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Structural characterization of globulin from common buckwheat (*Fagopyrum esculentum* Moench) using circular dichroism and Raman spectroscopy

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Abstract

Raman and far-UV circular dichroism (CD) spectroscopy was used to study the conformation of globulin from common buckwheat (*Fagopyrum esculentum* Moench) (BWG) under the influence of various buffer environments and heat treatments. Secondary structural analysis of BWG by CD spectroscopy yielded 15.0% α-helical, 25.8% β-sheet, 28.9% β-turn and 30.3% random coil contents. Raman spectrum also showed β-sheets as the major secondary structure in native BWG. Chaotropic salts caused band shifts and intensity changes in Raman amide III vibration, indicating transitions from β-sheet to disordered structure following the lyotropic series of anions. Extreme pHs and several protein structure perturbants led to changes in CD and Raman spectral characteristics, demonstrating protein unfolding and denaturation. Increasing heating time at 100 °C induced the appearance of anti-parallel β-sheet (1235–1237 cm^{-1}) and caused a progressive increase in random coil content, suggesting protein denaturation and aggregation. Both non-covalent and covalent interactions play important roles in stabilizing the conformation of BWG.

Keywords: *Fagopyrum esculentum* Moench; Buckwheat globulin; Secondary structure; Raman spectroscopy; Circular dichroism

1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is an underutilized pseudocereal and its seed storage proteins are nutritionally important because of their high and balanced essential amino acids contents and making their biological value much higher than that of other cereal proteins. According to net protein utilization (NPU), buckwheat protein is classified close to animal proteins and has excellent supplementary value to cereal grains (Sure, 1953). Unlike other cereal storage proteins, the salt soluble globulin in common buckwheat (BWG) is the predominant protein fraction. BWG has been classified as a legumin-like storage protein (Derbyshire, Wright, & Boulter, 1976), containing six non-identical monomers that interact non-covalently. Each monomer is composed of disulfide-linked acidic and basic polypeptides with molecular weight ranging from 57.5 to 23.5 kDa (Dunaevsky & Belozersky, 1989). BWG has been found to possess good functional properties comparable to that of a commercial soy protein product (Zheng, Sosulski, & Tyler, 1998). BWG has been studied with respect to chemical composition, physicochemical characterization, polypeptides profiles and genetic background (Belozersky, 1975; Dunaevsky & Belozersky, 1989; Radović, Maksimović, & Varkonji-Gašić, 1996; Rout & Chrungoo, 1996, 1997; Rout, Chrungoo, & Rao, 1997; Tomotake, Shimaoka, Kayashita, Nakajoh, & Kato, 2002). However, there have been few studies on the structure and conformation of BWG. A detailed understanding of the structure–function relationship in BWG is essential for its full utilization in food industry.

Conformational changes of BWG under the influence of various buffer conditions and heat treatments have been

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studied by differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR) (Choi & Ma, 2005). In the present investigation, circular dichroism (CD) and Raman spectroscopy will be used to further study the effect of environmental conditions and heat treatments on the conformation of BWG.

CD spectroscopy is an optical technique that allows the detection and quantitation of the chirality of molecular structures and provides information about the secondary and tertiary structures of proteins. Optical activity of α-helix in the far-UV region permits the use of CD spectroscopy to investigate conformational changes in proteins, and the CD band positions for various structures, the α-helix, the interchain hydrogen bonded β-structure and a fully extended parallel or antiparallel arrangement of peptide chains have been reported (Chen, Yang, & Martinez, 1972; Matsuura & Manning, 1994; Sarkar & Doty, 1966). Empirical methods have been developed which utilized reference databases consisting of spectra of proteins with known structures, allowing the decomposition of the CD spectrum of an unknown protein, and providing information on secondary structural features (Sreerama & Woody, 1993). It is often used to complement the more detailed structural information available from other techniques. CD measurements are fast and simple and have been used frequently to calculate the relative proportions of secondary structures. However, it has a limitation that only clear and highly diluted samples can be analyzed. Interference due to absorbance of various salts and buffer substances in the far-UV region also limits the use of CD spectroscopy in studying the effects of environmental conditions such as chaotropic salts and some protein structure perturbants on protein conformation (Stanley & Yada, 1992).

On the contrary, Raman spectroscopy can be used to monitor changes in protein solution even at higher concentration. Raman spectroscopic analysis is based on the inelastic scattering of photons resulting from vibrational transitions of the functional groups of the molecules. Both the frequency and intensity of molecular vibrations can provide information on the microenvironment of various amino acid side chains as well as on the conformation of the polypeptide backbone, as indicated by changes in Raman spectrum (Li-Chan, Nakai, & Hirotsuka, 1994; Tu, 1986). Raman spectroscopy has the advantage of being applicable to solid, liquid or aggregated food systems for investigating *in situ* protein structural changes during denaturation, aggregation and gelation. It is usually more suitable for *in vivo* or *in situ* study of foods that are primarily aqueous in nature since water has weak Raman scattering properties and thus less interference in Raman spectroscopy (Li-Chan, 1996a; Li-Chan et al., 1994). Hence, Raman spectroscopy has a distinct advantage over FTIR spectroscopy which cannot be used in aqueous systems due to strong interference of the water band in the IR spectrum. Another important feature of the Raman technique is that spectral vibrational modes tend to be sharper when compared to those of an IR spectrum.

2. Materials and methods

2.1. 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 (Osborne & Mendel, 1914). The protein content of BWG was 93.9% (dry basis), determined by the micro-Kjeldahl method (Kjeltec, 1986) using a nitrogen-to-protein conversion factor of 5.53 (Tkachuk, 1969). BWG was found to contain 0% crude fat, 5.49% carbohydrate and 0.607% ash.

