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Conformational Study of Globulin from Common Buckwheat (*Fagopyrum esculentum* Moench) by Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry

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Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC) were used to study changes in the conformation of globulin from common buckwheat (*Fagopyrum esculentum* Moench) (BWG) under various environmental conditions. The IR spectrum of the native BWG showed several major bands from 1691 to 1636 cm^{-1} in the amide I′ region, and the secondary structure composition was estimated as 34.5% β-sheets, 20.0% β-turns, 16.0% α-helices, and 14.4% random coils. Highly acidic and alkaline pH conditions induced decreases in β-sheet and α-helical contents, as well as in denaturation temperature (T_d) and enthalpy of denaturation (ΔH), as shown in the DSC thermograms. Addition of chaotropic salts (1.0 M) caused progressive decreases in ordered structures and thermal stability following the lyotropic series of anions. The presence of several protein structure perturbants also led to changes in IR band intensities and DSC thermal stabilities, suggesting protein unfolding. Intermolecular antiparallel β-sheet (1620 and 1681 cm^{-1}) band intensities started to increase when BWG was heated to 90 °C, suggesting the initiation of protein aggregation. Increasing the time of the preheat treatment (at 100 °C) caused progressive increases in T_d and pronounced decreases in ΔH, suggesting partial denaturation and reassociation of protein molecules.

KEYWORDS: ***Fagopyrum esculentum*** **Moench; buckwheat globulin; FTIR spectroscopy; differential scanning calorimetry; protein conformation; aggregation; denaturation**

INTRODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench) is an underutilized pseudocereal with high nutritional value. Buckwheat seeds contain 10−12.5% protein, 65−75% starch, 2−2.5% fat, and 2−2.5% minerals (*1*). Buckwheat protein is relatively high in essential amino acids, particularly lysine (∼6 mg/100 g of protein), which is generally the first limiting amino acid in plant proteins, and arginine (*2*, *3*), and according to net protein utilization (NPU), it is classified close to animal proteins and has excellent supplementary value to cereal grains (*4*). However, it has not been extensively characterized. Salt soluble globulins, the main component of buckwheat seed proteins, are represented mainly by the 13S protein fraction with molecular weight ranging from 280 to 300 kDa (*5*, *6*). Buckwheat globulin (BWG) has been classified as a leguminlike storage protein (*7*). It is an oligomeric protein composed of six nonidentical monomers that interact noncovalently. Each monomer contains one large acidic polypeptide and one small basic polypeptide with molecular weight of around 57.5 and 23.5 kDa, respectively, and the two polypeptides are linked by disulfide bonds (*6*). BWG has been found to possess functional properties comparable to that of a commercial soy protein product (*8*). A better understanding of the structure−function relationship in BWG is essential for its utilization in food applications.

In this study, Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC), two techniques that have been extensively used to study the structural and thermal behavior of various food proteins (*9*−*13*), will be employed to monitor conformational changes in BWG under various buffer conditions and heat treatments. FTIR is a vibrational spectroscopic technique that can be used to determine the secondary structures of peptides and proteins (*14*, *15*) and can be used to investigate protein structural changes during aggregation/gelation, since the technique can be applied to liquid, semisolid, and solid samples (*14*, *16*). On the other hand, DSC will be used to study thermal characteristics of BWG and to detect thermally induced denaturation of proteins, which is critical to their suitability for certain functions such as gelation, emulsification, and foaming (*17*). The two techniques should provide complementary information on the conformation of BWG. By following conformational changes under the influence of various buffer environments and heat treatments, the chemical forces involved in stabilizing the secondary and tertiary structures of the protein can be elucidated.

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MATERIALS AND METHODS

Preparation of Buckwheat Globulin. Buckwheat flour was obtained from Nikkoku Flour Milling Co. Ltd. (Japan) and defatted by Soxhlet extraction with hexane. BWG was extracted from the defatted buckwheat flour using 0.5 M NaCl buffer in a ratio of 1:10 (w/v) according to the Osborne fractionation scheme (*18*). The protein content of BWG was 93.9%, determined by the micro-Kjeldahl method (*19*) using a nitrogen-to-protein conversion factor of 5.53 (*20*). All chemicals used were of analytical grade. Deuterated reagents (D_2O and DCl) were purchased from Aldrich (Milwaukee, WI).

Sample Preparation. For FTIR experiments, BWG dispersions (10%, w/v) were prepared in 0.01 M deuterated phosphate buffers. D_2O was used instead of water, since D_2O has greater transparency in the infrared amide I′ region (*21*). To ensure complete hydrogen−deuterium exchange, sample dispersions were incubated at 4 °C for 1 day before experiments and equilibrated to room temperature prior to measurements. To study the effect of pH, the desired pD (pD = pH + 0.4) was obtained by the addition of 1 M NaOD (dissolving NaOH in D_2O) or 1 N DCl with magnetic stirring, and the protein mixtures were stirred for 1 h at room temperature to allow the pD to equilibrate. Chaotropic salts (sodium chloride, bromide, iodide, and thiocyanate) and protein structure perturbants (sodium dodecyl sulfate, *N*-ethylmaleimide, and dithiothreitol) were prepared in deuterated phosphate buffer at pD 7.4 to give the desired concentrations. The selected concentrations were based on previous studies (*12*, *13*) which were sufficient to elicit conformational changes in proteins (oat and red bean globulins) with similar molecular structures. For DSC experiments, 0.01 M phosphate buffer at pH 7.0 (or with the desired pH) was used instead of D_2O for preparing different buffers.

