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Conformational study of globulin from rice (*Oryza sativa*) seeds by Fourier-transform infrared spectroscopy

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Abstract

The conformation of rice globulin (10%, w/v, in deuterated phosphate buffer, pD 7.4) under the influence of pH, chaotropic salts, several protein structure perturbants and heat treatments was studied by Fourier-transform infrared (FTIR) spectroscopy. Rice globulin exhibited seven major bands in the region of 1700–1600 cm^{-1} and the spectrum suggests high α-helical content with large quantities of β-sheet and β-turn structures. Highly acidic and alkaline pH conditions induced changes in band intensity attributed to intermolecular β-sheet structure (1681 and 1619 cm^{-1}). Addition of chaotropic salts led to progressive changes in band intensity, following the lyotropic series of anions, whereas several protein structure perturbants caused shifts in band positions. Heating at increasing temperature led to progressive decreases in α-helical content and increases in random coil structures, suggesting protein denaturation. This was accompanied by intensity increases in the intermolecular β-sheet transitions.
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Keywords: Rice globulin; FTIR spectroscopy; Protein conformation; Denaturation; Aggregation

1. Introduction

Rice globulin is the second major protein [1] in rice seeds, which accounts for 10–12% of the total endosperm storage proteins [2]. The structure of rice globulin is known to be similar to wheat seed, glutenin, a polymeric protein which has many intermolecular disulfide bonds. Two rice globulin polypeptides have been identified: the 26 and 16 kDa polypeptides [3,4]. The 26 kDa fraction, α-globulin, is the major polypeptide of rice globulin [5,6]. Rice globulin has been studied with respect to chemical composition, physicochemical characterization, polypeptides profile and genetic background [7,8], but there have been few studies on the structure and conformation of rice globulin. A previous study revealed that the secondary structure of rice globulin is composed of α-helix as the main ordered structure [9].

The conformation of a protein is a critical determinant of its functional properties [10]. Hence, a detailed study of the secondary structure of the proteins will provide knowledge of structure–function relationships to improve quality of existing foods or assist the fabrication of new food products [11]. However, researchers have found it is difficult to follow conformational changes of large proteins, such as seed globulins in food systems due to low solubility and lack of biological activities [12].

Fourier-transform infrared (FTIR) spectroscopy is a vibrational spectroscopic technique that can be used to monitor protein structural changes at any physical state: liquid, semisolid or solid and in native, denatured or aggregated state [13–15]. The use of FTIR technique for the study of protein secondary structure is based on the amide I region, composed of C=O stretching vibrations in the region of 1700–1600 cm^{-1} [16]. The characteristic absorption band is generally a composite, consisting of overlapping components representing α-helices, β-sheets, β-turns and random coils. This technique is extremely sensitive to changes in hydrogen bonding and is therefore useful for obtaining subtle changes in protein structure [17]. It provides a global insight into the overall secondary structure of proteins, without the need to

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locate the precise three-dimensional arrangement of individual structural components [18].

FTIR spectroscopy has been used to study the conformation of various food proteins, such as oat globulin [19], whey proteins [20,21], soy glycinin [11] and wheat gluten [22]. Moreover, it has been extended to study changes in protein conformation induced by temperature [15,23], pH [23], pressure [24,25], chemicals [26] and various buffer conditions [19]. A previous FTIR spectroscopic study revealed that the native conformation of rice globulin possessed high α-helical structures [27]. This study aims to study rice globulin conformation under the influence of various buffer conditions and heat treatments and to evaluate its structural changes quantitatively by FTIR spectroscopy.

