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THERMAL PROPERTIES OF GLOBULIN FROM BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)
Effects of salts and protein perturbants

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The thermal properties of buckwheat (*Fagopyrum esculentum* Moench) proteins with different lipid contents (2.5, 6.5 and 17.8%) were studied by differential scanning calorimetry (DSC) under various medium conditions. From DSC curves, many DSC characteristics including denaturation temperature (T_d), enthalpy change (ΔH) and the width at half peak height ($\Delta T_{1/2}$) of endothermic peaks were obtained and evaluated. The DSC curves of various buckwheat proteins (BWPs) in the 0.05 M phosphate buffer (pH 7.0) showed a major endotherm at about 102°C and a minor endotherm at about 80°C, attributed to thermal transitions of 13S and 8S globulins, respectively. T_d and ΔH of the globulins of BWPs were independent of their lipid contents, while the presence of high lipid content (17.8%) to some extent increased the $\Delta T_{1/2}$. The progressive increase in T_d of 13S globulins with increase in NaCl concentration, suggests a more compact conformation with higher thermal stability. The influence of chaotropic salts on the DSC characteristics of 13S globulins was also independent of their lipid contents.

Thermal analysis of the 13S globulins in the presence of protein perturbants (including urea, sodium dodecyl sulfate, ethylene glycol, dithiothreitol and N-ethylmaleimide) indicated that hydrophobic and hydrogen bondings are the major interactions for stabilizing protein conformation of buckwheat 13S globulins and the SS–SH interchange also attributes to the stabilization of the protein conformation.

Keywords: *buckwheat proteins, denaturation, DSC, Fagopyrum esculentum Moench, globulin*

Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is mostly consumed in the form of flour, as a material for bread, pancakes and other food items, and also known as a valuable source of protein (12–15% w.b.), the amino acid composition of which is nutritionally superior to that of cereals [1]. The buckwheat protein is relatively poorly digested, and even some components have been reported to be major allergens [2], however, it has many physiological activities which are potential for human health, such as hypocholesterolemic activity in rats, suppression in body fat, constipation, mammary carcinogenesis and colon carcinogenesis and in the formation of cholesterol gallstones in hamsters [3–7]. Furthermore, the buckwheat protein, as a vegetable protein, has some superior functional properties, such as higher nitrogen solubility index and higher water holding, emulsifying and foaming capacities (as compared to soy protein isolates), contributing to its application in food formulations and processing [8–10]. A better understanding of biological and physicochemical properties of buckwheat protein, including its thermal properties, can greatly enhance its potential utilization as a food ingredient.

Differential scanning calorimetry (DSC) has been widely used to monitor protein unfolding or denaturation as affected by various environmental factors, or calorimetric changes in proteins as a function of temperature [11]. Thermally induced denaturation or changes of proteins may be caused by disruption of various chemical forces. Usually, endothermic changes are associated with the rupture of hydrogen bonds, while exothermic changes result from the breaking-up or weakening of hydrophobic interactions and aggregation of proteins [12, 13]. The peak transition or denaturation temperature, T_d, is a measure of thermal stability, while the enthalpy change (ΔH), measured as area under the endothermic peak, represents the proportion of undenatured protein in a sample, or extent of ordered structure [12]. The sharpness of the transition peak, measured as width at half peak height ($\Delta T_{1/2}$), is an index of the cooperativity of the transition from native to denatured state [13].

Using the DSC analysis technique, thermal denaturation of some food proteins, such as muscle proteins [14, 15], egg and bovine serum albumin [16, 17], soybean proteins [18], α-lactalbumin and β-lactoglobulin [19], fababean proteins [20], oat globulins [21, 22], red bean globulins [23] and flaxseed proteins [24] has been widely studied. To date, there

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is very limited information about the denaturation or thermal properties of buckwheat proteins (particularly the globulins).

The objective of our investigation was to study the thermal properties of buckwheat proteins using DSC. The DSC characteristics of various buckwheat protein products with different lipid content, produced by various processing, were analyzed and compared to gain some insight into the effect of the lipids. The effects of salts and protein structure perturbants on the DSC curves were also investigated, to elucidate the role of covalent and non-covalent chemical forces in stabilizing protein conformation of buckwheat globulins with different lipid contents.

Experimental

Materials

The buckwheat seeds were purchased in a retail outlet in Guangzhou (China), cultivated in Ganshu Province (China). Molecular mass (*MW*) marker kit (97 to 24 kDa) was from DingGuo Biol. Co. (China). The electrophoresis reagents and ß-mercaptoethanol (2-ME) were purchased from Shanghai BOAO Biochemical Co. (China). All other chemicals were of analytical or better grade.

