogen solubility may be due to organic solvent-induced protein denaturation and/or aggregation, especially for low molecular weight sunflower albumins. However, the changes in protein−polyphenol interactions also partially account for the influence of the removal of the polyphenols on the physicochemical and/or structural properties of the proteins. Thus, it is difficult to ascertain which influence would play a predominant role. In a more recent work, González-Pérez et al. (*28*) proposed a new process for obtaining nondenatured and free phenol sunflower protein, where the extraction with cold aqueous organic solvents (95% ethanol, 70% 2-propanol, and 80% methanol, v/v) was applied. This work facilitates the investigation of the influence of the removal of the polyphenols from the sunflower flour on the physicochemical and structural properties of sunflower protein products, by neglecting the influence of organic solvent-induced protein denaturation and/or aggregation. Nevertheless, the related works are still less investigated.

On the basis of the above-mentioned, the main objective of this work was undertaken to investigate the influence of the removal of the polyphenols from buckwheat flour with the same aqueous organic solvents as those applied by González-Pérez et al. (*28*) on the physiochemical and conformational properties of BPI. The dephenolization efficiency with various organic solvents was also evaluated.

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 (China). Prior to analysis, the seeds were milled and ground to pass through a 60 mesh screen to produce the whole flour and kept in refrigerator at 4 °C until used. Rutin and 1,8-anilinonaphthalenesulfonate (ANS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Low molecular weight protein markers were purchased from Shanghai DINGUO Biotech. Co., Ltd. (China). All other chemicals were of analytical reagent or better grade.

Preparation of Various BPI Products. Buckwheat protein isolate (BPI; control) was prepared from the buckwheat seed flour according to the process described in a previous paper (*18*) with slight modifications. Briefly, the flour (unextracted) was fully dispersed in 10-fold volume of deionized 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 7000*g* for 20 min, and the resultant supernatant was adjusted to pH 4.5 using 1.0 N HCl to precipitate the proteins. The precipitate was obtained by centrifugation at 4000*g* for 15 min and redispersed in deionized water. Finally, the dispersion was adjusted to pH 7.0 and freeze-dried to obtain the control BPI.

BPI products with low polyphenol contents were prepared from organic solvent-extracted buckwheat seed flours, according to the same above-mentioned process. The organic solvent-treated flours were prepared according to the method of Gonzalez-Pérez et al. (*28*) with slight modifications. The whole flour was extracted with cold (4 °C) mixtures of organic solvents and water, namely, 95% (v/v) ethanol, 70% (v/v) 2-propanol, and 80% (v/v) methanol, at a flour-to-solvent ratio of 1:10 (w/v), under mechanical stirring condition for 4 h. After the suspensions were centrifuged at 8000*g* for 20 min at 4 °C, the extraction was repeated until the supernatant no longer developed a yellow color upon the addition of 0.5 N NaOH. Finally, the dephenolized buckwheat flours were dried at room temperature overnight and then applied to prepare the BPI products with low polyphenol contents. These BPI products obtained from ethanol, methanol, and 2-propanol-extracted flours were further denoted as BPI-E, BPI-M, and BPI-P, respectively.

Quantification of Total, Free, and Protein-Bound Polyphenols. The contents of total, free, and protein-bound polyphenols were determined according to the method described by Carbonaro et al. (*29*), 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 the 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 N NaOH. The latter was estimated using a bovine serum albumin (BSA) 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 g of the sample.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed on a discontinuous buffered system according to the method of Laemmli (*30*) 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% (w/v) SDS, 2% (v/v) 2-mercaptoethanol (2-ME), 5% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, and heated for 5 min in boiling water before electrophoresis. For each sample, 10 μL was applied to each lane. After the electrophoresis, the gel was stained in 0.25% Coomassie blue (R-250) in 50% TCA and then destained in 7% 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) followed 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 buffered saline (PBS; pH 7.2) containing 50 mM NaCl was centrifuged at 10,000*g* for 10 min, and then the obtained supernatant was filtered with a Millipore membrane (0.2 μm). The following chromatographic conditions were applied: (1) injection volume, 20 μL; (2) eluting rate, 0.8 mL/min; (3) elution solvent, 50 mM PBS (pH 7.2) containing 50 mM NaCl. The absorbance was recorded at 280 nm. All data were collected and analyzed by Breeze software (Waters, Division of Millipore, Milford, MA, USA).

Surface Aromatic Hydrophobicity (H_o). H_o was determined with the fluorescence probe ANS according to the method of Haskard and Li-Chan (*31*). Serial dilutions in 0.01 M PBS (pH 7.0) were prepared with the protein isolates (stock solutions; 1.5%, w/v) to a final concentration of 0.004−0.02% (w/v). ANS solution (8.0 mM) was also prepared in the same phosphate buffer. Twenty microliters of ANS solution was added to 4 mL of each dilution, and the fluorescence intensity (FI) of the mixture was measured at 390 nm (excitation) and 470 nm (emission) using an 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.