Fourier Transform Infrared Spectroscopy. Infrared spectra were recorded by an Excalibur FTIR spectrometer (system FTS 3000) equipped with a deuterated triglycine sulfate (DTGS) detector (Bio-Rad Laboratories, Cambridge, MA). Samples were held in an IR cell with 25 μm path length CaF_2 windows. A total of 32 scans were performed at 4 cm^{-1} resolution and were averaged. Preliminary data showed that increasing the number of scans from 32 to 64, 128, 256, and 512 did not significantly improve the resolution of the IR spectra. Hence, 32 scans were used for taking the spectra, which was particularly important for monitoring changes during heat treatments.

Deconvolution of the infrared spectra was performed using the Merlin Software, version 1.2 (Bio-Rad Laboratories, Inc., Cambridge, MA) according to Byler and Susi (*22*). The half-bandwidth used for deconvolution was 10 cm^{-1}, and the enhancement factor, γ, was 2.0. To ensure that the spectra were not over-deconvoluted, the acquired spectra were judged by evaluating the second derivative spectra, comparing the number and positions of the bands with those of the deconvoluted spectra. Band assignment in the amide I′ region (1600−1700 cm^{-1}) was according to Byler and Susi (*22*) and Kavanagh et al. (*11*). Quantitative estimation of secondary structure components was performed using Gaussian peaks and curve fitting models according to Byler and Susi (*22*). All FTIR experiments were performed in duplicates with standard deviations of less than 10%. For heating experiments, an Omega temperature controller (Omega Engineering, Stanford, CA) was used. The samples prepared in 0.01 M deuterated phosphate buffer, pD 7.4, were heated from 30 °C to 110 °C at 10 °C intervals (with an accuracy of ± 0.5 °C) and allowed to equilibrate for 3 min at each temperature prior to data acquisition.

Differential Scanning Calorimetry. The thermal properties of BWG were examined by a TA 2920 modulated DSC thermal analyzer (TA Instruments, New Castle, DE). An aliquot (10 μL) of buffer solution was added to approximately 1 mg of protein in a preweighed DSC pan, hermetically sealed, and equilibrated for 15 min. The samples were heated at a rate of 10 °C/min from 25 °C to 140 °C. A sealed empty pan was used as a reference. Indium standards were used for temperature and energy calibration. For BWG receiving preheat treatments, the protein samples in sealed pans were heated at 100 °C in a boiling water bath for fixed time periods, followed by rapid cooling in an ice bath for 15 min. The cooled samples were then analyzed by DSC as described above. Thermal transition characteristics including denaturation temperature (T_d), enthalpy (ΔH), and the width at half-peak height ($\Delta T_{1/2}$) were measured. All DSC measurements were performed in triplicate with standard deviations less than 2% for T_d and $\Delta T_{1/2}$ and 10% for ΔH.

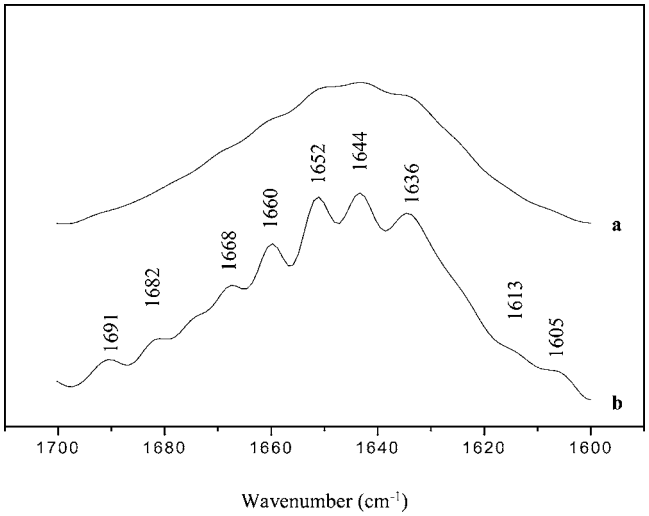

Figure 1. Original (**a**) and deconvoluted (**b**) infrared spectra of 10% buckwheat globulin in D_2O.

Table 1. Assignments of Deconvoluted Amide I′ Bands in the FTIR Spectrum of Buckwheat Globulin[a]

wavenumber (cm^{-1})	assignment
1605	side chain vibration
1613	antiparallel β-sheet, aggregated strands
1627	β-strand
1636	β-sheet
1644	random coil
1652	α-helix
1660	β-turns
1668	β-turns
1682	antiparallel β-sheet, aggregated strands
1691	β-type

[a] Ten percent in 0.01 M deuterated phosphate buffer, pD 7.4.

Statistical Analysis. Analysis of variance and Duncan's multiple range tests were performed where appropriate to establish the significance of different treatments (buffer conditions or heating) at the $p \leq 0.05$ level, using a standard statistical software package.

RESULTS AND DISCUSSION

Spectral Assignment. Figure 1 shows a typical infrared spectrum (1600−1700 cm^{-1}) of a BWG dispersion (10% in deuterated phosphate buffer, pD 7.4) before and after deconvolution. **Table 1** presents the assignments of each deconvoluted amide I′ component of BWG. The deconvoluted spectrum of BWG (**Figure 1b**) revealed several bands at 1691, 1682, 1668, 1660, 1652, 1644, and 1636 cm^{-1} and shoulders at 1613 and 1605 cm^{-1}. The secondary structure composition of BWG was estimated as 34.5% β-sheets (1627 and 1636 cm^{-1}), 20.0% β-turns (1660 and 1668 cm^{-1}), 16.0% α-helices (1652 cm^{-1}), and 14.4% random coils (1644 cm^{-1}). Our results are close to the circular dichroism data of Marcone et al. (*23*), which showed that BWG, similar to most plant globulins, has a large quantity of β-sheets and random coil structures and low α-helical content.