2. Materials and methods

2.1. Preparation of rice seed globulin

Thailand grown, dehulled rice (*Oryza sativa* var. *Xiang Mi*) seeds were obtained from local market. Globulin was extracted from milled, defatted rice flour with 0.7 M NaCl following the procedure of Osborne fractionation [28]. The extracted globulin was purified using the isoelectric precipitation method [3] with slight modifications. The crude globulin was dissolved in 2% acetic acid at pH 2.5. NaOH (0.5N) was gradually added with vigorous stirring to bring the pH to 4.5. A white, cloudy precipitate was formed. The precipitate (pH 4.5 insoluble fraction) was separated by centrifugation at 14,000 × *g* for 20 min and recovered by dialyzing against distilled water and freeze-drying. This globulin fraction was further purified by repeating the precipitation at pH 4.5 three times. The protein content was 95%, determined by Lowry assay [29]. The purity of this purified globulin fraction was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [30], and a single band was observed with an estimated molecular weight of 26 kDa, corresponding to α-globulin [5,6]. Deuterated regents (D_2O and DCl) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals used were of analytical grade.

Table 1
Assignment of deconvoluted amide I′ bands in the FTIR spectrum of rice globulin in deuterated phosphate buffer

Frequency (cm^{-1})	Band assignment
1611–1612	Side chain vibrations
1618–1620	Anti-parallel β-sheet, aggregated strands
1623	Anti-parallel β-sheet
1629–1633	β-Strand
1637	β-Sheet
1643–1645	Unordered (random coil)
1650–1652	α-Helix
1660	β-Turns
1668	β-Turns
1680–1682	Anti-parallel β-sheet, aggregated strands
1690	β-Type structure

2.2. Sample preparation

Rice globulin dispersions (10%, w/v) were prepared in 0.01 M deuterated phosphate buffer (pD 7.4). All protein

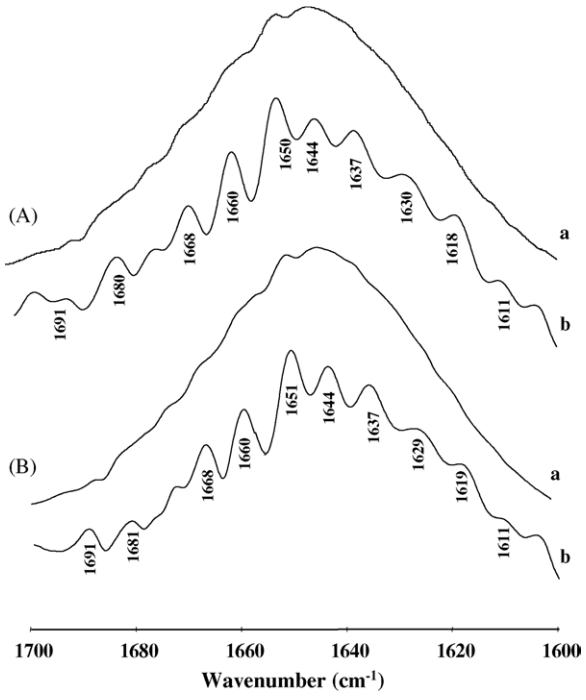

Fig. 1. FTIR spectra of crude rice globulin (A) and purified rice globulin (B) (10% in 0.01 M deuterated phosphate buffer, pD 7.4): (a) original spectrum and (b) deconvoluted spectrum.