Preparation of buckwheat proteins (BWPs)

The buckwheat seeds were ground to pass through a 60-mesh screen to produce fine flour in a disintegrator. Various BWPs were prepared from the buckwheat flour by different processing (e.g., usual mechanic and ultrasonic-assisted extraction and/or delipidated treatment). Briefly, five hundreds grams of buckwheat flour were dispersed in 5 L of 0.05 M tris-HCl buffer (pH 8.0), then extracted with usual mechanical stirring at ambient temperature for 4 h, or with ultrasonic-assisted treatment for 30 min (the ultrasonic frequency and power are 15 kHz and 500 W, respectively) (XingDongLi Ultrasonic Electron Equipment Co. Ltd., China). The resulting slurry was separated into the supernatant and residue by a centrifuge (9000 g for 20 min) at 4°C. The pH of the supernatant was adjusted to 4.5 by the addition of 0.1 N HCl to precipitate the protein isoelectrically. The isoelectric precipitate was obtained by centrifuge (9000 g, 20 min). After a washing with an adequate amount of de-salted water, the precipitates were neutralized and dissolved with 0.1 N NaOH. The protein solution was dialyzed against de-ionized water (24 h, three changes, 4°C), and then freeze-dried.

As for the additional delipidated pretreatment, the buckwheat flour was defatted with hexane (1:1 *w*/*v*, three changes). The chemical compositions (including protein, lipid, ash and moisture) of the buckwheat flour and protein products were determined according to AOAC procedures [25].

Methods

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

SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli [26] using 12% separating gel and 4% stacking gel. The samples [the enzyme mixture mixed with sample buffer, 1:1 *v*/*v*] were heated for 5 min in boiling water before electrophoresis. Every sample (8 μL) was applied to each lane. Before the sample entering the separating gel, electrophoresis was performed at 6 mA, and the other was at 10 mA. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid, and de-stained in 7% acetic acid (methanol:acetic acid:water, 227:37:236 *v*/*v*/*v*).

Differential scanning calorimetry (DSC)

The thermal properties of various buckwheat proteins under various medium conditions were examined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, Delaware 19720 USA). The procedure was according to that of Meng and Ma [23], with some modifications. Approximately 2.3~2.5 mg of samples (containing 1.5~2.0 mg protein) were accurately weighed into the aluminium pan suitable for liquid sample analysis, and 10 μL of 0.05 M phosphate buffer (pH 7.0), was added. The pan was hermetically sealed and heated from 20 to 130 or 140°C at a rate of 5°C min^{-1}. A sealed empty pan was used as a reference. Onset temperature (T_m), peak transition or denaturation temperature (T_d), enthalpy of denaturation (ΔH) and cooperativity, represented by the width at half-peak height ($\Delta T_{1/2}$), were computed from the curves by the Universal Analysis 2000, Version 4.1D (TA Instruments-Waters LLC). All experiments were conducted in triplicate and the coefficient of variation ranges from 0.3 to 0.6% for T_m and T_d, and 5–10% for ΔH. Some of the thermograms were comprised of large and small over-lapping peaks, and only the ΔH of the combined transitions were measured, due to difficulties in accurately estimating the partial areas of the overlapping transitions. For experiments involving additives (e.g., salts and protein structure perturbants), buffers containing the additives were added to the pans, which were then sealed and equilibrated at 25°C.

Statistical analysis

The data was analyzed by one-way ANOVA analysis of variance followed by the inspection of the difference ($P \leq 0.05$, or $P \leq 0.1$) of different means by Duncan's multiple-range test.

Results and discussion

Characterization of BWPs

The chemical composition of the buckwheat flour used in the present study was as follows (*w*/*v*%, wet basis): protein, 12.7; lipids, 2.95; moisture, 13.0; and ash, 1.59. This data is almost the same as that of Tomotake *et al*. [10]. From this flour, various BWPs with different levels of lipid were prepared using various processing, e.g., selectively choosing the extraction method (mechanical stirring or ultrasonic-assisted treatment) and defatting treatment. Table 1 shows chemical compositions of various BWPs (including M-BWP, U-BWP and DU-BWP).

As expected, various BWPs had different levels of protein and lipid. M-BWP, prepared by usual mechanical method, had much higher protein and lower lipid content (81.4 and 6.5% respectively) than U-BWP (66.1 and 17.8%), prepared by ultrasonic-assisted extraction (Table 1). The difference may be due to action of ultrasonic-induced emulsification in a protein–lipid system. Of course, the ultrasonic-induced strong oscillation may also lead to the release of lipid fraction from the flour, and the released lipids would be associated with proteins, thus accumulating in the proteins prepared by acid-precipitation. In the ultrasonic-assisted cases, additional de-fatting treatment of the whole-meal flour with hexane significantly increased the protein content and at the same time decreased the lipid content of buckwheat protein ($P \leq 0.05$; Table 1). The lipid content (2.5%) of DU-BWP from the defatted flour was even much less than that of M-BWP.

After stored at normal temperature and relative humidity of about 70–80%, various BWPs showed moisture content of 4.0~5.0% (Table 1). The moisture content seems to be negatively associated with the lipid content. The data suggests that the presence of lipid may change hydration property of buckwheat proteins, and surface hydrophobic and/or hydrophilic nature of proteins. The ash content of BWP was nearly unaffected by extraction method and de-fatting treatment.