BWG exhibited one endothermic peak in the DSC thermogram with T_d at 100.4 °C and ΔH of 17.6 J/g (**Table 2**), showing that BWG has relatively high thermal stability and a large content of ordered structure, when compared to other plant globulins (*23*). The peak or denaturation temperature can be used as a measure of thermal stability of proteins (*24*), whereas

Table 2. Effect of Some Protein Structure Perturbants on Thermal Transition Characteristics of Buckwheat Globulin[a]

perturbants	T_d (°C)[b]	ΔH (J/g)[c]	$\Delta T_{1/2}$ (°C)[d]
control	100.4 ± 0.1 a	17.6 ± 0.5 a	12.7 ± 0.2 a
10 mM SDS	104.8 ± 0.2 b	8.53 ± 0.4 b	17.2 ± 0.3 b
10 mM NEM	103.2 ± 0.5 c	14.3 ± 0.0 c	16.2 ± 0.5 c
10 mM DTT	101.9 ± 0.0 a	15.0 ± 0.9 d	15.2 ± 0.5 d

[a] Averages and standard deviations of triplicate determinations. Means in a column bearing the same letter are not significantly different ($p > 0.05$). [b] Denaturation temperature. [c] Denaturation enthalpy. [d] Width at half-peak height.

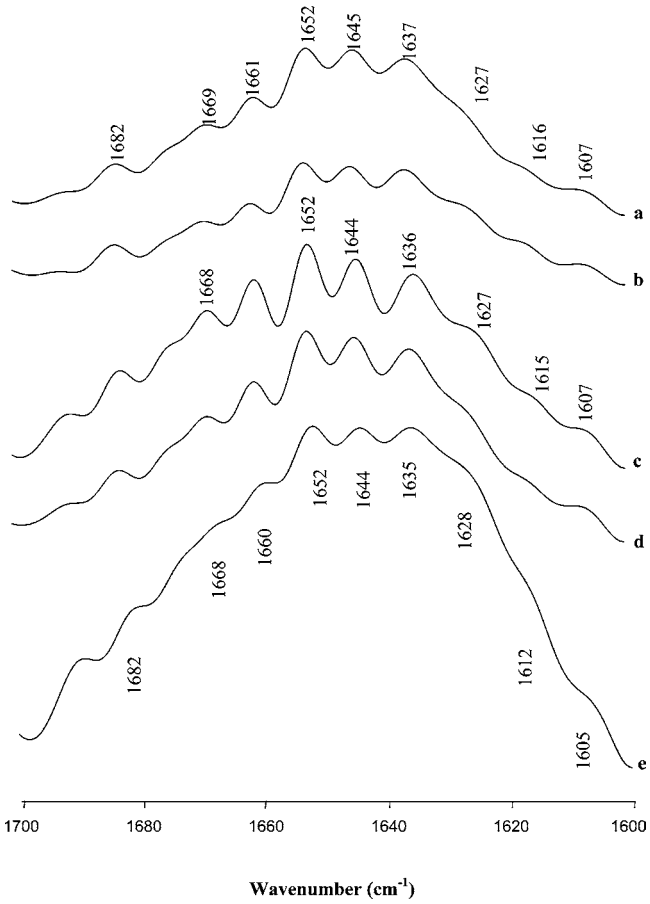

Figure 2. Stacked plot of deconvoluted infrared spectra of 10% buckwheat globulin (in 0.01 M deuterated phosphate buffer, pD 7.4) at different pH values. (**a**) pH 11; (**b**) pH 9; (**c**) pH 7; (**d**) pH 5; (**e**) pH 3.

the enthalpy value is correlated with the content of ordered secondary structure of a protein (*25*). The high content of disulfide linkages in BWG (36.4 μM/g protein) (data not shown) may contribute to the relatively high thermal stability of BWG. This is consistent with the observation that proteins containing disulfide bonds show higher temperatures and enthalpies of denaturation (*26*). In the present study, the denaturation temperature of BWG was higher than that (94.5 °C) observed by Marcone et al. (*23*). This could be due to differences in the heating rate used and in sample preparation. Heating rate is a factor known to shift the DSC curves to higher temperatures and has been shown to affect T_d measurements (*24*). Differences in protein purity could also lead to different DSC responses.

Effect of pH. Figure 2 shows the infrared spectra of BWG dispersions at different pH values. A highly acidic pH (pH 3) condition induced shifts in bands attributed to β-sheets (1636 cm^{-1}) and antiparallel β-sheets (1615 cm^{-1}) to lower wavenumbers. Such shifting of IR peak positions has been shown to

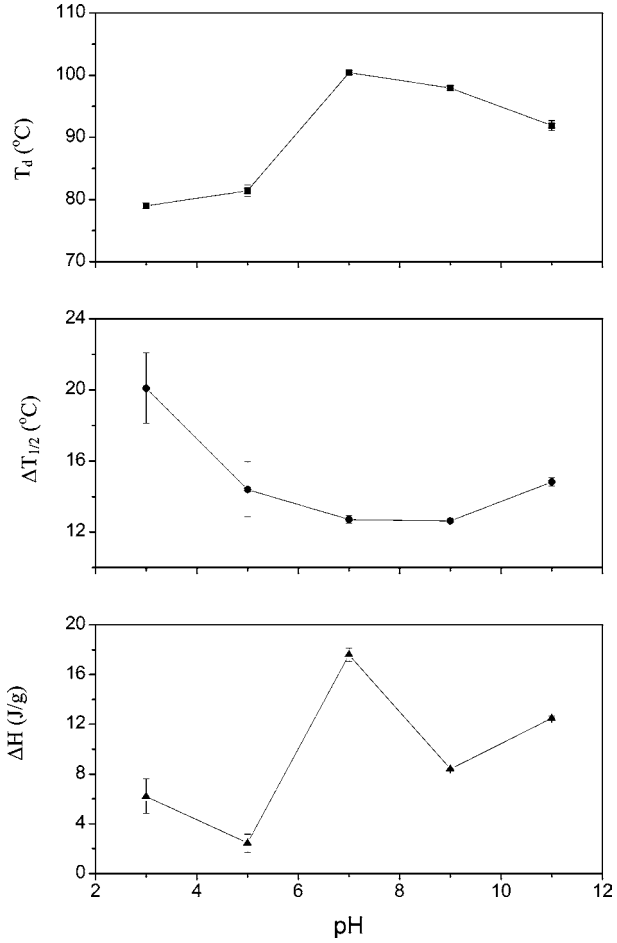

Figure 3. Effect of pH on thermal transition characteristics of buckwheat globulin. Error bars represent standard deviations of triplicate determinations. T_d, denaturation temperature; $\Delta T_{1/2}$, width at half-peak height; ΔH, enthalpy.