2.2. Circular dichroism spectroscopy

BWG solutions (0.01%) were prepared in 0.01 M phosphate buffer (pH 7.4) and were filtered by 0.02 μm membrane prior to CD measurements. To study the effect of pH, 0.01 M phosphate buffers with desired pH (3, 5, 7, 9, 11) were prepared by adding 0.1 N HCl or 0.1 N NaOH. The changes in secondary structure upon addition of 10 mM and 20 mM sodium dodecyl sulfate (SDS) and 20%, 40% and 60% ethylene glycol (EG) were also studied. The effect of chaotropic salts on BWG conformation was not shown since preliminary experiments resulted in noisy CD spectra when these salts were added. For the heating experiments, aliquots of protein solutions in covered test tubes were heated at 100 °C for various time intervals (5, 10, 30 and 60 min) and then cooled immediately in an ice bath. The heated solutions were centrifuged at 10,000*g* for 10 min, and the clear supernatant, containing the buffer-soluble aggregates, was subjected to CD measurement.

The CD spectra were obtained using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) calibrated at 290.5 nm with ammonium *d*-10-camphorsulphonate. The spectra were recorded from 200 to 250 nm in 0.1 cm quartz cells with resolution of 0.2 nm, scan speed of 20 nm/min, time constant of 2.0 s, 1.0 nm band width and sensitivity of 20 mdeg. CD spectra were corrected for solvent contributions and were expressed in terms of specific ellipticities versus wavelength. Estimation of secondary structure composition was performed using the Jasco SSE-338 Protein Secondary Structure Estimation Program (Japan Spectroscopic Co. Ltd., Tokyo, Japan), which was based on CD spectra of reference proteins of known secondary structures (Yang, Wu, & Martinez, 1986). Analyses were performed in triplicate.

2.3. Raman spectroscopy

BWG dispersions (5% w/v) were prepared in different buffers. For the control, freeze-dried protein samples were dispersed in distilled water. Protein dispersions with desirable pH were prepared by adding 0.1 N HCl or 0.1 N NaOH. Chaotropic salts (1.0 M) and some protein structure

perturbants (10 mM) such as sodium dodecyl sulfate (SDS), *N*-ethylmaleimide (NEM) and dithiothreitol (DTT) were added as solids to the protein dispersions. To study effect of heat treatments, BWG solutions (1%, w/v) were prepared in 0.01 M phosphate buffer containing 1.0 M NaCl at pH 7.4 and were heated at 100 °C for different time intervals (5, 10, 30 and 60 min). After heating, the samples were immediately cooled in an ice bath for 5 min, and the solutions were centrifuged at 10,000*g* for 20 min at 4 °C to separate into buffer-soluble and insoluble aggregates. The supernatant containing soluble aggregates (SA) was dialyzed exhaustively against distilled water at 4 °C, while the precipitate containing the insoluble aggregates (ISA) was washed several times with distilled water. Both soluble and insoluble aggregates were then freeze-dried as solid form for Raman analysis. Preliminary experiments showed that freeze-dried protein samples exhibited Raman spectra identical to those in dispersions or wet pellets (not shown), indicating that freeze-drying did not affect the conformation of BWG.

Raman spectra were collected on a Renishaw-Raman Imaging Microscope (system 1000) equipped with a 514 nm argon ion laser (Spectra Physics Co., Mountain View, CA, USA). Frequency calibration was performed using the silicon line at 520 cm^{-1}. Protein dispersions were introduced into glass capillary tubes as pellet and tubes were then held on microscope slides. Raman spectra were recorded at room temperature under the following conditions: laser power, 100 mW; number of scan, 20; exposure time, 30 s and spectral resolution, 2 cm^{-1}. All experiments were performed in dark to reduce the possibility of light interfering spectrograph and overwhelming the Raman spectra by cosmic ray events. The spectral data were baseline-corrected using the GRAMS/32 AI Software (Galatic Industries Corporation, Salem, NH, USA), and the spectral intensities were normalized to the intensity of the phenylalanine band at 1001 ± 1 cm^{-1} as internal standard (Howell & Li-Chan, 1996; Li-Chan et al., 1994; Tu, 1986). The Raman spectra were plotted as relative intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}). All analyses were performed in triplicates.

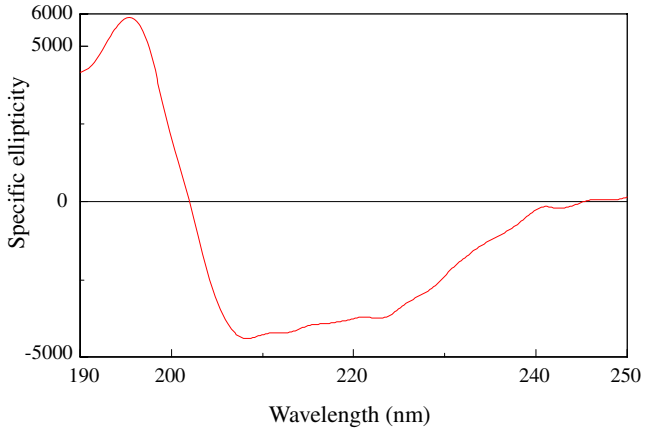

Fig. 1. Circular dichroism spectrum of buckwheat globulin (0.01% in 10 mM phosphate buffer, pH 7.4).

2.4. Statistical analysis

Analysis of variance and Duncan's multiple range tests were performed on CD data at $p \leqslant 0.05$ level, using a standard statistical software package.

3. Results and discussion

3.1. Secondary structure analysis of buckwheat globulin

The CD spectrum of BWG in the far-UV region (Fig. 1) shows a negative minimum at 208 nm and a shoulder around 215–220 nm, indicating involvement of α-helical conformation in BWG for stabilizing the molecular structure. A positive band of comparable magnitude was also observed at 196 nm, the characteristic CD features of β-sheet, suggesting prominent β-sheet content in BWG (Fig. 1). The BWG spectrum is consistent with the general features of α-helices and β-sheets, in which the CD spectrum of α-helices has an intense positive band at about 190 nm and two negative bands at about 208–210 nm and 222 nm, while that of β-sheets has a fairly intense positive band at about 198 nm and a negative band at about 215–220 nm (Woody, 1996). As shown in Table 1, quantitative estimation of the relative amounts of α-helical, β-sheet, β-turn and random coil fractions in BWG was 15.0%, 25.8%, 28.9% and 30.3%, respectively. Since the α-helical, β-sheet and β-turn fractions were found to account for

Table 1
Circular dichroism analysis of buckwheat globulin under the effect of two protein structure perturbants