The protein constituents of various BWPs were analyzed by reducing SDS-PAGE, and there were no obvious variations of protein constituent pattern among various BWPs (Fig. 1). The mobility of various polypeptides agrees with that of Fujino *et al*. [27], but is a bit different from that of Rout *et al*. [28] and Milisavljević *et al*. [29]. Like previous studies, BWPs prepared in the present study were composed of globulins (including 8S and 13S globulins) and 2S albumins and 13S globulins were major component consisting of two kinds of polypeptides, acidic and basic subunits, with *MW* of 38~30 and 20 kDa, respectively. According to the density analysis by scanning technique, relative protein contents of 8S and 13S globulins were about 20 and 68% total protein content respectively (in this case, the total protein content only include 8S and 13S globulins and 2S albumins; data not shown). The data is different from that of another literature which showed the 8S and 13S globulins represented about 6.5 and 33% of total seed proteins, respectively [30].

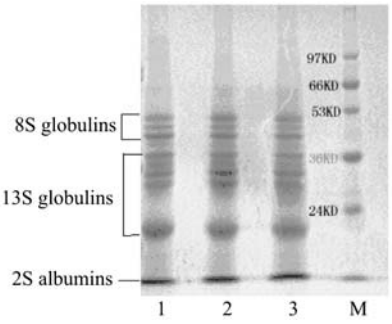

Fig. 1 SDS-PAGE patterns of various buckwheat proteins (BWP). 1 – M-BWP, 2 – U-BWP and 3 – DU-BWP; M – protein markers

Table 1 The chemical compositions of buckwheat protein products, prepared by different processing. Means and standard deviations of triplicate samples were given

Protein product[a]	Chemical composition (wet basis, *w*/*v*%)			
	protein[b]	lipid	moisture	ash
M-BWP	81.4 ± 0.3^{B}	6.5 ± 0.2^{B}	4.5 ± 0.1^{B}	1.0 ± 0.1^{A}
U-BWP	66.1 ± 0.4^{C}	17.8 ± 0.2^{A}	3.9 ± 0.1^{C}	0.8 ± 0.2^{A}
DU-BWP	82.3 ± 0.3^{A}	2.5 ± 0.2^{C}	5.1 ± 0.0^{A}	0.8 ± 0.1^{A}

[A, B, C]significant ($P \leq 0.05$) difference among various BWPs, within a same column; [a]M-BWP – BWP obtained by usual mechanical extraction, from the non-delipidated flour; U-BWP – BWP obtained by ultrasonic-assisted extraction, from the non-delipidated flour; DU-BWP – BWP obtained by ultrasonic-assisted extraction plus the de-fatting pretreatment of the flour; [b]determined by Kjeldahl method, N×6.25.

Typical DSC curve of BWP

A typical DSC curve of BWP (M-BWP) dispersed in 0.05 M phosphate buffer (pH 7.0) is shown in Fig. 2. The protein sample was heated from 20 to 130°C, at a rate of 5°C min^{-1}. In this DSC profile of M-BWP, there were two distinct endothermic events, with denaturation temperatures (T_{d1} and T_{d2}) of about 80 and 101.7°C, respectively. These two thermal transitions observed may be attributed to different protein components with different T_d. Evidently, 13S and 8S globulins would correspondingly contribute to the major and minor endotherms, since these two protein components were the major protein components of buckwheat proteins (Fig. 1). As expected, the T_d of 13S and 8S globulins of buckwheat proteins were reasonably higher than that of 11S (90–100°C) and 7S (77–78°C) globulins of soy proteins, respectively [31, 32].

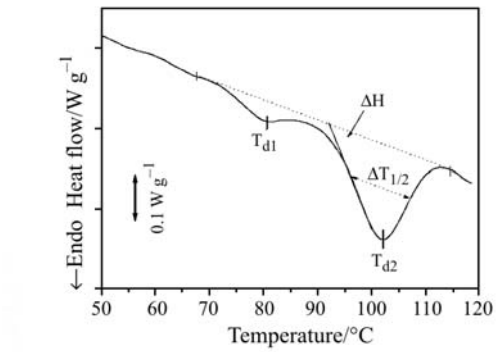

Fig. 2 A typical DSC curve of buckwheat protein (M-BWP). The protein sample (1.5–2.0 mg of protein in 10 μL 0.05 M phosphate buffer, pH 7.0) was heated at a rate of 5°C min^{-1}. T_{d1} and T_{d2} represent thermal denaturation temperatures of two main endotherms. The ΔH indicates combined enthalpy of two endothermic peaks, while $\Delta T_{1/2}$ represents the width at half peak height of the major endothermic peak

From the DSC curve, many DSC characteristics including denaturation temperature (T_d), onset temperature of denaturation (T_m) and width at half peak height of major endotherm ($\Delta T_{1/2}$), and enthalpy change of combined endotherms or major endotherm (ΔH) could be obtained. These DSC characteristics of various BWPs (including M-BWP, U-BWP and DU-BWP) in 0.05 M phosphate buffer (pH 7.0) are summarized in Table 2. There were no significant variations of T_m, T_d and ΔH (of major endotherm) among various BWPs (Table 2), suggesting that thermal denaturation of buckwheat globulins in a certain medium be unaffected by the presence of lipid (2.5–17.8%). However, the presence of high level of lipid (e.g. 17.8% in the U-BWP) to some extent increased the $\Delta T_{1/2}$ (Table 2), suggesting a decline of the extent of ordered structure [12].