indicate a change in hydrogen bonding (*27*) or loss of secondary structure possibly due to partial unfolding (*10*). Decreases in β-sheets (1627 and 1636 cm^{-1}) and α-helical (1652 cm^{-1}) contents were obtained at acidic conditions, while random coil (1644 cm^{-1}) content was increased at both extreme acidic and alkaline pHs, suggesting protein unfolding (data not shown). At pH 3, the carboxyl group of the Asp residue was not ionized, and this would tend to decrease self-association, which may explain the decrease in β-sheet content at acidic pH. The intensity of the β-turn components (1660 and 1668 cm^{-1}) was decreased, suggesting changes of secondary structure. The infrared spectra at pH 5 and 7 (**Figure 2**) were similar to that of the control (**Figure 1**) (in D_2O buffer without pH adjustment), since the isoelectric pH range of BWG was found to be at the acidic pH of 4−6.8 (*28, 29*). The present data suggest that BWG exhibited native conformation at the isoelectric pH range (pH 5−7) and the secondary structure conformations of BWG were altered at both extreme acidic and alkaline pHs.

DSC results (**Figure 3**) show that highly acidic and alkaline pHs caused decreases in T_d and ΔH, suggesting loss of thermal stability and protein denaturation. However, the changes in thermal characteristics were more pronounced at highly acidic conditions. Hence, both FTIR and DSC data demonstrate destabilization of native conformation in BWG at extreme pH conditions. Most proteins are stable near the isoelectric pH when the repulsive forces are low and the proteins remain in a native state. At extreme pHs, large net charges are induced and

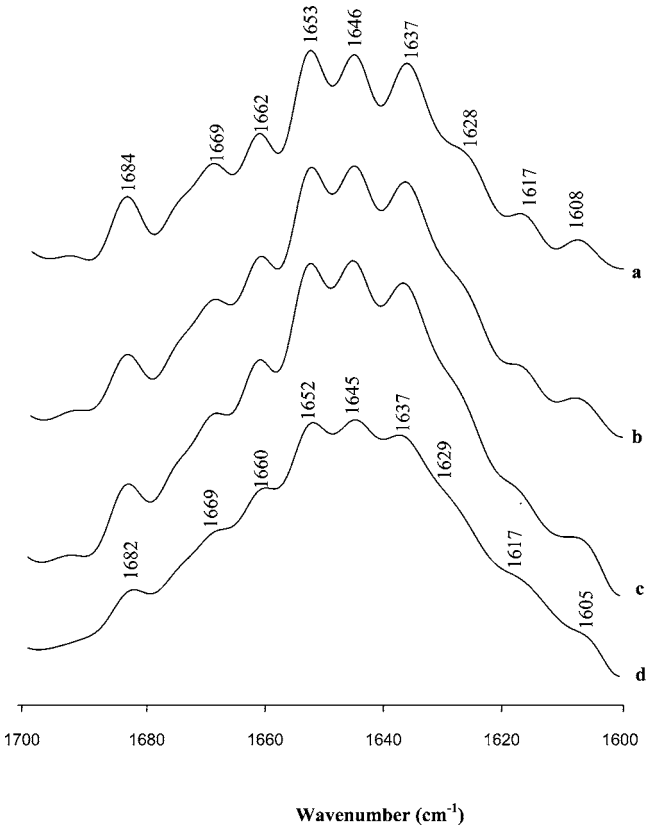

Figure 4. Stacked plot of deconvoluted infrared spectra of 10% buckwheat globulin (in 0.01 M deuterated phosphate buffer, pD 7.4) in the presence of 1.0 M chaotropic salts. (**a**) Sodium chloride; (**b**) sodium bromide; (**c**) sodium iodide; (**d**) sodium thiocyanate.

repulsive force increase, resulting in protein unfolding (*30*). The unfolding of proteins at extreme pHs may also be attributed to rupture of hydrogen bonds and a breakup of hydrophobic interactions (*31*). The pH-induced changes in conformation may lead to a less cooperative system, as indicated by higher $\Delta T_{1/2}$ values (**Figure 3**). Similar changes in thermal characteristics with pH were also reported for globulins from oats (*32*) and fababean (*33*).

Effect of Chaotropic Salts. In the presence of 1.0 M sodium salts, the β-turn (1662 cm^{-1}), α-helix (1653 cm^{-1}), and random coil (1646 cm^{-1}) bands were shifted to lower wavenumbers (**Figure 4**). Progressive decreases in β-sheet and α-helical contents were observed, indicating progressive protein unfolding, following the order Cl^-, Br^-, I^-, and SCN^- according to the lyotropic series of anions (*34*). The spectral data are consistent with the DSC results, which show progressive reductions in both T_d and ΔH in the presence of chaotropic salts (**Figure 5**), again indicating progressive protein unfolding and denaturation following the lyotropic series.