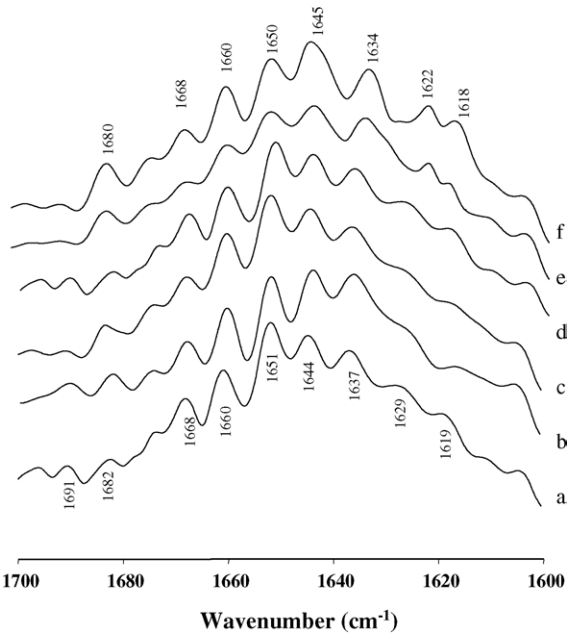

Fig. 2. Stacked plot of deconvoluted infrared spectra of rice globulin (10% in 0.01 M deuterated phosphate buffer) at different pH: (a) control (no pH adjustment); (b) pH 3; (c) pH 5; (d) pH 7; (e) pH 9; (f) pH 11.

dispersions were prepared in D_2O instead of H_2O since D_2O has greater transparency in the infrared region, 1600–1700 cm^{-1} [19]. In order to ensure complete hydrogen-deuterium exchange, samples were prepared 1 day before, kept at 4 °C and were equilibrated to room temperature prior to infrared measurements [19].

To study the effect of pH, protein dispersions with the desired pD (pD = pH + 0.4) were prepared by the addition of 1 M NaOD (dissolving NaOH in D_2O) or 1N DCl with mixing in a magnetic stirrer, and the dispersions were stirred for an hour at room temperature to allow pD to equilibrate. Chaotropic salts including sodium chloride, bromide, iodide and thiocyanate, and some protein structure perturbants including sodium dodecyl sulfate, *N*-ethylmaleimide (NEM), ethylene glycol (EG), dithiothreitol (DTT) and urea were prepared in 0.01 M deuterated phosphate buffer, pD 7.4 to give the desired concentrations. The concentrations selected were based on preliminary experiments which showed conformational changes in rice globulin. For heating experiments, an Omega temperature controller (Omega Engineering, Stanford, CA) was used. The samples prepared in 0.01 M deuterated phosphate buffer with 0.2 M NaCl, pD 7.4, were heated from 40 to 110 °C at 10 or 20 °C increments (with an accuracy of ±0.5 °C) and allowed to equilibrate for 4 min at each temperature prior to data acquisition.

2.3. FTIR spectroscopy

Infrared spectra of rice globulin dispersions were recorded in a Bio-Rad Excalibur FTIR spectrometer (Bio-Rad Laboratories, Cambridge, MA) equipped with a deuterated triglycine sulphate (DTGS) detector. Samples were held in an IR cell