	Secondary structure content (%)			
	α-Helix	β-Sheet	β-Turn	Random coil
Control	15.0 ± 0.13[A]a	25.8 ± 0.50a	28.9 ± 0.48a	30.3 ± 0.88a
20% EG	20.1 ± 0.30b	15.6 ± 1.80b	32.3 ± 0.20b	32.0 ± 0.50a
40% EG	22.3 ± 0.75c	12.3 ± 1.50b	32.9 ± 0.90b	32.5 ± 0.25a
60% EG	23.1 ± 1.20c	16.3 ± 0.20b	30.5 ± 0.55a	30.1 ± 0.15a
10 mM SDS	7.55 ± 0.85d	35.9 ± 1.50c	14.4 ± 0.25c	42.2 ± 0.90b
20 mM SDS	7.25 ± 1.35d	37.1 ± 2.60c	13.6 ± 0.30c	42.1 ± 1.50b

EG, ethylene glycol; SDS, sodium dodecyl sulfate.
[A] Mean of triplicate determinations ± SD. Means in a column bearing the same letter are not significantly different ($p > 0.05$).

approximately 70% of the secondary structure, the native BWG can be considered as a protein with highly ordered and stable protein conformation, similar to other 11S globulins.

The calculated percentages of β-sheets and β-turns in BWG differ somewhat from those obtained from a previous CD study (Marcone, Kakuda, & Yada, 1998) which showed that globulin from buckwheat contained 32.0% β-sheets, 22.0% β-turns, 29.3% random coils, and 16.8% α-helical structures. A higher percentage of β-turn structure was obtained in the present study, but was still within the range (23.7–32.0%) shown in other plant globulins (Marcone et al., 1998). Differences in the proportion of the secondary structure composition may be due to the differences in protein source and extraction method. Both CD data support that β-sheets and β-turns are a predominant portion of BWG secondary structure (54.0–54.7%), which is in good agreement with earlier FTIR estimation of 54.5% β-sheets and β-turns in native BWG (Choi & Ma, 2005).

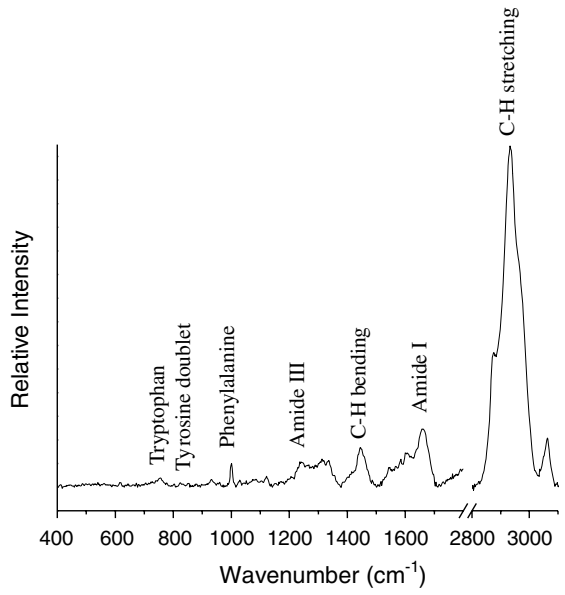

Fig. 2. Raman spectrum (400–3100 cm^{-1}) of buckwheat globulin dispersion (5%, w/v) in distilled water (wet pellet).

Fig. 2 shows a typical Raman spectrum of 5% BWG dispersion in distilled water. Table 2 summarizes the tentative assignment of some Raman bands based on comparison with Raman spectral data reported by previous workers (Careche & Li-Chan, 1997; Li-Chan & Nakai, 1991; Li-Chan & Qin, 1998; Li-Chan et al., 1994; Nonaka, Li-Chan, & Nakai, 1993; Peticolas, 1995; Tu, 1986). The –CO–NH– amide or peptide bond has several distinct vibrational modes, with the amide I band near 1650 cm^{-1} and the amide III band near 1250 cm^{-1} being the most easily characterized secondary structure of protein. Due to the possible overlap of bands from the solvent water in the amide I region and from various C–H bending and aromatic ring vibrations in the amide III region, both regions are recommended to be analyzed in order to obtain more reliable interpretations of the composition and changes in the protein secondary structure (Bouraoui, Nakai, & Li-Chan, 1997; Li-Chan et al., 1994). Seven major bands in the Raman spectrum of BWG (Fig. 2) were identified and were centered at 757 cm^{-1} (tryptophan band), 826 cm^{-1} (tyrosine doublet), 849 cm^{-1} (tyrosine doublet), 1239 cm^{-1} (amide III), 1446 cm^{-1} (C–H bending), 1663 cm^{-1} (amide I) and 2933 cm^{-1} (C–H stretching). The location of amide III and amide I bands indicated that β-sheet and disordered structures were the major secondary structures in BWG, which are consistent with the CD and FTIR data (Choi & Ma, 2005).

Our findings showed that BWG, similar to most plant globulins, has a large quantity of β-sheet and random coil structures and low α-helical content, in good agreement with previous CD analysis which showed seed storage proteins have consistently low α-helical and high β-sheet contents (Jacks, Barker, & Weingang, 1973). Analysis with 21 plant seed globulins from both monocotyledonous and dicotyledonous plants again revealed a typically low α-helical content and a large quantity of β-type and random coil structures. In addition to these general observations, striking similarities were also noted in the amounts of β-turn and random coil fractions (23.7–32.0%) in each globulin (Marcone et al., 1998). It should be noted that α-helical and β-sheet fractions have the tendency to be deeply buried within the polypeptide chain (Hopp, 1986; Hopp & Woods, 1982). The observed similarity in the amounts of α-helix

Table 2
Tentative assignment of some bands in the Raman spectrum of buckwheat globulin (in distilled water)

Assignment	Wavenumber (cm^{-1})	Structural information
Tryptophan	760	Sharp intense line for buried residue; intensity diminished on exposure or environmental change
Tyrosine	830, 850	State of phenol – OH (exposed or buried, hydrogen bond donor or acceptor)
Phenylalanine	1001	Conformation insensitive; useful as an internal intensity standard
Amide III	>1275	α-Helix
	1235 ± 5	Antiparallel β-sheet
	1245 ± 4	Disordered structure
C–H bending	1450	Microenvironment, polarity
Amide I	1655 ± 5	α-Helix
	1670 ± 3	Antiparallel β-sheet
	1665 ± 3	Disordered structure
C–H stretching	2930	Microenvironment, polarity

and β-sheet structures shared by all seed globulins may, therefore, indicate that their interior conformations are very similar or highly conserved.