It has been demonstrated that salts perturb protein conformational structures by influencing the physical state of water, breaking the hydrogen-bonded structure of water, and affecting the electrostatic and hydrophobic interactions of protein (*35*, *36*). The degree to which water structure is affected depends on the nature of anions or cations, following the lyotropic series (*34*). Chloride and bromide ions can promote salting-out and aggregation due to higher molar surface tension, which may stabilize protein conformation. On the other hand, iodide and thiocyanate ions are destabilizing anions which could weaken intramolecular hydrophobic interactions and thus promote unfolding, dissociation, and salting-in of proteins (*37*).

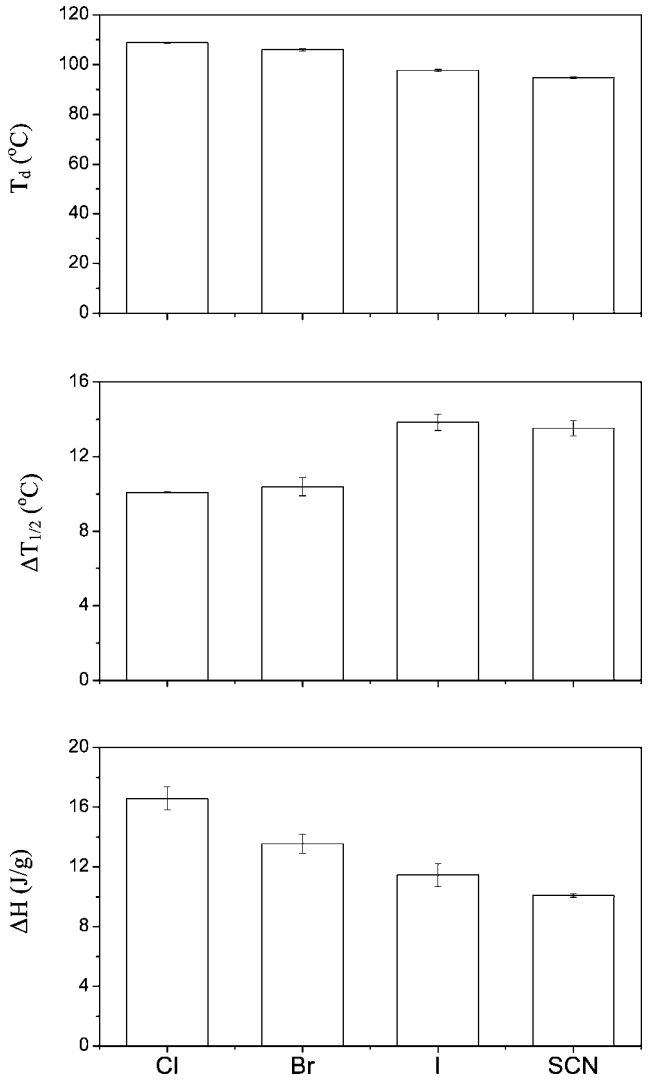

Figure 5. Effect of chaotropic salts (1.0 M) on thermal transition characteristics of buckwheat globulin. Error bars represent standard deviations of triplicate determinations. T_d, denaturation temperature; $\Delta T_{1/2}$, width at half-peak height; ΔH, enthalpy; Cl, chloride; Br, bromide; I, iodide; SCN, thiocyanate.

Effect of Protein Structure Perturbants. The effect of some protein structure perturbants on the IR spectral characteristics of BWG dispersions is shown in **Figure 6**. Peak shifts were observed in the presence of 10 mM sodium dodecyl sulfate (SDS), *N*-ethylmaleimide (NEM), and dithiothreitol (DTT), suggesting changes in protein conformation. β-Sheet and β-turn structures seem to be particularly sensitive to changes in buffer conditions, which may be attributed to the predominance of β-type conformation in BWG. In the presence of SDS, increases in β-sheet (1637 cm^{-1}) and antiparallel β-sheet (1617 and 1684 cm^{-1}) band intensity and decreases in β-turn (1662 and 1669 cm^{-1}) band intensity were observed (**Figure 6**), indicating protein unfolding and reorganization of protein structures. Such unfolding or denaturation may lead to the formation of intermolecular antiparallel β-sheet (1617 and 1684 cm^{-1}) structure at the expense of β-turn structure, accompanied by a slight increase in random coil structure. DSC data show that the addition of 10 mM SDS caused a significant ($p \leq 0.05$) decrease in ΔH and increase in T_d value (**Table 2**). This further confirms the changes in secondary structure by the addition of 10 mM SDS. SDS is an anionic detergent which can bind to protein molecules by noncovalent forces causing ionic repulsion