Determination of the Free Sulfhydryl Group (SH) and Disulfide Bond (SS) Contents. The SH and SS contents of samples were determined by the method of Beveridge et al. (*32*). Protein samples (75 mg) were dissolved in 10 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, and 0.04 M EDTA, pH 8.0) containing 8 M urea. The solution was gently stirred overnight until a homogeneous dispersion was achieved. For SH content determination, 4 mL of the Tris-Gly buffer was added to 1 mL of protein solution. Then, 0.05 mL of Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/mL) was added, and absorbance was measured at 412 nm after 5 min. For total SH content [SH + reduced SS] analysis, 0.05 mL of β-ME and 4 mL of Tris-Gly buffer were added to 1 mL of the protein solution. The mixture was incubated for 1 h at room temperature. After an additional hour of incubation with 10 mL of 12% TCA, the mixtures were centrifuged at 5000*g* for 10 min. The precipitate was twice resuspended in 5 mL of 12% TCA and centrifuged to remove β-ME. The precipitate was dissolved in 10 mL of Tris-Gly buffer. Then 0.04 mL of Ellman's reagent was added to 4 mL of this protein solution, and the absorbance was measured at 412 nm after 5 min. The calculation was as follows: μM SH/g = $73.53 \times A_{412} \times D/C$; where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), D is the dilution factor, 5 and 10 are used for SH and total SH (SH + reduced SS) content analysis, respectively, and 73.53 is derived from 10^6 / (1.36×10^4); 1.36×10^4 is the molar absorptivity (*33*), and 10^6 is for the conversion from molar basis to μM/mL basis and from mg solids to g solids. Half of the value after subtracting the SH value from the total SH value was defined as the SS content.

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 desired values. After 30 min of stirring, the pH was readjusted if necessary. Then, it was centrifuged at 8000*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 (*34*) using BSA as the standard. The PS was expressed as grams of soluble protein/100 g of sample. All determinations were conducted three times.

Differential Scanning Calorimetry (DSC). The thermal transition of the protein in various BPI products was examined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, Delaware 19720 USA), according to the procedure of Meng and Ma (*35*) with some modifications. Approximately 2.0 mg of the samples was weighed into aluminum liquid pans (Dupont), and 10 μL of 0.05 M PBS (pH 7.0) was added. The pans were hermetically sealed and heated from 20 to 110 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. Onset temperature (T_m), peak transition or denaturation temperature (T_d), enthalpy change of the endotherm (ΔH), and cooperativity, represented by the width at half-peak height ($\Delta T_{1/2}$), were computed from the thermograms by the Universal Analysis 2000, Version 4.1D (TA Instruments-Waters LLC). All experiments were conducted in triplicate. The sealed pans containing protein isolate samples and buffers were equilibrated at 25 °C for more than 6 h.

Intrinsic Fluorescence Spectroscopy. Intrinsic emission fluorescence spectra of various BPI products were determined in an RF-5301 PC fluorophotometer (Shimadzu Corp., Kyoto, Japan). Protein dispersions (0.15 mg/mL) were prepared in 10 mM PBS (pH 7.0). Protein solutions were excited at 295 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission.

Far-UV and Near-UV Circular Dichroism (CD) Spectroscopy. Far-UV and near-UV CD spectra were obtained using an MOS-450 spectropolarimeter (BioLogic Science Instrument, France). The far-UV CD spectroscopy measurements were performed in a quartz cuvette of 2 mm with a protein concentration around 0.1 mg/mL in 10 mM PBS (pH 7.0). The sample was scanned from 190 to 250 nm. The near-UV CD spectroscopy measurements were performed in a 1 cm quartz cuvette with a protein concentration around 1.0 mg/mL. The sample was scanned over a wavelength range from 250 to 320 nm. For both measurements, the spectra were an average of eight scans. The following parameters were used: step resolution, 1 nm; acquisition duration, 1 s; bandwidth, 0.5 nm; sensitivity, 100 mdeg. The cell was thermostatted with a Peltier element at 25 °C, unless specified otherwise. The concentration of the proteins was determined by the Lowry method (*34*) using BSA as the standard. Recorded spectra were corrected by subtraction of the spectrum of a protein-free buffer. A mean value of 112 for the amino acid residue was assumed in all calculations and CD measurements expressed as the mean residue ellipticity (θ) in deg·cm^{-2}·dmol^{-1}. The secondary structure compositions of the samples were estimated from the far-UV CD spectra using the CONTIN/LL program in CDPro software, using 43 kinds of soluble proteins as the reference set (*36*). Each data point was the mean of duplicate measurements.

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

RESULTS AND DISCUSSION

Yields and Protein and Polyphenol Content. In the untreated buckwheat seed flour, the protein content was about 15.1% (dry basis). The protein content was slightly increased to 18.1% after treatment with ethanol or 2-propanol extraction, while the methanol treatment only slightly increased the protein content of the flour (**Table 1**). Correspondingly, the solvent treatment resulted in loss of proteins and nonprotein components (e.g., polyphenols and sugars), but to a varying extent depending on the type of applied solvents. Among tested solvents, 2-propanol seems to be the most appropriate solvent to remove nonprotein components (about 14.1%) and retain the proteins (about 90%).