3.2. Effect of pH

The effect of pH on CD spectral characteristics of BWG is shown in Fig. 3. Secondary structural analysis of BWG samples at different pH showed that extreme acidic (pH 3) and alkaline (pH 11) pHs caused a marked decrease in β-sheet fraction and an increase in random coil content (Fig. 4), suggesting protein denaturation. Moreover, a noticeable decrease in α-helical fraction was observed at acidic pH (pH 3–5), but not at alkaline pH. In addition, the β-turn fraction was largely increased at acidic condition, suggesting that the denaturing effect was more pronounced at acidic condition (pH 3–5). The lowest random coil content was found at neutral pH. Similar observations have also been obtained by FTIR study (Choi & Ma, 2005), in which extreme pHs led to decreases in β-sheet and α-helical contents and an increase in random coil content, indicating protein unfolding. DSC results showed that highly acidic (pH 3) and alkaline (pH 11) pHs caused decreases in denaturation temperature (T_d) and enthalpy (ΔH) values, suggesting loss of thermal stability and protein denaturation. Moreover, the changes in thermal characteristics were more pronounced at acidic conditions (pH 3–5) (Choi & Ma, 2005).

The effect of pH on the Raman spectral characteristics of BWG was also studied. At pH 5–7, bands in the amide III region were centered at 1239 cm^{-1}, indicating a predominance of β-sheet structure. Since this pH region corresponds to the isoelectric pH range of BWG (pH 4–6.8) (Radović et al., 1996; Tomotake et al., 2002), the repulsive forces should be low and the proteins were expected to remain in their native conformation with predominantly ordered secondary structures. At extreme pHs, there were shifts in the amide I and III vibrations, which suggests a transition from β-sheets structure near neutral pH to disordered structures.

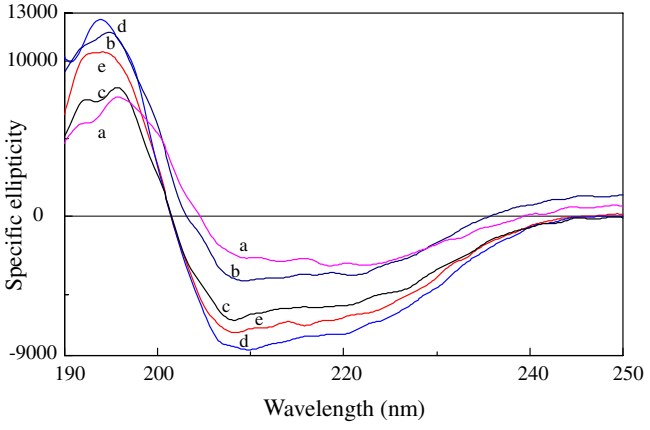

Fig. 3. Circular dichroism spectra of buckwheat globulin (0.01% in 10 mM phosphate buffer) at different pH: (a) pH 3; (b) pH 5; (c) pH 7; (d) pH 9; (e) pH 11.

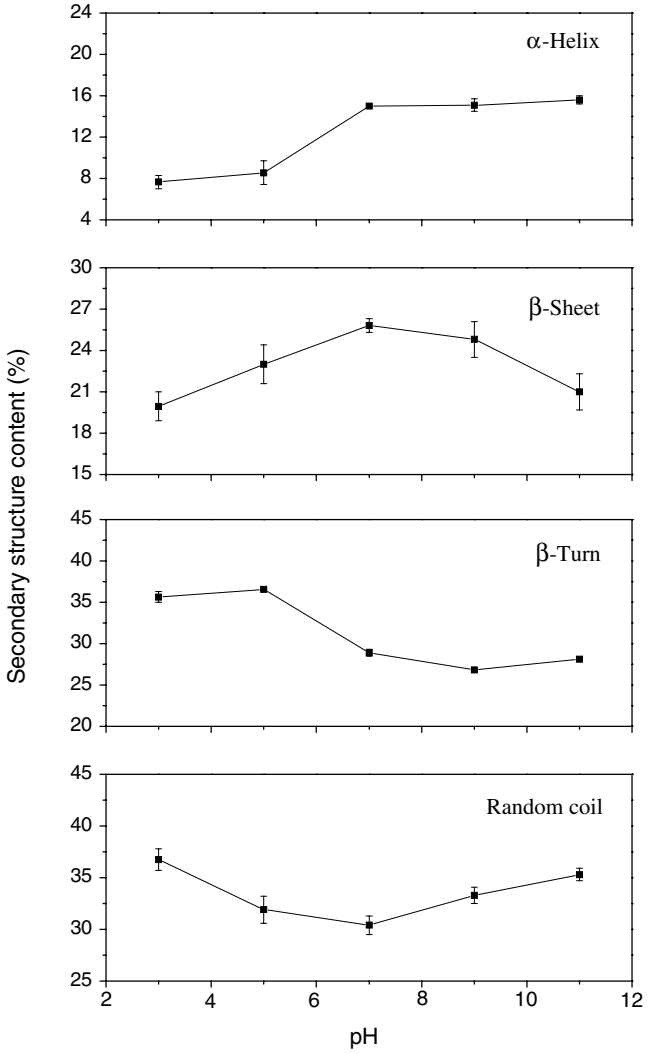

Fig. 4. Effect of pH on secondary structure content of buckwheat globulin examined by circular dichroism spectroscopy. The error bars represent standard deviations of the means.