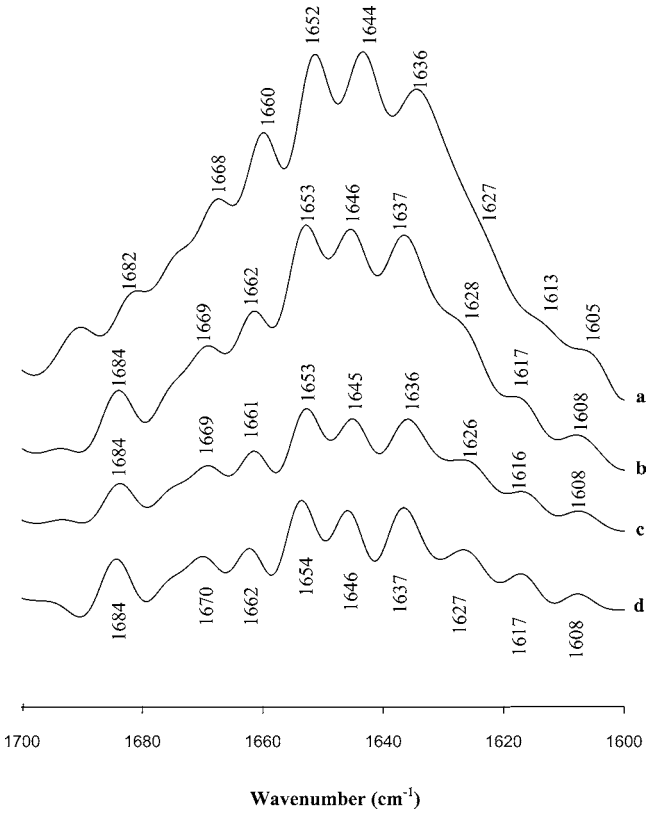

Figure 6. Stacked plot of deconvoluted infrared spectra of 10% buckwheat globulin (in 0.01 M deuterated phosphate buffer, pD 7.4) in the presence of some protein structure perturbants. (**a**) Control (no additive); (**b**) 10 mM sodium dodecyl sulfate; (**c**) 10 mM *N*-ethylmaleimide; (**d**) 10 mM dithiothreitol.

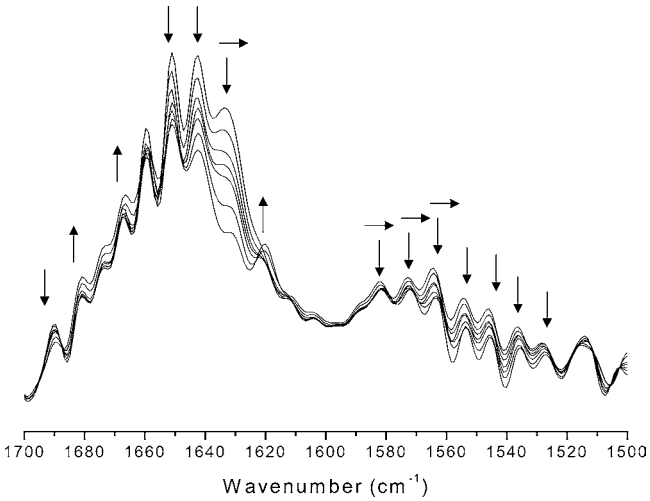

Figure 7. Stacked plot of deconvoluted infrared spectra (1700–1500 cm^{-1}) of 10% buckwheat globulin (in 0.01 M deuterated phosphate buffer, pD 7.4) heated from 50 to 110 °C at 10 °C intervals. The arrows show the increase or decrease of integrated intensities and shifts of band wavenumber with increasing heating temperature.

and the increase in negative charge leading to unfolding of the polypeptides (*38*), thus leading to denaturation (lower ΔH). The decrease in ΔH due to protein unfolding could be balanced by exothermic events such as aggregation (*39*), since endothermic changes are associated with the rupture of hydrogen bonds, while exothermic changes include weakening of hydrophobic interactions and aggregation of proteins (*40*). On the other hand, BWG may form some intermolecular β-sheet structures in the presence of 10 mM SDS or form the intermediate when the protein is undergoing the unfolding process, resulting in an increase in T_d value. The present result also implied the aggregation of BWG in the presence of low SDS concentration (10 mM).

The band intensity of β-strand (1626 cm^{-1}) and antiparallel β-sheet (1616 and 1684 cm^{-1}) was also increased in the presence of 10 mM NEM (**Figure 6**) and the β-turn content was decreased, suggesting protein unfolding and rearrangement of protein molecules. The increased antiparallel β-sheet intensity may be attributed to protein aggregation following the unfolding of polypeptides, at the expense of β-turn structure. Addition of NEM caused a significant ($p \leq 0.05$) reduction in ΔH suggesting protein unfolding and/or aggregation, whereas T_d was significantly increased (**Table 2**). Protein aggregation, an exothermic reaction, may decrease the overall ΔH values in DSC (*31*). The increase of T_d could be attributed to the formation of a complex between BWG and NEM assuming a conformation which is more heat stable. NEM, a sulfhydryl-blocking reagent, blocks free thiol groups on protein molecules and thus prohibits sulfhydryl−disulfide (SH−SS) interchange reaction (*41*). BWG was found to have relatively high disulfide content (36.4 μM/g protein) but low sulfhydryl content (3.2 μM/g protein). In this case, BWG appeared to aggregate when the sulfhydryl groups were blocked by NEM. The enhanced aggregation may be attributed to increased molecular flexibility and interactions via nonspecific bonding between protein molecules in the presence of NEM. Both IR and DSC data show that the conformation of BWG was affected after blocking of the sulfhydryl groups by NEM, implying that SH−SS interchange reactions are important in stabilizing the conformation of BWG.

In the presence of 10 mM DTT, increases in antiparallel β-sheet (1617 and 1684 cm^{-1}) band intensity and a reduction in β-turn content (**Figure 6**) were observed, indicating changes in protein conformation. Prominent increases in 1617 and 1684 cm^{-1} band intensity suggest the formation of hydrogen bonded β-sheet structures associated with aggregate formation. DTT is a reducing agent which can break up disulfide bonds in the BWG oligomers to create a destabilized conformation and lead to aggregation. In the DSC study, DTT seems to show a less pronounced influence in thermal characteristics, with a slight but significant decrease in ΔH but no significant ($p > 0.05$) change in T_d (**Table 2**). The addition of each of the three chemicals led to marked increases in the $\Delta T_{1/2}$ value (**Table 2**), suggesting that BWG would denature in a less cooperative fashion in the presence of these perturbants.

Both FTIR and DSC data show that noncovalent interactions such as hydrophobic and ionic interactions and hydrogen bonding interactions seem to be the major chemical forces contributing to the thermal stability and conformational stability of BWG. Conformational changes due to the blocking of sulfhydryl groups by NEM or the reduction of the disulfide bonds by DTT were also observed by FTIR spectroscopy, indicating the importance of covalent forces (disulfide bonding and SH−SS interchange reaction) in maintaining conformational stability in BWG. However, such changes were less pronounced when observed by DSC. Hence, FTIR spectroscopy seems to be a more sensitive technique than DSC in monitoring conformational changes in proteins, based on the instruments used in this study. Moreover, the spectroscopic technique can provide more detailed information on the secondary and tertiary structures of the protein, whereas DSC only measures overall changes in conformation.