Effect of NaCl concentration

The effect of NaCl concentration on DSC characteristics of 13S globulins of various BWPs in 0.05 M phosphate buffer (pH 7.0) is demonstrated in Table 2. In all BWP cases, increasing concentration of NaCl from 0 to 4.0 M progressively increased T_m of 13S globulins from about 93 to 117°C, and T_d from about 102 to 124°C (Table 2). Similarly, the T_m and T_d of the minor endothermic peak also progressively increased with NaCl concentration (data not shown). The data suggest stabilizing effect of higher ionic strength on thermal stability of globulins of BWPs. Similar results have been reported on oat globulins [21], fababean globulins [20], red bean globulins [23] and flaxseed proteins [24].

Table 2 Effects of NaCl concentration on DSC characteristics of globulins from buckwheat[a]

Protein product	NaCl/mol L^{-1}	T_m/°C[b]	T_d/°C[c]	ΔH/J g^{-1} protein[d]	$\Delta T_{1/2}$/°C[e]
M-BWP	0.0	92.4±1.2	101.7±0.2	6.0±0.5	9.8±1.0
	0.5	99.5±0.3	105.8±0.1	7.1±0.1	8.0±0.4
	1.0	103.0±0.6	109.3±0.1	8.7±0.9	7.7±0.5
	2.0	108.8±0.4	115.5±0.1	8.3±0.5	7.9±0.4
	4.0	117.0±0.2	124.6±0.2	9.5±1.2	8.3±0.3
U-BWP	0.0	93.1±0.3	102.6±0.6	6.2±0.1	11.2±0.1
	0.5	98.8±0.4	106.0±0.3	8.6±0.4	9.9±0.6
	1.0	102.1±1.4	109.2±0.2	9.3±0.6	9.2±0.2
	2.0	108.8±0.5	115.7±0.1	9.8±1.0	8.8±0.7
	4.0	118.0±2.0	124.8±0.0	10.1±0.5	8.2±1.2
DU-BWP	0.0	92.8±0.7	102.0±0.5	5.9±1.0	10.3±0.9
	0.5	99.61±0.1	105.8±0.4	6.9±0.7	8.8±0.6
	1.0	103.2±0.5	109.7±0.1	8.2±0.3	8.7±0.1
	2.0	108.9±0.3	115.7±0.0	8.8±0.1	8.6±0.5
	4.0	117.1±0.5	124.7±0.4	9.2±1.2	8.7±0.2

[a]Averages and standard deviations of triplicate determinations. Different concentrations of NaCl in pH 7.0 phosphate buffer were used; [b]on-set temperature of the major endotherm; [c]denaturation or peak temperature of the major endotherm; [d]enthalpy of the major endotherm and [e]width at half peak height of the major endotherm

The heat stability of proteins is controlled by the balance of polar and non-polar residues [33]. Protein conformation can be perturbed by the addition of salts (e.g. NaCl), which influence the electrostatic interaction with the charged groups and polar groups of proteins, and affect the hydrophobic interaction via a modification on the structure of water [21, 34]. The extent to which the protein conformation is affected depends on the nature and concentration of the salt. The stabilizing effect of low concentration of NaCl (<1.0 M) may be attributed to the improvement of hydration of protein molecules, due to a 'salting-in' phenomenon [35]. At high NaCl concentrations (>1.0 M), the proteins may aggregate or precipitate, due to the competition between the protein and ions for water, or a 'salting-out' phenomenon [36]. Therefore, the presence of high concentration of NaCl favors the formation of more compact conformation of buckwheat globulins, with increased thermal stability (higher T_m and T_d).

The ΔH of the major endothermic peak (13S globulins) of various BWPs, in some extent increased, while the width at half peak height ($\Delta T_{1/2}$) decreased with increasing NaCl concentration (Table 2), suggesting that the presence of high concentration of NaCl enhance the proportion of more compact proteins and the cooperativity of thermal transition process [12]. Li-Chan and Ma [24] also indicated that higher salt condition (1.0 M) resulted in higher enthalpy values and greater cooperativity of the transition of flaxseed proteins, at pH 3–11 (as compared to 0.01 M salt condition). Whereas in another previous study, the ΔH values of thermal denaturation of oat globulins were relatively unchanged in the presence of 0 to 1.0 M NaCl [21], which, in fact, were also consistent with our present data (Table 2). Interestingly, at NaCl concentrations higher than 0.5 M, the ΔH values of various BWPs became almost identical, suggesting that the presence of NaCl (at least higher than 0.5 M) may alleviate the extent of aggregation or breaking up of hydrophobic interactions of proteins, which is exothermic in DSC curves [12, 13]. In the presence of NaCl (1.0–4.0 M), the ΔH and $\Delta T_{1/2}$ of buckwheat proteins (e.g. 13S globulins) seem to be independent of their lipid content and NaCl concentration.