From the untreated and dephenolized flours, various protein isolates (denoted as BPI-C, BPI-M, BPI-E, and BPI-P for untreated, methanol, ethanol, and 2-propanol-extracted flours, respectively) were prepared using the conventional alkali solubilization and acid precipitation technique. The protein contents of BPI-M, BPI-E, and BPI-P were similar (95.1−97.6%, dry basis), much higher than that (88.4%) of BPI-C (control) (**Table 1**). The difference is clearly due to the removal of the lipid fraction from

Table 1. Yields (Solid and Protein) and Protein and Polyphenol Contents of Different Buckwheat Seed Flours and Protein Isolates[a]

		yields (%)		polyphenol contents (%)[c]		
samples[b]	protein content (%, dry basis)	solid	protein	total	free	protein-bound
BF (control)	15.1 (0.8)	100	100.0 (0.6)	0.60 (0.02)	0.34 (0.03)	0.26 (0.03)
BF-M	16.5 (0.6)	94.9	90.1 (0.8)	0.13 (0.04)	0.04 (0.03)	0.09 (0.01)
BF-E	18.1 (0.7)	81.8	85.4 (0.8)	0.34 (0.06)	0.16 (0.04)	0.19 (0.04)
BF-P	18.1 (0.6)	85.9	89.7 (1.2)	0.12 (0.03)	0.04 (0.02)	0.08 (0.02)
BPI-C (control)	88.4 (0.6)	4.3	21.9 (0.2)	2.93 (0.10)	0.42 (0.09)	2.51 (0.08)
BPI-M	97.6 (0.2)	3.8	21.4 (0.3)	0.40 (0.08)	0.15 (0.04)	0.25 (0.05)
BPI-E	95.1 (0.1)	4.0	21.9 (0.7)	0.69 (0.04)	0.32 (0.02)	0.37 (0.03)
BPI-P	96.5 (0.1)	3.7	20.6 (0.9)	0.24 (0.02)	0.08 (0.03)	0.16 (0.03)

[a] Each data (on a dry basis) is the mean (and standard deviation) of duplicate determinations. [b] BF, buckwheat seed flour; BF-M, methanol-extracted BF; BF-E, ethanol-extracted BF; BF-P, 2-propanol-extracted BF; BPI-C, BPI-M, BPI-E, and BPI-P, buckwheat protein isolates obtained from BF, BF-M, BF-E, and BF-P, respectively. [c] The polyphenol contents were expressed in grams of rutin equivalent per 100 g of the sample (dry solid).

the flours, after organic solvent extraction. However, the protein yields (21.0−22.0%; relative to total protein) were similar for all of the protein isolates.

In the untreated flour, total polyphenol content was about 0.60% rutin equivalent (dry basis). The data is much higher than that (0.28%) of our previous work (*20*) and that (0.37−0.40%; total flavonoid content) reported by Oomah and Mazza (*37*). The difference may be attributed to the differences in variety and/or cultivation conditions, as well as choice of the standard polyphenol compound. In this flour, about 57% of total polyphenols were in the protein-bound form (**Table 1**). However, in our previous work, it was shown that almost all of the polyphenols were in the protein-bound form (*20*). The underlying mechanism for this difference is still unknown. As expected, the dephenolization treatment resulted in remarkable reductions in total polyphenol content and free and protein-bound polyphenol content (**Table 1**). The efficiency (about 78−80%) of polyphenol removal with aqueous methanol and 2-propanol was similar but much better than that (about 43%) with aqueous ethanol. A similar phenomenon about the efficiency difference has been observed in sunflower flour by González-Pérez et al. (*28*). In comparison with the data of protein yield and protein-bound polyphenol contents, it can be crudely estimated that about 81−82% of protein-bound polyphenols were contained in about 5% of total proteins. This is an indirect indication that the polyphenols could preferentially associate with some specific proteins to form protein−polyphenol complexes, which can be to a great extent removed by extraction with aqueous organic solvents.

Relative to the flours, the total polyphenol content of individually obtained protein isolates to a varying extent increased by 1−2-fold (**Table 1**). This is consistent with our previous view point that there is high affinity between buckwheat proteins and polyphenols (*20*). The total polyphenol content (2.93%) of BPI-C was considerably higher than that (0.24−0.69%) of dephenolized BPI products. For BPI-C, the increase in total polyphenol content was mainly contributed by protein-bound polyphenols (**Table 1**). Compared to the extent of decrease (77−92%) in total polyphenol content, the extent of decrease (85−94%) in protein-bound polyphenol content for any dephenolized BPI was similar. In contrast, the extent of decrease in free polyphenol content was much less (24−81%). The data also indicated that the influence of the treatment with organic solvents on the polyphenol content of the protein isolates was mainly due to the reduction in protein-bound polyphenols.