Effect of Heating Temperature. Figure 7 shows the overlapping of BWG dispersions heated at increasing temperature

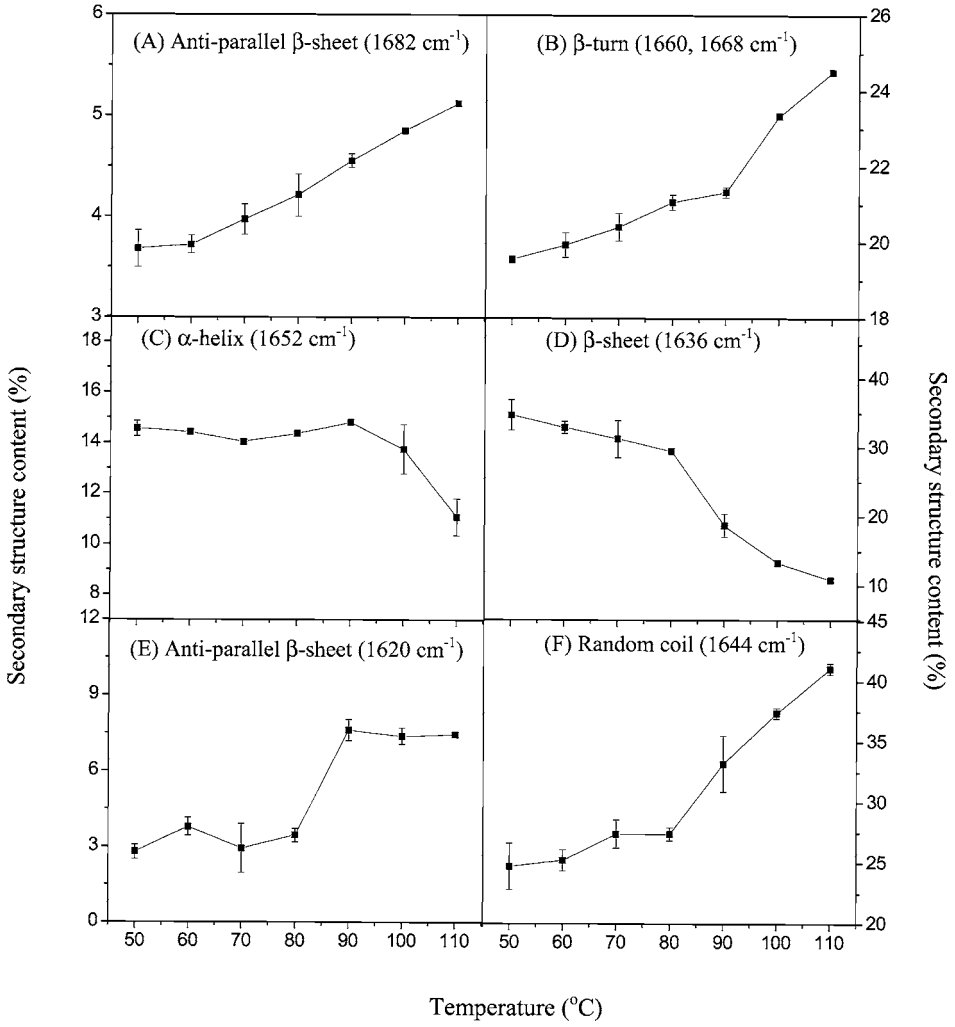

Figure 8. Effect of heating on the secondary structure contents of 10% buckwheat globulin (in 0.01 M deuterated phosphate buffer, pD 7.4). The bars represent standard deviations of the means.

from 50 °C to 110 °C. The major changes observed during heating were a pronounced decrease and shift of the β-sheet band (1636 cm^{-1}) to lower wavenumber (**Figure 8**), suggesting protein unfolding and denaturation. Upon heating, the antiparallel β-sheet band at 1620 cm^{-1} started to appear at 80−90 °C (**Figure 7**) and there was a marked increase in the intensity of the 1682 cm^{-1} band indicating onset of aggregation (*16*, *37*).

Pronounced changes in secondary structure contents were observed when BWG was heated above 80−90 °C (**Figure 8**). The contents of α-helical and β-sheet structures were not markedly changed when BWG was heated from 50 °C to 80 °C, but progressive decreases in the ordered structures (α-helix and β-sheets) were observed after reaching 90 °C. On the other hand, β-turn, antiparallel β-sheet, and random coil structures were markedly increased when BWG was heated to 90 °C. Turns can be a product of the unfolding of any higher order structures, where antiparallel β-strands can be formed as intermolecular β-sheets at the interphase of some aggregated molecules of the protein (*22*, *42*). The development of aggregates through the formation of antiparallel β-sheet structures is characterized by intensity increase in the 1620 cm^{-1} band indicating a net increase in aggregation upon heating (*16*).

The FTIR data show that thermal denaturation and aggregation of BWG were initiated at about 90 °C, which is well below its T_d (close to 100 °C) (**Table 2**) but close to its onset temperature (91.2 °C) (data not shown) determined by DSC. The results again suggest that FTIR is a more sensitive technique than DSC in detecting thermal denaturation of BWG, using the present instrumentation. In studying thermal denaturation of β-lactoglobulin by FTIR and DSC, the DSC onset temperature (T_m) rather than the peak temperature (T_d) was found to correlate with the denaturation temperature based on changes in IR amide I bands (*43*). For red bean globulin, protein unfolding and structural reorganization also occurred at temperatures well below the T_d and T_m by DSC, while thermal aggregation was preceded by thermal denaturation and occurred at temperature close to T_m (*13*).