Fig. 5 shows the effect of pH on the intensities of some major Raman bands. The intensities of amide I and III band located at disordered structures were higher at acidic pH than at neutral or alkaline pH, indicating more pronounced protein denaturation at acidic pH. Increases in intensities of C–H bending and C–H stretching vibrations were observed at extreme pH conditions. Increases in C–H bending band intensity may be attributed to increased exposure of hydrophobic groups to a more polar environment, whereas increases in C–H stretching band intensity may be due to increasing polarity at highly acidic and alkaline pH conditions (Bouraoui et al., 1997). The increase in the intensity of C–H stretching band has been suggested as an indication of protein denaturation (Ma, Rout, Chan, & Phillips, 2000). The tryptophan band intensity was decreased at either extreme acidic (pH 3) or alkaline (pH 11) pH conditions (Fig. 5), suggesting that the buried tryp-

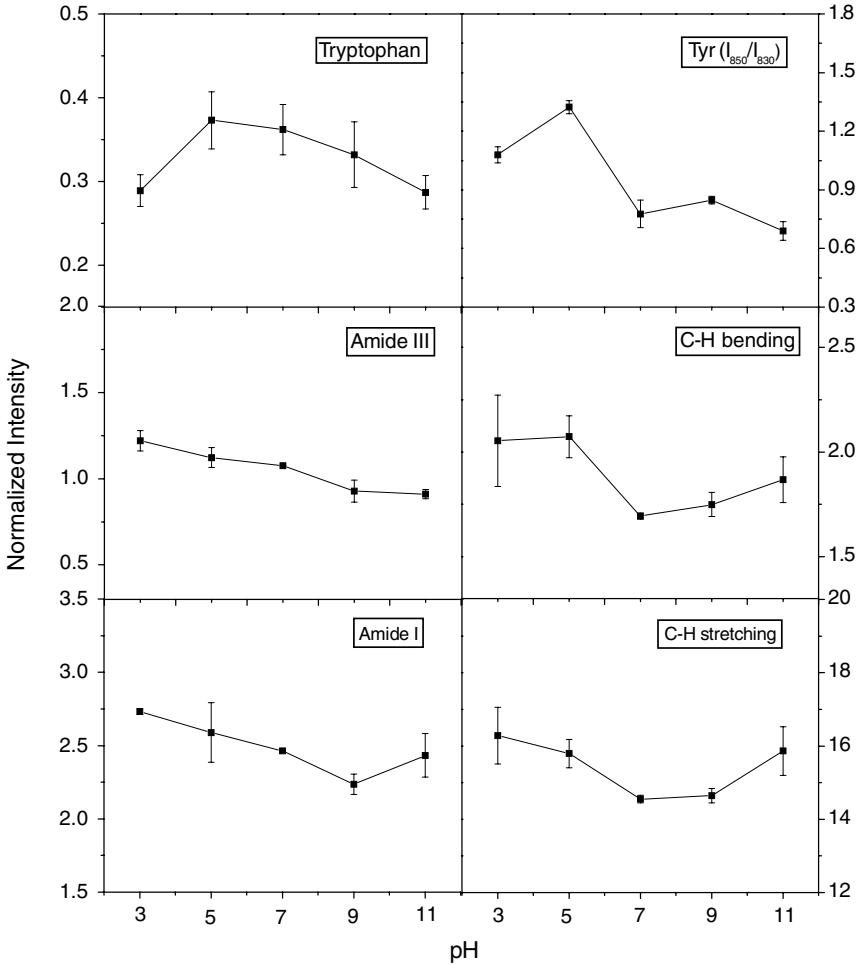

Fig. 5. Effect of pH on normalized intensity of several regions in Raman spectrum of buckwheat globulin. The error bars represent standard deviations of the means.

tophan residues in hydrophobic microenvironment became exposed to polar aqueous solvent (Li-Chan, 1996a). The intensity ratio of the tyrosine doublet band intensity (I_{850}/I_{830}) was increased at acidic pHs (pH 3 and 5), indicating exposure of tyrosine residues at extreme pH and their involvement as simultaneous hydrogen bond acceptors or donors (Li-Chan et al., 1994; Tu, 1986).

Both CD and Raman data demonstrated destabilization of native conformation in BWG at extreme pH conditions. In addition, the results showed more pronounced changes in secondary structures under acidic condition (pH 3–5), which were also observed by FTIR and DSC studies (Choi & Ma, 2005). Most proteins are stable near the isoelectric pH, when the repulsive forces are low and the proteins remain in a native state. At high or low pH, large net charges are induced and repulsive forces increase, resulting in protein unfolding (Morrissey, Mulvihill, & O'Neill, 1987). The unfolding of proteins at extreme pH conditions may also be attributed to rupture of hydrogen bonds and a breakup of hydrophobic interactions (Privalov & Khechinashvilli, 1974). Since pH is one of the important extrinsic factors affecting protein structure and function, food products containing BWG with desirable functional characteristics can be obtained by controlling the pH condition.

3.3. Effect of chaotropic salts

Fig. 6 shows the effect of several chaotropic salts (1.0 M) on Raman spectral characteristics of BWG. Shifts in amide III vibration to higher wavenumbers with progressive decreases in band intensity were observed, suggesting transitions from β-sheet structure (1236 and 1239 cm^{-1}) in the presence of chloride (Cl^-) or bromide (Br^-) to disordered structure (1246 and 1250 cm^{-1}) in the presence of iodide (I^-) or thiocyanate (SCN^-). The location of the amide I band showed that disordered structure (1664 cm^{-1}) is predominant in BWG under the influence of these anions. The tryptophan band (752 cm^{-1}) was intensified by the addition of SCN^-, indicating that trytophan residues became buried. The amide I band intensity was progressively increased when the sodium salt was changed from Cl^- to Br^-, I^- and SCN^-.