Physicochemical Characterization. *SDS−PAGE and SEC Analyses*. The protein compositions of various BPI products were evaluated using SDS−PAGE and SEC techniques, as shown in **Figure 1**. In preliminary experiments, we also evaluated the influence of the dephenolization treatment on the protein compositions of the buckwheat flour using reducing and nonreducing SDS−PAGE, and found that no significant changes were observed among the various flours (data not shown). In the SDS−PAGE profiles in the presence of reducing agent 2-ME, there were no observed changes of the electrophoretic bands for the polypeptides of 8 and 13S globulins and 2S albumins, among different protein isolates (**Figure 2A**, lanes 5−8). Under the nonreducing conditions, the major bands corresponding to the polypeptides of 8S globulins and 2S albumins as well as the combined acidic and basic subunits of 13S globulins were also unchanged by the dephenolization treatment (**Figure 2A**, lanes 1−4). However, it can be observed that in dephenolized BPI products, some aggregates with molecular weight (MW) of above 97 kDa were more distinct than the control. The formation of these aggregates is mainly maintained by disulfide bonds since they could be disrupted by 2-ME. This may be attributed to

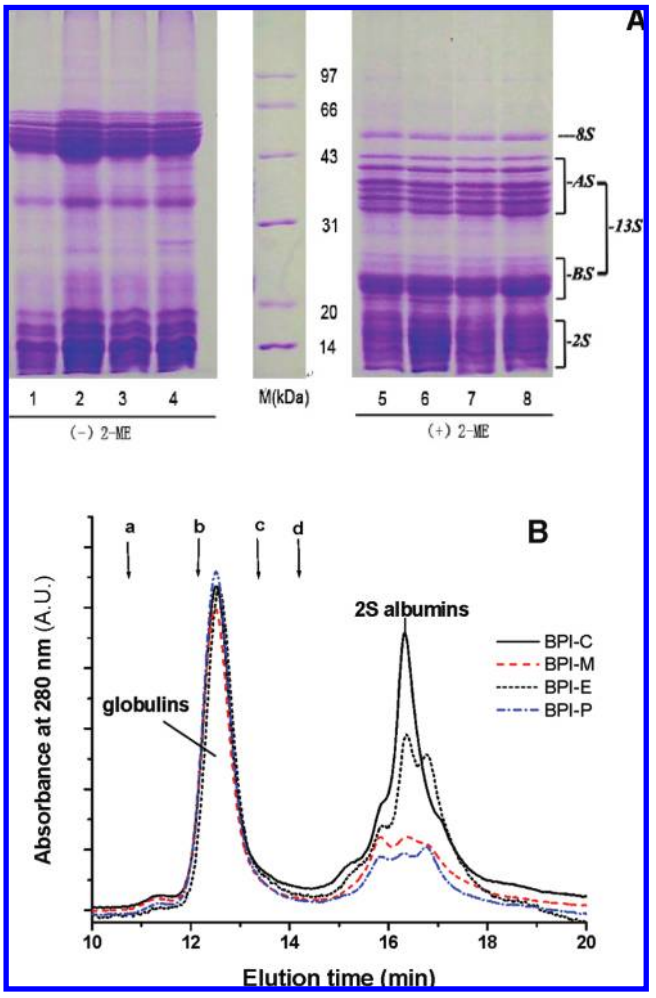

Figure 1. SDS−PAGE (**A**) and SEC (**B**) profiles of various BPI products. Lanes 1−4 and 5−8 correspond to BPI-C (control), BPI-M, BPI-E, and BPI-P, under nonreducing conditions and reducing conditions, respectively. The arrows (a−d) indicate the protein markers of 669, 440, 158, and 75 kDa, respectively. For legends, refer to **Table 1**.

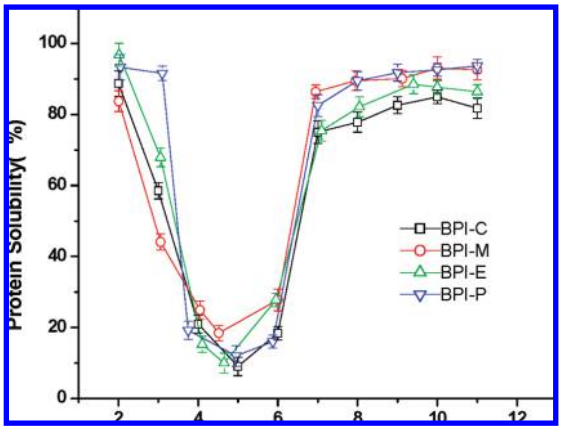

Figure 2. PS profiles of various BPI products, as a function of pH. Each data was the mean and standard deviation of three determinations. For legends, refer to **Table 1**.

organic solvent-induced changes in protein conformations and subsequent protein aggregation.