Effects of chaotropic salts

The effects of sodium salts of chloride, bromide, iodide and thiocyanate (1.0 M) on the thermal transition characteristics of buckwheat globulins (including 8S and 13S) are shown in Table 3. In any BWP sample, the T_d of 8S or 13S globulins of buckwheat proteins progressively declined with the order $Cl^- > Br^- > I^- > SCN^-$ (Table 3), following the lyotropic series of anions [37]. Similar effects of these chaotropic salts on thermal stability were observed in globulins from oats [21], red bean (Phaseolus angularis) [23] and flaxseed [24]. The combined enthalpy changes (ΔH) of buckwheat globulins decreased progressively when the anions were changed from Br^- to Cl^-, I^- and SCN^- (Table 3).

Chloride and bromide ions promote salting-out and aggregation due to high molar surface tension increments, which may stabilize protein conformation. High concentrations of these anions markedly resulted in higher T_d of 13S globulins of buckwheat proteins as compared to the control (without addition of salts), while the T_d of minor peak (8S globulins) was only a bit affected by bromide ions (Table 3). The difference of relative concentration of 13S and 8S globulins in BWPs (about 68 and 20%, respectively) may account for the influence of these anions on their thermal stability, since high concentration of protein is more favorable for the 'salting-out' and aggregation induced by anions. The combined ΔH of buckwheat proteins were significantly improved by 1.0 M of chloride and bromide ions ($P \leq 0.05$), while the thermal transition cooperativity of 13S globulins was also improved (Table 3). However, the effect of bromide ions on the ΔH of BWPs was significantly higher than that of chloride ions. In the present study, it was shown that lower T_d values of 13S globulins in the presence of bromide ions (1.0 M) were accompanied by higher ΔH values (as compared with chloride ions). However, in a previous study on thermal properties of flaxseed proteins, the ΔH was nearly unaffected by the presence of these anions (1.0 M), and on the contrary, lower thermal stability was accompanied by lower ΔH values [24].

On the other hand, iodide and thiocyanate ions are destabilizing anions because of their higher hydration energy and steric hindrance, which promote unfolding, dissociation and salting-in of proteins [38]. The destabilizing effects of these anions were reflected in lower T_d of 13S and 8S globulins and lower ΔH (Table 3). The cooperativity of the thermal transition of 13S globulins were decreased slightly by the presence of 1.0 M iodide and thiocyanate ions.

The influence extent of various chaotropic salts on the T_d, the ΔH and $\Delta T_{1/2}$ of different globulin proteins (including M-BWP, U-BWP and DU-BWP) can be considered to be similar. This suggests that the effect of chaotropic salts on the thermal properties of buckwheat globulins also be not related with their lipid content.

Effects of urea, sodium dodecyl sulfate (SDS) and ethylene glycol (EG)

The effects of denaturing agents, including urea and SDS, and EG on DSC characteristics of various BWPs

Table 3 Effects of anions on the DSC characteristics of buckwheat globulins[a]

Anion	M-BWP			U-BWP			DU-BWP		
	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C
Control (no anion)	80.4±0.8[c] 101.7±0.2[d]	7.8±0.4	7.8±0.9[e] 9.8±1.0[f]	79.7±0.2[c] 102.5±0.6[d]	7.3±0.3	10.0±0.8[e] 11.2±0.1[f]	79.9±0.5[c] 102.0±0.5[d]	8.7±0.47	8.2±0.49[e] 10.3±0.87[f]
Cl^-	83.8±0.1[c] 109.3±0.1[d]	9.0±0.4	9.5±0.6[e] 7.7±0.5[f]	85.3±1.4[c] 109.2±0.2[d]	10.7±0.5	8.7±0.2[e] 9.2±0.2[f]	84.3±1.1[c] 109.7±0.1[d]	9.8±0.10	9.0±0.59[e] 7.9±0.62[f]
Br^-	78.2±0.5[c] 105.5±0.1[d]	12.3±1.3	10.5±0.3[e] 8.8±0.1[f]	80.8±0.1[c] 105.2±0.1[d]	12.0±0.3	9.9±0.1[e] 10.4±0.3[f]	79.2±0.6[c] 105.6±0.1[d]	10.7±0.38	10.5±0.13[e] 9.2±0.01[f]
I^-	67.2±0.3[c] 96.1±0.1[d]	7.6±0.8	15.7±1.9[e] 10.9±0.5[f]	72.2±0.3[c] 95.2±1.0[d]	6.3±0.9	5.8±0.3[e] 13.0±0.1[f]	68.7±1.5[c] 96.2±0.1[d]	7.1±0.46	7.9±1.01[e] 11.3±0.81[f]
SCN^-	64.7±0.1[c] 90.5±0.1[d]	4.9±0.4	7.0±0.5[e] 11.8±0.1[f]	66.3±0.1[c] 92.5±0.3[d]	3.8±0.3	6.2±0.2[e] 12.1±0.3[f]	64.5±0.2[c] 90.8±0.2[d]	3.5±0.33	9.5±1.02[e] 12.0±0.58[f]