The gradual shifts in Raman band position and changes in band intensity indicate progressive protein unfolding, and the order of change follows the lyotropic series of

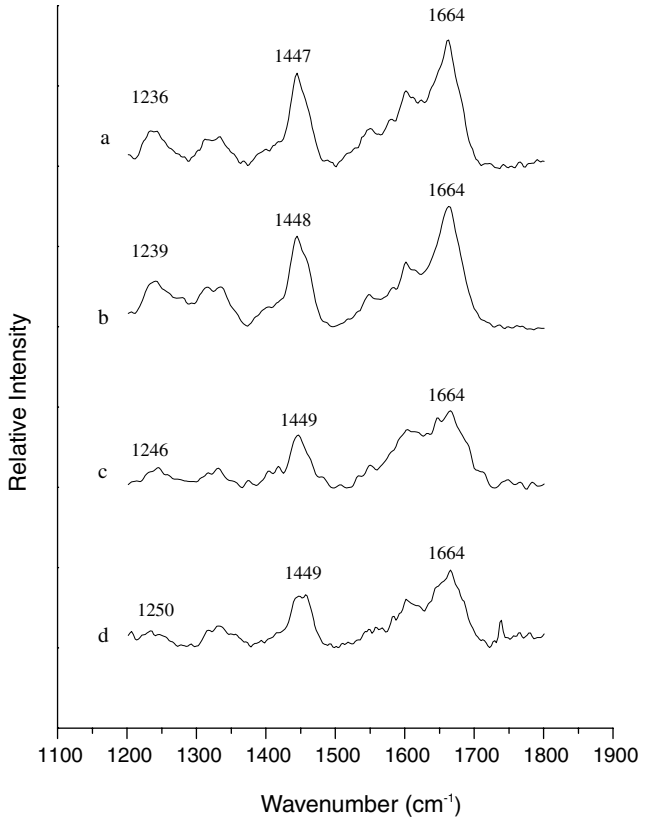

Fig. 6. Raman spectra of buckwheat globulin in the presence of 1.0 M chaotropic salts: (a) sodium chloride; (b) sodium bromide; (c) sodium iodide; (d) sodium thiocyanate.

anions (Hatefi & Hanstein, 1969). Protein conformation can be perturbed by the addition of salts which affect the electrostatic interactions with the charged groups and polar groups, and influence the hydrophobic interactions via a modification of water structure (Damodaran & Kinsella, 1982; von Hippel & Scheich, 1969). Chloride and bromide can promote salting-out and aggregation due to their high molar surface tension, which could stabilize the protein conformation. On the other hand, iodide and thiocyanatecan promote salting-in, unfolding and dissociation of proteins by their higher hydration energy and steric hindrance (Hatefi & Hanstein, 1969). The present results are in agreement with our previous DSC and FTIR study (Choi & Ma, 2005) that showed progressive decreases in T_d and ΔH values as well as reductions in β-sheet and α-helical contents of BWG, implying progressive protein unfolding and denaturation following the lyotropic series. Similar effect of these anions in perturbing the conformation of oat globulin has also been reported (Ma et al., 2000). By understanding the effect of salts on BWG conformation, the functional characteristics of the protein can be better controlled. For example, protein unfolding and denaturation caused by salts are critical for the process of aggregation and subsequent gelation which provides desired textural properties of food products such as gelling or thickening agents.

3.4. Effect of protein structure perturbants

The effect of two protein structure perturbants, EG and SDS on the conformation of BWG was studied by CD spectroscopy. The changes in secondary structure composition under different EG concentration are shown in Table 1. The presence of EG decreased a significant fraction of β-sheets and increased the α-helical and β-turn fractions, indicating conformational changes whereas the random coil content remained fairly constant. There was a progressive increase in α-helical content with increasing EG concentration, suggesting solvent induced the α-helix formation in BWG at the expense of β-sheets structure. The α-helix induction may be caused by reduced hydrogen bonding between protein and solvent. Then, the protein molecules tend to form intramolecular hydrogen bonds, thereby promoting the formation of α-helices. EG increased significantly the proportion of α-helical structure has also been reported in β-lactoglobulin (Dib, Chobert, Dalgalarrondo, & Haertlé, 1995). EG is a water-miscible solvent which could destabilize protein by lowering the dielectric constant of water and weakening the non-polar interactions between protein molecules (Damodaran & Kinsella, 1982; Tanford, 1962). The destabilizing effect of EG on BWG conformation was also observed by DSC study that showed decreases in T_d and ΔH values with increasing EG concentration.

CD data revealed the changes in secondary structure composition of BWG in the presence of 10 and 20 mM SDS (Table 1). With the addition of SDS, there was a dramatic increase in β-sheet and random coil fractions together with significant decreases in α-helical and β-turn fractions, suggesting conformational changes in protein molecules. The increase in β-sheet content indicated that BWG exhibited more stable structure at low concentrations (10 and 20 mM) of SDS. The β-sheet fraction was increased by about 25% at 20 mM SDS as compared to the control. The observed increase in β-sheet structure appeared to be at the expense of the α-helical and β-turn fractions (Table 1). Similar stabilizing effect of low SDS concentration was also observed in DSC study resulting in higher T_d and lower ΔH (Choi & Ma, 2005). Conformational changes of BWG in the presence of 10 mM SDS were also detected by Raman spectroscopy (Fig. 7), as indicated by shifts and increased intensities of amide I and III bands, C–H bending and C–H stretching as well as tyrosine doublet band. SDS is an anionic detergent which interacts with protein molecules by non-covalent forces, causing unfolding and destabilization (Steinhardt, 1975). Due to the amphiphilic character of these surfactants, their interaction with protein is expected to be concentration dependent. Low concentrations of SDS have been shown to stabilize protein against denaturation by highly specific interactions between the cationic groups of proteins and the anionic groups of SDS (Tanford, 1970). The spectral data show that SDS induced changes in the tertiary structure of BWG, possibly by the exposure of hydrophobic groups.