Figure 1B shows the SEC elution profiles of various BPI products, with UV detection at 280 nm. In all of the SEC profiles, there were two major peaks, centered at elution times of about

Table 2. Surface Aromatic Hydrophobicity (H_0), free SH and SS Contents, and DSC Characteristics of Various BPI Products[a]

		free SH and SS contents (μM/g protein)			DSC characteristics		
protein samples	H_o	SH	SS	T_0 (°C)	T_d (°C)	ΔH (J/g protein)	$\Delta T_{1/2}$ (°C)
BPI-C	544.8 (6.7) b	14.7 (0.6) a	58.5 (1.5) d	96.4 (0.3) a	102.6	2.3	7.4
BPI-M	522.8 (4.5) c	4.5 (0.3) b	79.5 (2.9) a	95.0 (0.2) b	102.8	5.2	8.8
BPI-E	564.3 (7.9) a	2.9 (0.2) d	70.2 (2.4) b	95.3 (0.2) b	102.9	4.8	8.3
BPI-P	540.3 (5.6) b	3.8 (0.3) c	62.9 (1.9) c	94.4 (0.1) c	102.0	8.4	9.7

[a] Each data is the mean and standard deviation of two determinations. Different letters (a−d) indicate the significant difference at $p < 0.05$ level in the same column. For the legends (BPI-C, BPI-M, BPI-E, and BPI-P), refer to **Table 1**. T_o and T_d, on-set temperature and the denaturation temperature of the major endotherm, respectively; ΔH, combined enthalpy change of the endotherms; $\Delta T_{1/2}$, the width at half-peak height of the major endotherm.

12.5 and 16.5 min, respectively. The MW of the former peak was appropriately estimated to be about 320−330 kDa, indicating that this is mainly attributed to the hexameric form of the 13S globulin. The MW of the peak in the elution time range 15.5−17.5 min was clearly attributed to the 2S albumin fraction. This is further confirmed by the fact that the elution pattern of these protein fractions was well in accordance with that of the isolated globulin and albumin fractions from buckwheat seeds (data not shown).

The integrated areas of the globulin (< 14 min) and albumin (> 14 min) fractions for various BPI products were estimated, and it was observed that the relative proportion of the albumin to the globulin fraction was distinctly decreased when the dephenolization treatment was applied, but the extent of decrease depended on the type of the solvents (**Figure 1B**). The decrease in relative albumin-to-globulin proportion by the dephenolization treatment is well consistent with the data of total (or protein-bound) polyphenol contents (**Table 1**). This observation may suggest that the 2S albumin fraction was the major protein that preferentially interacted with the polyphenols and that the removal of polyphenols with the dephenolization treatment was mainly from those of 2S albumin−polyphenol complexes. A similar SEC pattern has been observed for the proteins (11S globulins and 2S albumins) of unextracted and methanol-extracted sunflower flours, where the methanol extraction led to a reduction in the proportion of the 2S albumin fraction (*24*).

Surface Hydrophobicity (H_o). The H_0 values of various BPI products, as determined using ANS emission fluorescence spectra at varying protein concentrations, are shown in **Table 2**. The H_0 of BPI was 544.8, less than that (678) reported in our previous paper (*20*). The difference may be attributed to the difference in the applied pH at which the proteins were extracted (pH 8.0 in the present study versus pH 8.5 in the previous study), subsequently resulting in the difference in extent of exposure of hydrophobic clusters to the exterior of the proteins. It has been previously observed that the enzymatic hydrolysis led to remarkable decreases in H_0 of BPI, and at the same time, only the globulin fraction was degraded, while the albumin fraction was nearly unaffected by the hydrolysis (*20*). Thus, it can be suggested that the H_0 of BPI was mainly due to the contribution of hydrophobic clusters at the surface of the globulins.

The removal of the polyphenols from the flours resulted in a diversity in the H_0 values of the obtained BPI products (**Table 2**). The treatment with aqueous methanol slightly but significantly decreased the H_0, while the H_0 was increased by the treatment with aqueous ethanol. However, the relative difference between various BPI products was slight (< ±5%). The data suggest that the tertiary conformation of the globulin fraction in BPI was nearly unaffected by the dephenolization treatment with cold organic solvents.

SH and SS Contents. The SH and SS content data of various BPI products are also included in **Table 2**. BPI-C has a SH content of 14.7 μM/g protein and a SS content of 58.5 μM/g protein. This suggests that most cysteine residues exist as disulfide linkages rather than as free SH groups. The data are much higher than that (3.2 and 36.4 μM/g protein for SH and SS contents, respectively) of the globulins extracted from buckwheat flour with 0.5 M NaCl buffer (*38*).

The dephenolization treatment with aqueous organic solvents resulted in significant decreases in SH content and concomitant increases in SS content, though the extent of decrease or increased varied with the type of applied organic solvents (**Table 2**). The data suggest that the removal of the polyphenols would be favorable for the formation of SS bonds, at the expense of free SH groups. Factually, in a number of polyphenol−protein models, it has been well confirmed that the reactive nucleophilic sites of the proteins, including the side chains of cysteine, can react with the electrophilic *o*-quinone (oxidative products of the polyphenols) and thus cause a significant decrease in their contents (*21*, *22*). Thus, high protein-bound polyphenols in BPI-C (**Table 1**), as the result of covalent interactions between the proteins and the polyphenols, might account for the much lower SS content in BPI-C relative to that in other BPI products. However, it should also be pointed out that besides the protein−polyphenol interaction, organic solvent-induced protein conformational changes may also affect the formation of disulfide bond formation in the protein isolates.