A similar observation was obtained in the current study; the DSC onset temperature of BWG was determined to be 91.2 °C (data not shown), close to the temperature at which denaturation was observed by FTIR. The results suggest that the DSC onset temperature was correlated to the denaturation temperature based on FTIR data. However, since aggregation was also observed by FTIR near the onset temperature (increases in peak intensity of the two aggregation bands), it is difficult to determine whether the DSC peak temperature (∼100 °C) corresponds to the aggregation temperature of BWG. Despite the relatively high thermal stability, BWG can aggregate under relatively mild heat treatments (at ∼10 °C below the T_d).

There was a general decrease in band intensities in the amide II′ region (1600−1500 cm^{-1}) of BWG (**Figure 7**) with shifts of bands to lower wavenumbers, indicating progressive unfold-

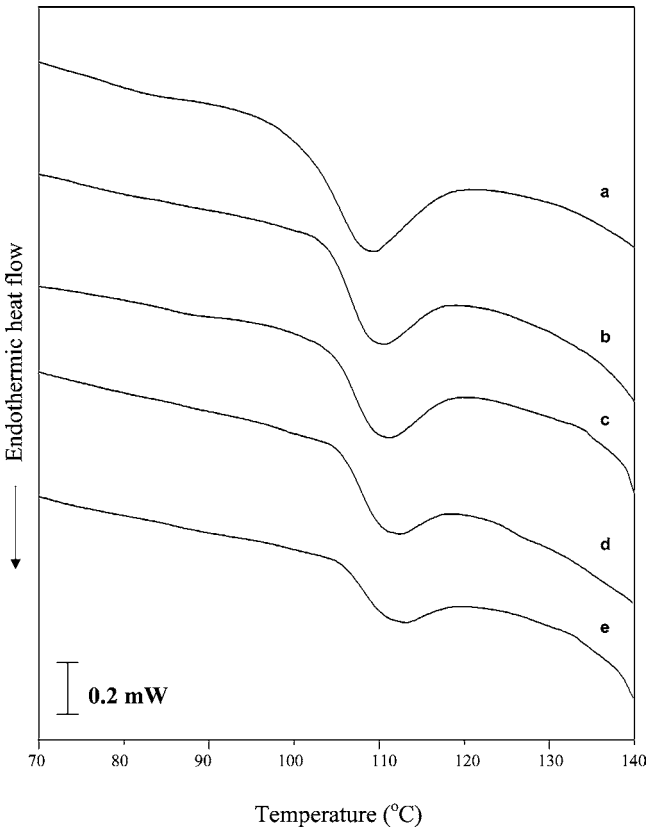

Figure 9. Differential scanning calorimetric thermograms of buckwheat globulin preheated at 100 °C for (**a**) 0 min; (**b**) 5 min; (**c**) 10 min; (**d**) 30 min; (**e**) 60 min.

ing of protein (*9*, *16*). The presence of amide II′ absorption indicates that not all of the protein's peptide hydrogen atoms have been exchanged for deuterium, probably due to the compact inaccessible form of the native, folded protein molecules (*11*, *16*). Decreases in amide II′ band intensities show that such exchange was accelerated by heat treatment as heat-induced protein unfolding increased solvent accessibility.

Effect of Heating Time. The DSC thermograms of BWG preheated at 100 °C for different time periods are shown in **Figure 9**. There were progressive decreases in the size of the endothermic peaks and increases in T_d suggesting partial denaturation with increasing heating time. Preheated BWG showed pronounced decreases in ΔH and $\Delta T_{1/2}$ values, suggesting protein denaturation and aggregation by preheat treatment in a highly cooperative manner. Moreover, the breakup of hydrophobic interactions and protein aggregation are exothermic reactions which could lower the net endothermic contribution causing a decrease in ΔH (*39*, *40*, *44*). For oligomeric proteins with complex quaternary structures such as BWG, heating may cause association/dissociation of the oligomer and disruption of the quaternary structure resulting in aggregation (*45*−*49*).

The increases in thermal stability (as indicated by higher T_d) in the preheated BWG may be due to the rearrangement of the protein to assume a more compact conformation or the association of the protein molecules to a complex network structure of aggregates which have higher thermal stability. On the other hand, unfolding of native protein may cause the exposure of buried apolar groups, enhancing protein−protein interactions by hydrophobic association. Subsequent heating of the heat-associated BWG would require the rupture of more hydrophobic groups than in the native protein, resulting in higher T_d.

CONCLUSION

The present study demonstrated that the secondary structure conformation of BWG was influenced by pH, chaotropic salts, some protein structure perturbants, and heat treatments, indicating the involvement of both covalent and noncovalent forces in stabilizing the conformation of BWG. Heat-induced aggregation of BWG was observed in the heat-treated samples as indicated by prominent bands associated with aggregated strand structures, at a temperature well below the DSC denaturation temperature. As a potential food ingredient, BWG will be subjected to various processing conditions during food manufacturing, leading to conformational and structural changes. Monitoring of these changes is important as they may impact on the nutritional and functional properties of the processed foods (*14*). As demonstrated in this study, both FTIR and DSC are appropriate analytical techniques for monitoring structural changes in BWG and other plant proteins with limited solubility. Moreover, these techniques can also be used to monitor heat-induced protein aggregation/precipitation in situ.

ABBREVIATIONS USED

FTIR, Fourier transform infrared; DSC, differential scanning calorimetry; BWG, buckwheat globulin; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; T_d, denaturation temperature; ΔH, enthalpy; $\Delta T_{1/2}$, width at half-peak height; T_m, onset temperature; SH−SS, sulfhydryl−disulfide.

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