[a]Averages and standard deviations of triplicate measurements were given. Cl^-, Br^-, I^- and SCN^- in 0.05 M phosphate buffer (pH 7.0) were used in 1.0 M concentration. [b]Enthalpy change of the combined endotherm. [c]Denaturation temperature of minor endothermic peak. [d]Denaturation temperature of major endothermic peak. [e]Width at half peak height of minor endothermic peak. [f]Width at half peak height of major endothermic peak.

Table 4 Effects of various concentrations of urea, SDS and ethylene glycol on DSC characteristics of buckwheat globulins[a]

Additives		M-BWP			U-BWP			DU-BWP		
Name	Conc.	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C
Control		101.7±0.2	6.0±0.5	9.8±1.0	102.5±0.6	6.2±0.1	11.2±0.1	102.0±0.5	5.9±1.0	10.3±0.9
Urea	1 M	99.7±0.3	4.1±0.2	8.7±0.3	98.3±0.1	4.5±0.1	10.3±0.3	98.6±0.0	5.2±0.3	11.2±0.9
	2 M	96.8±0.5	3.9±0.2	10.9±0.4	97.6±0.2	4.3±0.1	11.9±0.5	97.7±0.5	6.6±0.6	12.3±0.5
	4 M	93.4±0.1	2.9±0.5	13.4±1.0	94.7±0.8	1.2±0.2	15.0±1.0	89.8±1.0	1.8±0.3	13.5±0.8
	6 M	92.2±0.6	1.4±0.1	17.5±1.5	–[f]	–	–	–	–	–
	8 M	–	–	–	–	–	–	–	–	–
SDS	5 mM	100.3±0.3	5.5±0.1	8.8±0.9	101.7±0.2	6.0±1.4	11.6±1.1	102.1±0.7	5.8±0.7	10.5±0.1
	10 mM	100.5±0.2	4.7±0.4	10.8±0.8	101.5±0.2	4.8±0.1	11.8±1.0	103.0±1.1	5.7±0.2	13.6±0.8
	20 mM	99.4±1.0	4.7±0.9	12.8±0.6	100.6±0.1	2.7±0.1	10.9±0.5	101.4±0.3	5.5±0.4	12.6±0.6
	40 mM	100.4±0.1	5.1±0.6	14.0±0.1	–	–	–	99.7±0.6	4.9±0.3	10.1±0.6
Ethylene glycol (*w*/*v*)	20%	95.0±1.9	5.9±0.3	9.7±0.1	97.0±0.4	4.3±0.3	9.7±1.1	97.1±0.2	5.3±0.8	10.5±0.4
	40%	93.0±0.2	6.4±0.6	10.5±0.4	93.5±0.3	4.7±0.2	9.2±0.1	93.6±0.4	5.4±0.3	9.8±0.5
	60%	88.7±0.1	4.6±0.6	11.1±0.4	89.6±0.5	3.6±0.1	9.6±0.2	89.3±0.1	4.0±0.3	10.0±0.1

[a]Averages and standard deviations of three determinations. Various concentrations of additives in 0.05 M phosphate buffer (pH 7.0) were used. [b]Denaturation temperature of major peak.
[c]Enthalpy of the major peak endotherm. [d]Width at half peak height of the major peak endotherm. [f]The symbol '–' indicates no observable endothermic peak.

are shown in Table 4. In all cases, with increasing concentration of urea (in the range from 0 to 8 M), both T_d and ΔH values of 13S globulins of buckwheat proteins (including M-BWP, U-BWP and DU-BWP) were progressively decreased (Table 4), indicating both a decrease in thermal stability and gradual denaturation of buckwheat globulins by action of urea. The T_d and ΔH values of 8S globulins also decreased with the urea concentration from 0 to 8 M (data not shown). At 6.0 M urea, no discernible endothermic responses were observed in U-BWP and DU-BWP, indicating extensive protein denaturation, while in the M-BWP case, it needed 8.0 M urea (Table 4). Urea effectively disrupts hydrogen bonding, and facilitates protein unfolding by weakening hydrophobic interactions [39]. Urea also increases the 'permittivity' of water for apolar residues causing loss of protein ordered structure and thermal stability [21]. Therefore, it is suggested that the conformation or molecular stability of buckwheat proteins is maintained by hydrogen bonding and hydrophobic interactions, and the role of disulfide bonds in thermal response by DSC is insignificant or negligible, since disulfide bonds are not expected to be cleaved by 8.0 M urea. Apart from the decreases in T_d and ΔH, the $\Delta T_{1/2}$ values of various BWPs significantly increased with the urea concentration (Table 4), suggesting a gradual reduction in the cooperativity of thermal transition process. Effects of urea on DSC characteristics were similarly reported in oat globulins [21] and red bean globulins [23].