Protein Solubility-pH profiles. In **Figure 2**, the PS profiles of various BPI products are given as a function of pH in water. Minimum PS (10−28%) was observed between pH 3.8 and pH 6.0 and maximum PS (75−98%) at below pH 3.0 and above pH 7.0. The data agree with our reported values, especially those of freeze-dried BPI products (*18*). The PS at around the isoelectric point is much lower, but that at below 3.0 and above pH 7.0 is significantly higher than those reported by Tomotake et al. (*17*). The difference may be due to the differences in the protein content (or content of nonprotein components), methods of preparation, and PS determination.

The pretreatment of the flours with aqueous organic solvents to a varying extent increased the PS of BPI at pH values above 7.0 (**Figure 2**). In the range pH 7.0−11.0, the PS of BPI-E was comparable to or even higher than that of BPI-C (control), while that of BPI-M and BPI-P was significantly higher. Interestingly, the PS data in this pH range are closely in accordance with the data of total polyphenol content (**Table 1**), suggesting that the removal of the polyphenols from the flours increased the PS of the obtained BPI products, at neutral and alkali pH. At high pH values, the PS of a protein is usually related to the nature of net negative charge on the protein. However, it is generally recognized that protein−polyphenol interactions, especially covalent and hydrogen bonds, would be favored at high pH values (*22*). Thus, the improvement of PS (at high pH values) by the removal of the polyphenols could be considered to be partially due to the masking of the charged amino acid residues by the association between the protein and the polyphenols. This explanation seems to be also suitable for the influence of the pretreatment with the

solvents on the pH profiles in a highly acidic pH range except BPI-M (**Figure 2**), though in this case the net positive charge contributes to protein solubility.

In contrast, the PS profiles at around the isoelectric point (pH 3.8−6.0) of various BPI products were nearly superimposable, except for the BPI obtained from methanol-extracted flour, where the PS at pH 4.0−4.5 was slightly higher than that of other BPI products (**Figure 2**).

Conformational Characterization. *DSC Thermograms.* DSC has been widely employed to monitor thermally induced denaturation of proteins, which is related to its conformational changes of proteins during heat treatments (*19*, *39*). In the DSC profiles of all tested BPI products, there was a prominent endothermic peak with an on-set temperature of denaturation (T_o) and a denaturation temperature (T_d) of about 94.5−96.5 and 102 °C, respectively. The T_o and T_d values of the prominent endotherm are consistent with that of buckwheat 13S globulins as reported in our previous work (*19*).

The T_d of the 13S globulins, indicative of the thermal stability of the protein, was nearly unaffected by the removal of the polyphenols with cold organic solvents. This observation is consistent with the fact that the polyphenols in the protein isolates would be preferably present in the 2S albumins, as evidenced by the analyses of the polyphenol content and SEC (**Table 1** and **Figure 1B**). In our previous work, it was also observed that the T_d of the 13S globulins was unaffected by the presence of lipids, though there was strong affinity between buckwheat proteins and lipids (*19*). However, the combined enthalpy change (ΔH) of the endotherms (8 and 13S globulins) was considerably and significantly increased by the removal of polyphenols (**Table 2**), indicating that the proportion of undenatured globulins in a sample or the extent of ordered structure (*40*) was remarkably increased. The remarkable decrease in ΔH for BPI-C relative to that of other BPI products seems to be associated with high free polyphenol content in unextracted flour (**Table 1**) since free polyphenolic substances may be readily oxidized in alkaline solutions (e.g., at pH 8.0 for the present alkali solubilization) to the respective quinones, which in turn can react with nucleophiles such as protein-bound lysine and tryptophan and cysteine residues (*21*, *23*). From this point of view, the ΔH data are consistent with the polyphenol data of various flour and BPI products (including free and protein-bound polyphenols) (**Tables 1** and **2**).

However, the influence of organic solvent-induced denaturation of the proteins on its structure should be taken into consideration. The much higher ΔH (8.4 J/g protein) for BPI-P relative to that (4.8−5.2 J/g protein) for BPI-M and BPI-E (**Table 2**) suggests that the solvent 2-propanol was a more appropriate solvent to produce less denatured buckwheat proteins, at least for the 13S globulins. Additionally, the dephenolization treatment resulted in increased width at half peak height of the endotherm ($\Delta T_{1/2}$) (**Table 2**), indicating less cooperativity of the transition from native to the denatured state.