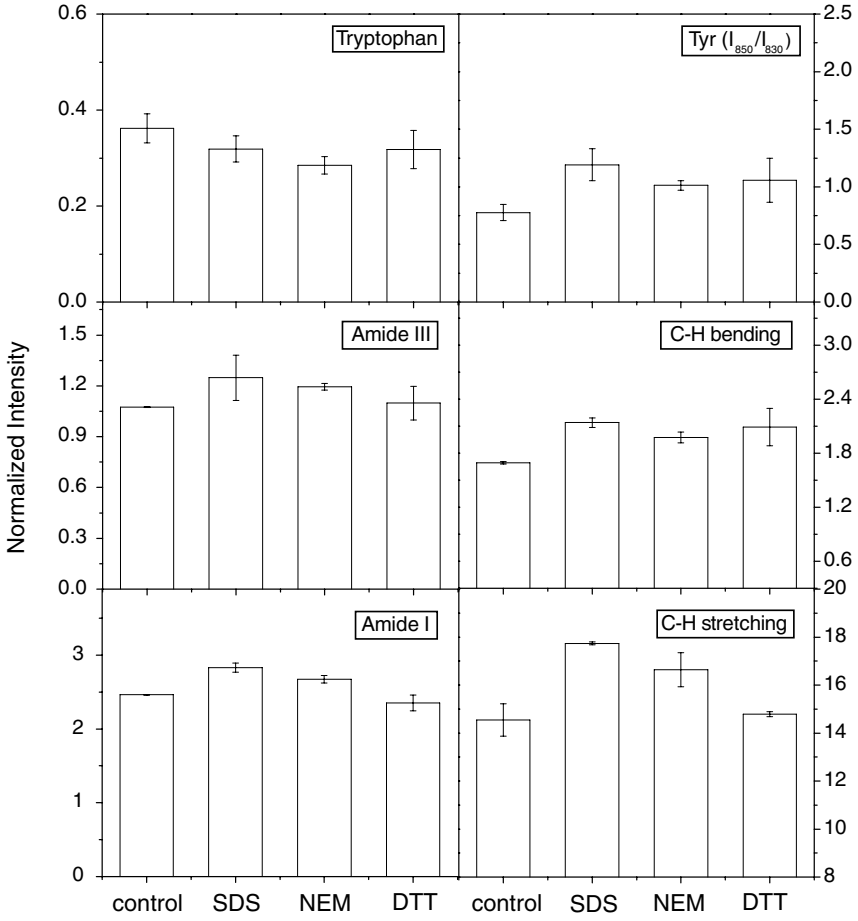

Fig. 7. Effect of some protein structure perturbants on normalized intensity of several regions in Raman spectrum of buckwheat globulin. Control: no additive; SDS: 10 mM sodium dodecyl sulfate; NEM: 10 mM *N*-ethylmaleimide; DTT: 10 mM dithiothreitol. The error bars represent standard deviations of the means.

The effect of some protein structure perturbants on Raman spectral characteristics of BWG was also examined. Addition of NEM led to shifts in the amide I band to higher wavenumbers, suggesting alteration of BWG conformation by NEM. The C–H bending, C–H stretching and tyrosine doublet intensities were increased in the presence of 10 mM NEM (Fig. 7), implicating the perturbation of hydrophobic interactions or interference with the hydrogen bond system and indicating changes in tertiary structure (Li-Chan, 1996b). On the other hand, the tryptophan band intensity was decreased by NEM, suggesting that the tryptophan residues were exposed during the dissociation of protein and protein denaturation resulted (Li-Chan, 1996a). NEM is a sulfhydryl-blocking agent that can block the sulfhydryl–disulfide interchange reactions in protein molecules (Matsudomi, Rector, & Kinsella, 1991). The present data show that blocking the sulfhydryl groups in BWG led to changes in the secondary and tertiary conformation.

Addition of 10 mM DTT caused peak shifts in the amide III region, indicating a transition from β-sheet (1239 cm^{-1}) to disordered structures (1243 cm^{-1}). In the presence of DTT, the tyrosine doublet ratio and C–H bending intensity were increased (Fig. 7), suggesting exposure of hydrophobic groups, protein denaturation and changes in the tertiary structure. DSC data showed a reduction in ΔH value with the addition of 10 mM DTT, suggesting the occurrence of dissociation or partial denaturation of BWG (Choi & Ma, 2005). DTT is a disulfide reducing agent which can break up the disulfide bonds into sulfhydryl groups and destabilize the tertiary structure, allowing conformational changes and exposure of hydrophobic groups (Li-Chan & Nakai, 1991).

The present data show that some protein structure perturbants led to remarkable changes in BWG conformation. Such changes could be attributed to the perturbation of the tertiary and/or quaternary structures of the oligomeric protein by destabilizing the covalent (disulfide bonds and sulfhydryl–disulfide interchange reactions) and non-covalent (hydrogen bonds, electrostatic interactions and hydrophobic interactions) chemical forces that are important for the stabilization of BWG conformation. The results are consistent with those obtained by DSC and FTIR study (Choi & Ma, 2005).

3.5. Effect of heat treatments

Fig. 8 shows the changes in the CD spectral characteristics of BWG heated at 100 °C for different time intervals. With increasing heating time, there was a progressive increase in the random coil content and decrease in α-helical structure in BWG. The fraction of random coil structure was increased at the expense of the ordered α-helical structure, indicating unfolding of protein molecules and protein denaturation during the heat treatment. However, no marked changes were observed in β-type structures. This could be attributed to the balance of a decrease in β-sheet content caused by protein denaturation and an increase in anti-parallel β-sheet conformation from protein aggregation, resulting in relatively constant β-type content. On the other hand, the negative minimum shifted to 206 nm and the shoulder appeared around 217 nm after 60 min heating, suggesting that β-sheet is the major secondary structure in the heated protein sample. The results demonstrated heating of BWG at 100 °C led to both protein denaturation and aggregation. Similar effects of heating on phaseolin of dry beans (*Phaseolus vulgaris* L.) and vicilin of green peas (*Pisum sativum*) studied by CD spectroscopy have been reported (Deshpande & Damodaran, 1989). The α-helical content was lowered upon heating. However, heating did not cause a complete unfolding of phaseolin and vicilin. Instead, both proteins maintained a high degree of structural integrity suggesting possible involvement of strong hydrophobic interactions in stabilizing their structures.

Preliminary experiments showed that the buffer-insoluble aggregate fraction produced very noisy Raman spectra. Hence, only the freeze-dried buffer-soluble aggregates were analyzed in this study. The effect of heating at 100 °C for different time intervals on Raman spectral characteristics of BWG is presented in Fig. 9. Upon heating, shifting of amide III band position was observed, suggesting a transition from predominantly β-sheet (1239 cm^{-1}) conformation to largely disordered (1240–1243 cm^{-1}) structures. A small proportion of the β-sheet structure was located near