SDS is an anionic detergent, which interacts with hydrophobic regions of protein molecules through its dodecyl hydrocarbon chain, causing unfolding and destabilization [40]. The addition of SDS (5–40 mM) led to a slight decrease in T_d of the 13S globulins but caused a significant change in ΔH, in all BWP cases (Table 4). Similar effects of SDS on the thermal properties of the globulins from oat [21], fababean [41], red bean [23] and flaxseed [24] have been reported. The extent of influence of SDS addition on the ΔH of BWPs decreased with the order U-BWP, M-BWP and DU-BWP, which was consistent with the order of their lipid content (Table 1). The ΔH of 13S globulins of U-BWP samples (containing 17.8% lipid) was progressively decreased from 5.0 to 2.2 J g^{-1}, with SDS concentration increasing from 0 to 20 mM. Further increase in SDS concentration (e.g., 40 mM) led to no discernible endotherm of U-BWP. And in M-BWP and DU-BWP cases, the ΔH values of the 13S globulins in the presence of SDS (5–40 mM) were slightly lower than that of control (without SDS) (Table 4). These results suggest that the presence of lipid disturb protein–protein interactions of buckwheat proteins. Our previous experiments showed that, some insoluble aggregates or precipitates appeared in 2 *w*/*v*% BWP dispersions (especially in the cases of U-BWP and DU-BWP), and the precipitates were mainly composed of 13S globulins as shown by SDS-PAGE (data not shown). Based on these observations, we assume that 1) the precipitates in the U-BWP are composed of lipids and globulins (especially 13S globulins), and in this case, the hydrophobic interactions between lipids and hydrophobic regions of proteins are prominent, while in the DU-BWP cases, the precipitates are formed from the aggregation of different hydrophobic cores of proteins and 2) the hydrophobic interactions between lipids and proteins could be disrupted by the presence of SDS, and the protein aggregates or precipitates were nearly unaffected by high concentration of SDS (40 mM).

By contrast, the thermal transition of 8S globulins was markedly affected by the presence of SDS. For example, the T_d of 8S globulins in the M-BWP was decreased from 81 to 67°C, with the SDS concentration increasing from 0 to 40 mM (data not shown). This result suggests that the extent of hydrophobic interactions maintaining protein conformation within 8S globulins be much lower than that of 13S globulins. The presence of basic subunits among 13S globulins [28] may account for this difference of influence of SDS on the thermal properties of 8S and 13S globulins. From this point, we presume that the lipid in the buckwheat proteins may peculiarly interact with basic subunits of 13S globulins.

The effect of ethylene glycol (EG) on the DSC characteristics of buckwheat globulins was also investigated. EG, a water-miscible solvent, could lower dielectric constant of medium, weaken non-polar interactions between protein molecules, and enhance hydrogen-bonding and electrostatic interactions [34, 42]. The T_d values of various BWPs (with different lipid contents) were all progressively decreased with increase in EG concentration (0, 20, 40 and 60%) (Table 4), indicating gradual decrease of thermal stability. Similar lowering in thermal stability was also observed for oat globulins [21], red bean globulins [23] and flaxseed proteins [24]. Addition of EG up to 40 *w*/*v*% did not cause a significant change of ΔH of 13S globulins of M-BWP and DU-BWP, while the ΔH for U-BWP was progressively decreased from 5.0 to 2.9 J g^{-1} with the EG concentration increasing from 0 to 60 *w*/*v*% (Table 4). Furthermore, the $\Delta T_{1/2}$ values for M-BWP and DU-BWP increased slightly after addition of EG up to 60 *w*/*v*%, while in the U-BWP case, the $\Delta T_{1/2}$ values, on the contrary declined significantly (Table 4). These results further confirmed that the presence of lipid in buckwheat proteins might affect interaction pattern among different proteins. The weakening of hydrophobic interactions by EG may be insufficient to

Table 5 Effects of DTT and NEM concentration on DSC characteristics of buckwheat globulins[a]