Intrinsic Emission Fluorescence Spectra. The intrinsic emission fluorescence spectra of various BPI products are given in **Figure 3A**. All of the BPI samples exhibited a fluorescence emission spectrum with a maximum at about 333−334 nm, which is a characteristic fluorescence profile of tryptophan residues in a relatively hydrophobic environment, such as the interior of the globulins (*41*). In our preliminary experiments, we also determined the intrinsic fluorescence spectra of fractionated buckwheat globulin and 2S albumin fractions and found that the fluorescence quantum yield of the globulin fraction was much higher than that of the albumin fraction and that the fluorescence emission maximum of these two fractions was at about 332 and

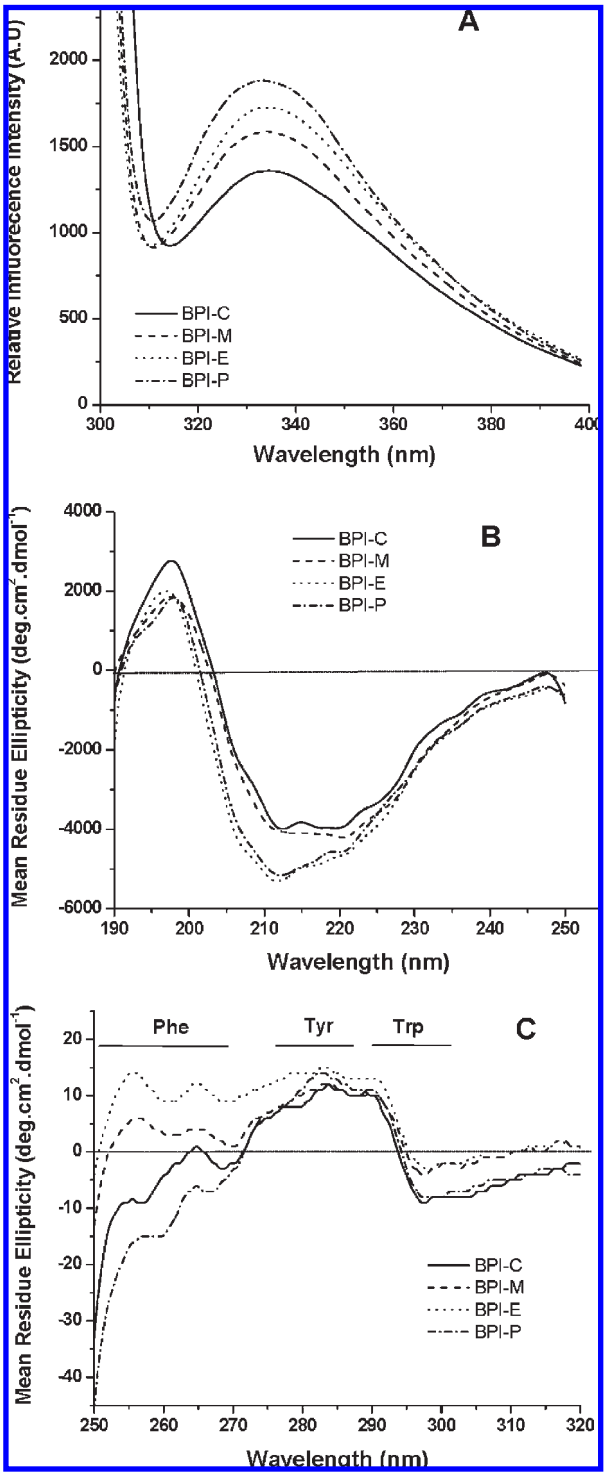

Figure 3. Intrinsic fluorescence (**A**) and far-UV (**B**) and near-UV (**C**) CD spectra of various BPI products, solubilized in 10 mM phosphate buffer (pH 7.0). For legends, refer to **Table 1**.

340 nm, respectively (unpublished data). The data is consistent with the H_o data (**Table 2**), further confirming that the fluorescence spectrum of BPI, especially at around 333−334 nm, was mainly contributed by the globulin fraction.

The fluorescence emission maximum of BPI was nearly unchanged by the dephenolization treatment (**Figure 3A**), suggesting that the environment of the main chromophores, especially those of the globulins was nearly unaffected (*42*). However, the pretreatment with aqueous organic solvents resulted in significant increases in fluorescence intensity to a varying extent (**Figure 3A**).

This can be greatly attributed to the difference in their polyphenol content since the interaction of the aromatic ring of the polyphenols with aromatic residues, such as tryptophan and tyrosine of the protein, would lead to the quenching of fluorescence intensity (*21*,*43*). A similar phenomenon of fluorescence emission spectra has been observed in proteins from unextracted, acetone-, ethanol- and methanol-extracted sunflower flours (*24*).

The increased fluorescence intensity could not be explained to be solely due to the removal of the polyphenols. For example, BPI-E had a total polyphenol content of 0.69%; yet its fluorescence intensity was expectedly higher than that of BPI-M whose total polyphenol content was 0.40%. This phenomenon reflects that in this case, the influence of organic solvent-induced protein unfolding and/or denaturation should be considered. In contrast, the fluorescence intensity of BPI-P was strongest among all tested BPI products. This is mainly due to the quenching effect of the polyphenols present in higher concentration in other samples. However, it also partially reflects that the extent of organic solvent-induced protein unfolding and/or denaturation in this sample might be the least, as suggested by the DSC data (**Table 2**).