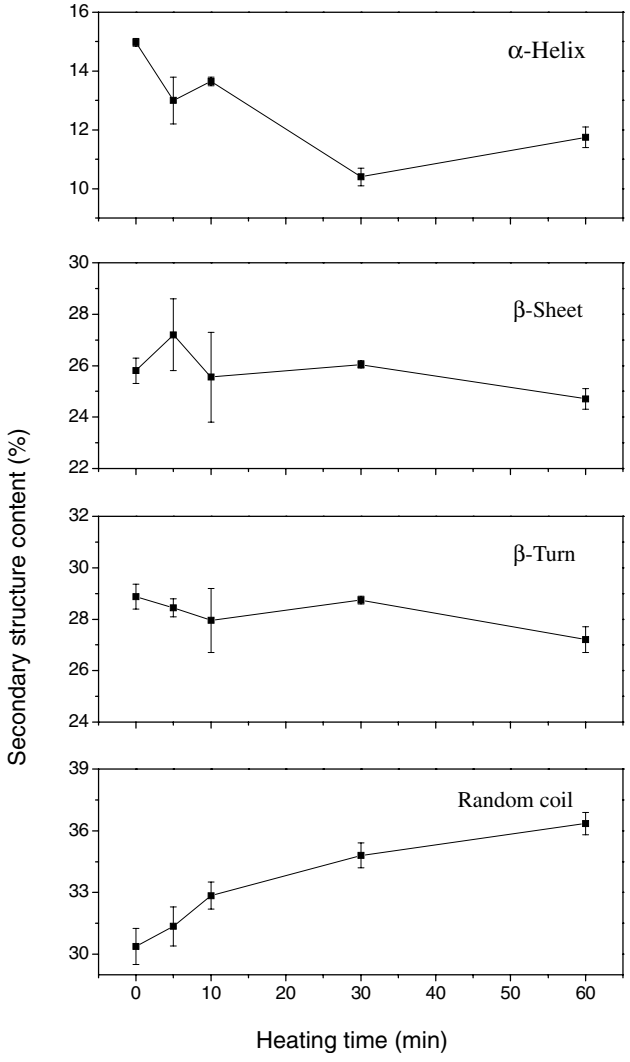

Fig. 8. Effect of heating time at 100 °C on secondary structure content of buckwheat globulin examined by circular dichroism spectroscopy. The error bars represent standard deviations of the means.

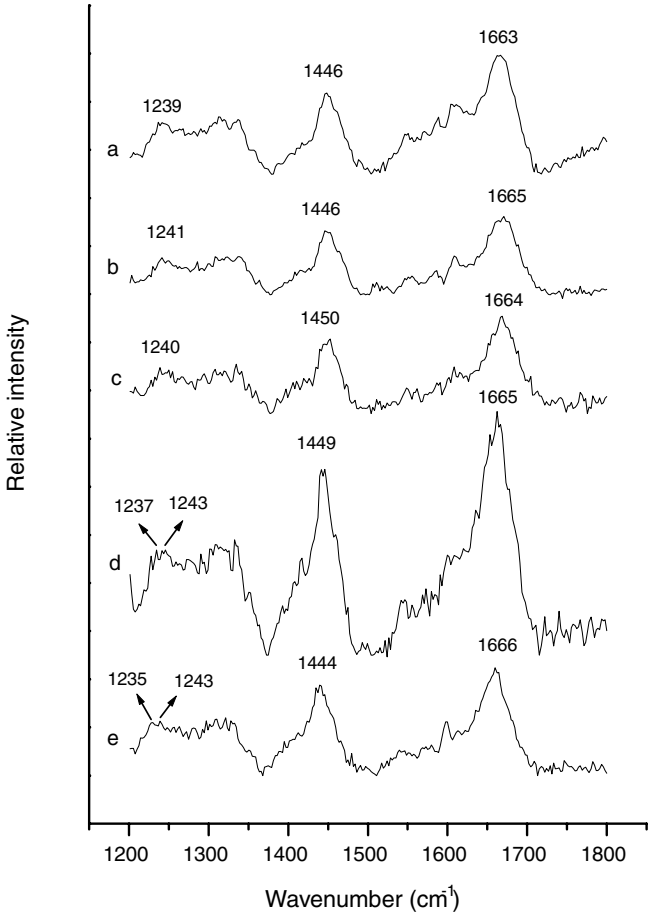

Fig. 9. Raman spectra of buckwheat globulin buffer-soluble aggregates heated at 100 °C for different time intervals: (a) 0 min; (b) 5 min; (c) 10 min; (d) 30 min; (e) 60 min.

1235–1237 cm^{-1}, indicating the presence of anti-parallel β-sheet structures which have been associated with aggregate or gel formation (Clark, Saunderson, & Suggett, 1981; Jackson & Mantsch, 1992). The amide I band (1663 cm^{-1}) was shifted to higher wavenumbers with increasing heating time, showing the predominance of disordered structure (Fig. 9). Changes in the band intensities of amide I and III, C–H bending and C–H stretching during heating suggests conformational changes and partial protein denaturation.

The present data demonstrate that heating of BWG at 100 °C resulted in both protein denaturation and aggregates formation, which are in agreement with the thermal aggregation studied by FTIR (Choi & Ma, 2005) and laser light scattering (Choi & Ma, 2006). The spectroscopic data also indicated that β-sheet is an important conformational component in the aggregated protein. The involvement of β-sheets in the secondary structure of protein aggregates may be attributed to the relatively large surface areas for ordered hydrogen bonding. Moreover, the weaker water hydration strength to β-sheet than to α-helical structures may play a role in the aggregate and network formation. This is caused by different geometry of the water–carbonyl group interactions in these conformations (Przybycien & Bailey, 1989, 1991). Similar to FTIR spectroscopy (Choi & Ma, 2005), both CD and Raman spectroscopy seems to be a sensitive technique in monitoring conformational changes in BWG during heat treatments.

4. Conclusions

The present CD and Raman data show that BWG, similar to most seed globulins, contains a low level of α-helical and a large quantity of β-sheets and disordered structures. The conformation of BWG was influenced by pH, chaotropic salts, some protein structure perturbants and heat treatments, suggesting the involvement of both covalent and non-covalent forces in stabilizing the conformation of BWG. The findings are concomitant with previous FTIR and DSC data (Choi & Ma, 2005). This study also demonstrated that Raman spectroscopy is a useful technique for investigating the secondary and tertiary structures of plant proteins with limited solubility, such as BWG and it is applicable for monitoring conformational changes in proteins under different buffer environments and heat treatments. Complementary information on the secondary structure and conformational changes of BWG were obtained.

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