Additive agents	M-BWP			U-BWP			DU-BWP		
	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C	T_d/°C	ΔH/J (g protein)$^{-1}$	$\Delta T_{1/2}$/°C
Control	101.7±0.2	6.0±0.5	9.8±1.0	102.5±0.6	6.2±0.1	11.2±0.1	102.0±0.5	5.9±1.0	10.3±0.9
20 mM DTT	101.1±0.1	5.5±0.3	9.9±0.5	101.5±0.7	4.5±0.1	11.0±0.3	102.3±1.4	4.3±0.6	9.8±1.6
100 mM DTT	98.8±0.1	6.0±0.6	9.8±0.1	98.7±0.4	4.2±0.6	10.2±0.3	97.6±0.1	2.8±1.1	8.7±0.1
20 mM NEM	99.8±0.2	6.5±1.0	13.2±0.9	101.6±0.7	6.8±0.5	13.8±1.3	100.8±0.7	4.9±1.0	11.2±0.2
100 mM NEM	96.2±0.3	6.3±1.1	12.7±0.1	95.2±0.2	3.8±0.3	12.3±0.5	95.1±1.8	4.6±1.1	11.6±0.2

[a]Averages and standard deviations of three determinations. Various concentrations of reducing agents in 0.05 M phosphate buffer (pH 7.0) were used. The NEM was dispersed in 50 *v*/*v*% alcohol, and then mixed with 0.05 M phosphate buffer (pH 7.0) (the alcohol concentration of final buffer was lower than 5 *v*/*v*%). [b]Denaturation temperature of the major endotherm. [c]Enthalpy of the major endotherm. [d]Width at half peak height of the major peak.

cause significant disruption of non-polar interactions between protein molecules, but it can affect those hydrophobic interactions between lipids and proteins. Therefore, EG seems to be able to denature most protein constituents of U-BWP, and unable to denature those of M-BWP and DU-BWP.

Effects of reducing or blocking agents

To determine contribution of disulfide bonds to the thermal properties of buckwheat globulins, the effects of sulfhydryl reducing or blocking agents dithiothreitol (DTT) and N-ethylmaleimide (NEM), on the DSC characteristics of the 13S globulins were investigated (Table 5). DTT is a reducing agent, and can reduce the disulfide bond of cystinyl residues to sulfhydryl groups in the proteins, thus causing protein destabilization. Like other legumin storage proteins, the 13S globulins of buckwheat proteins are composed of non-identical subunits with one subunit consisting of one acidic and one basic polypeptide linked by a disulfide bond [27, 30]. The presence of disulfide linkages should account for the thermal stability of 13S globulins of buckwheat proteins. However, by comparison with other oligomeric proteins, such as soy glycinin and flaxseed proteins [24, 43], the thermal stability of buckwheat globulins was much less affected by addition of DTT. Only a slight decrease in thermal stability of 13S globulins was observed in the presence of 20 mM DTT, and relatively marked reduction in thermal stability only resulted with 100 mM DTT (Table 5).

ΔH and $\Delta T_{1/2}$ of 13S globulins of M-BWP were not lowered by addition of DTT (20–100 mM), while that of U-BWP and DU-BWP was progressively decreased with increasing DTT concentration (Table 5). This result suggests that the thermal denaturation of 13S globulins in M-BWP seem to be unaffected by DTT, and on the contrary, the disulfide bonds play a major role in thermal transition of 13S globulins of U-BWP and DU-BWP. To better understand this difference among various BWPs, we suppose that presence of a certain amount of lipid (e.g., 6.5% in the M-BWP) be beneficial to maintain the protein stability or compact protein conformation of 13S globulins. If excess of lipids (e.g., 17.8% in the U-BWP) are present, high affinity between lipids and basic subunits of 13S globulins may disturb the disulfide linkages between acidic and basic subunits of 13S globulins. Of course, absence of lipids (as in the DU-BWP) may also in some extent destabilize protein conformation, and increase the chance of 'attraction' between different basic subunits of 13S globulins due to strong hydrophobic interactions, thus leading to disruption of some disulfide linkages.

NEM, a sulfhydryl-blocking reagent, also led to progressive decreases of T_d of 13S globulins of BWPs, especially at higher concentrations (Table 5), suggesting that SS-SH interchange reactions may play a major role in stabilizing conformation of protein molecules. Like in the DTT cases, the enthalpy change of 13S globulins of M-BWP was nearly unaffected by addition of NEM, and that of U-BWP and DU-BWP was progressively decreased with increasing NEM concentration. In addition, the cooperativity of thermal transition of 13S globulins of buckwheat proteins slightly declined by addition of NEM. Similar effects of DTT and NEM on the thermal denaturation were observed in globulins from flaxseed proteins [24].

Conclusions

The present data show that buckwheat proteins exhibit two endothermic transitions, attributed to 13S and 8S globulins, respectively. The presence of various concentrations of lipids unaffected the thermal stability of 13S globulins of buckwheat proteins, but in some extent, led to the diversity in the cooperativity of its thermal transition. The DSC characteristics of the 13S and 8S globulins could be influenced by many environmental factors, including salts and protein perturbants. The DSC analyses suggest that the hydrophobic and hydrogen bondings are major interactions for stabilizing the conformation of protein molecules of buckwheat globulins, and the protein conformation of 13S globulins is to a large extent dependent upon their lipid content.

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