Near-UV and Far-UV CD Spectra. The secondary and tertiary conformation or structure of various BPI products were further evaluated and compared using far- and near-UV CD spectra, respectively. Secondary structure elements such as α-helices and β-sheets have dichroic activity in the wavelength range from 190 to 260 nm (*44*). The far-UV CD spectra of various BPI products are presented in **Figure 3B**. All of the spectra show the dominance of two major negative bands, at 211−212 and 220 nm, a positive one between 195 and 200 nm, and a minor shoulder at 225 nm. These features are sufficient indicators of a highly ordered structure, most probably of the β type (*45*, *46*). In the spectrum of a typical β-pleated sheet, e.g., $(Lys)_n$, the magnitude of the positive band between 195 and 200 nm was even higher than that of the negative one at about 218 nm (*46*). However, in the present study, the magnitude of the positive band was much lower (**Figure 3B**). The lower magnitude of the positive band relative to the negative one may reflect that there was also a considerable amount of unordered form structures in these protein isolates since the spectrum of the unordered form generally shows a strong negative band near 200 nm (*46*).

The dephenolization treatment resulted in similar decreases in the intensity of the positive band centered at about 197 nm and, to a varying extent, concurrent increases in the ellipticity of the negative bands (**Figure 3B**). This phenomenon suggested changes in secondary structure compositions of the proteins, by the dephenolization treatment. The secondary structure compositions (including α-helix, β-strand, turns, and random coil) of various BPI products were calculated according to the CONTIN/LL program in CDPro software (*36*) and the results displayed in **Table 3**. In the BPI-C (control), the secondary structure composition was 6.2% α-helix, 36.8% β-strand, 23.0% turns, and 33.9% random coil, confirming that β-strand and random coil were the major secondary structures. The dephenolization treatment with various organic solvents led to a similar but slight increase in random coil (from 33.9 to 34.7%), at the expense of the β-strand (**Table 3**). Concomitantly, the α-helix content also to a variable extent increased after the dephenolization treatment. The extent of increase in α-helix content (or the extent of decrease in β-strand content) for BPI-E was much higher than that for other BPI products, indicating that the solvent ethanol was a more severe protein perturbant than other applied organic solvents. Venktesh and Prakash (*27*) also observed that the acidic butanol treatment caused increases in negative ellipticity of the low molecular weight proteins from sunflower seed, and the relative content of α-helix was increased from 19 to 27% by the treatment.

Table 3. Secondary Structure Compositions of Various BPI Products As Calculated from Far-UV CD Spectra Using the CONTIN/LL Program in CDPro Software

	secondary structure composition (%)			
protein samples	α-helix[a]	β-strand[b]	turns	random coil
BPI-C	6.2	36.8	23.0	33.9
BPI-M	6.3	36.0	23.0	34.7
BPI-E	7.2	35.5	22.7	34.6
BPI-P	6.4	36.0	22.9	34.7

[a] Combined regular and distorted α-helix. [b] Combined regular and distorted β-strands.

The CD spectra in the region 250−320 nm arise from the aromatic amino acids. Each of the amino acids tends to have a characteristic wavelength profile. Trp shows a peak close to 290 nm with fine structure between 290 and 305 nm; Tyr shows a peak between 275 and 282 nm, with a shoulder at longer wavelengths; and Phe shows weaker but sharper bands with fine structure between 255 and 270 nm (*44*). The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of each type of aromatic amino acid present, their mobility, and the nature of their environment (H-bonding, polar groups, and polarizability) as well as their spatial disposition in the protein (*44*). The near-UV CD spectra of various BPI products are given in **Figure 3C**.

The near-UV CD spectrum of KPI (control) consisted of two main positive dichroic bands between about 273 and 292 nm, clearly attributed by Tyr and Trp amino acid residues. The shape and magnitude of the spectrum of BPI was remarkably affected by the dephenolization treatment, but to a varying extent depending on the type of applied organic solvents (**Figure 3C**). By comparison, the Phe bands were much more easily affected by the treatment than the Tyr or Trp bands. The treatment with methanol and ethanol greatly increased the intensity of the Phe bands, while that with 2-propanol decreased it. The increases may be attributed to the exposure to the solvent of the Phe residues initially buried in the interior of the proteins or removal of the polyphenols, which might interact with the aromatic amino acid residues of the proteins. In the 2-propanol case, the decrease in intensity of the Phe bands should be due to the reduced extent of protein denaturation, as evidenced by much higher ΔH relative to other cases (**Table 2**).

In conclusion, the extraction treatment of buckwheat flours with aqueous cold organic solvents produced their protein isolate products, with high protein contents, much low polyphenol contents, and high proportion of undenatured globulins. The efficiency of the removal of the polyphenols with 2-propanol was much better than that with ethanol and methanol. The dephenolization treatment led to a reduction in the albumin fraction of BPI, which preferably interacts with polyphenols. The protein solubility (at neutral and alkali pH values) and the proportion of undenatured globulins of BPIs were to a variable extent improved by the removal of polyphenols and the extent of the improvements related to the efficiency of polyphenol removal. The dephenolization treatment also resulted in distinct changes in secondary and/or tertiary conformations of the proteins, especially the tertiary conformation. Thus, it can be concluded that the physicochemical and conformational properties of BPI are closely related to its polyphenol content and that there is a close relationship between its physicochemical functions and conformational features.

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