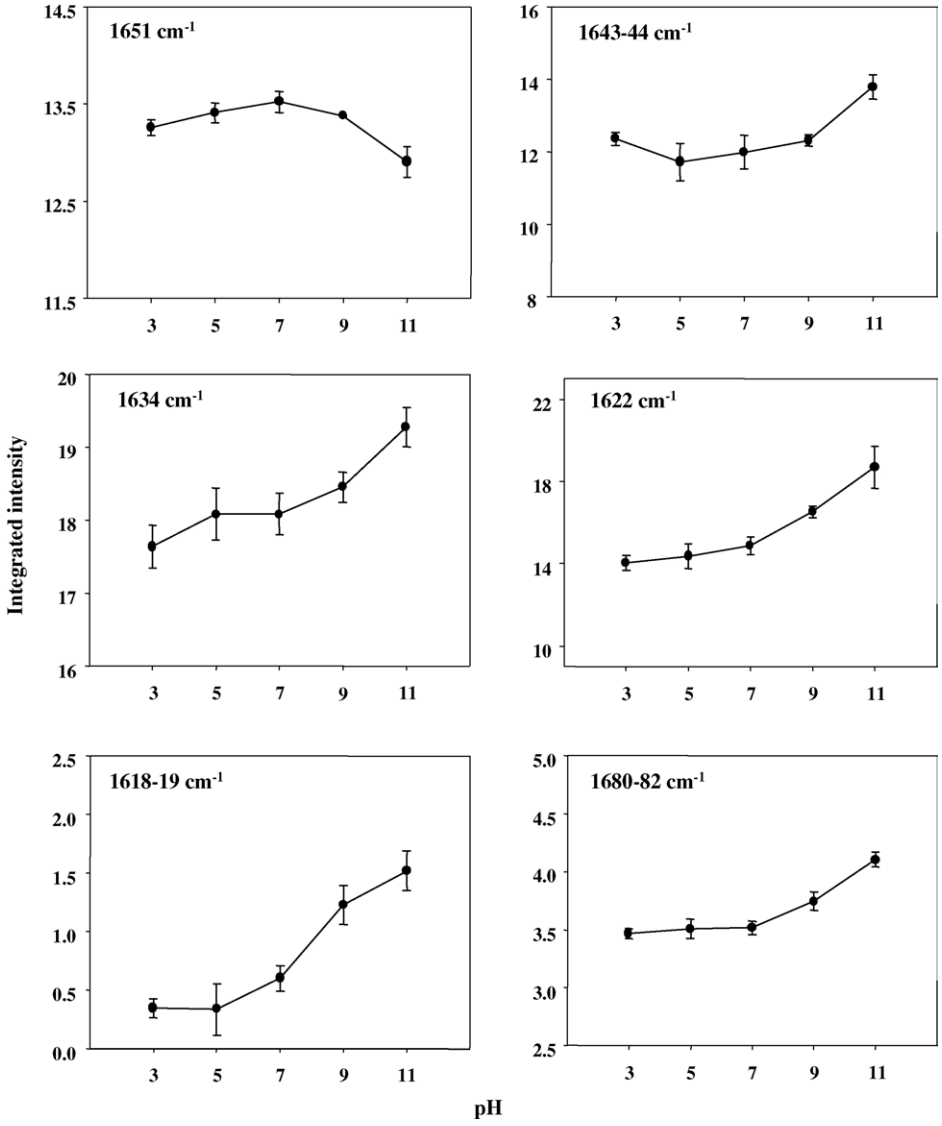

Fig. 3. Plots of integrated intensity of several infrared bands of rice globulin (10% in 0.01 M deuterated phosphate buffer) at different pH. Error bars represent standard deviations of the means.

with 25 μm path length CaF_2 windows. A total of 32 scans were averaged at 4 cm^{-1} resolution. Preliminary data (not shown) showed that increasing the number of scans from 32 to 512 did not significantly improve the resolution of the IR spectra. Hence, 32 scans were selected for all the heating experiments to minimize the time duration. Deconvolution of infrared spectra was performed using the Merlin Software, Version 1.2 (Bio-Rad Laboratories Inc., Cambridge, MA, USA) according to Kauppinen et al. [31]. The half width used for deconvolution was 10 cm^{-1} and the narrowing factor was 2.0. To ensure that the spectra were not overdeconvoluted, the acquired spectra were judged by evaluating the second derivative spectra, comparing the number and position of bands with the deconvoluted spectra. Band assignment in the amide I′ region (1600–1700 cm^{-1}) was according to Susi and Byler [16]. Quantitative analysis of secondary structure components was performed using Gaussian peaks and curve fitting models according to the method of Byler and Susi [32] and the least squares analysis described by Jackson and Mantsch [33]. All FTIR experiments were performed in duplicates and reproducible data (with standard deviations < 10%) were obtained.

3. Results and discussion

3.1. Spectral assignment

Fig. 1 shows the infrared spectra (1600–1700 cm^{-1}) of crude and purified rice globulin dispersions (≈10% in deuterated phosphate buffer) before and after deconvolution. Seven major bands were observed in the amide I′ region. Table 1 shows the frequencies (cm^{-1}) and assignments of deconvoluted amide I′ components of rice globulin [16,34]. Both crude and purified globulin samples exhibited similar spectrum having all the major bands in the region of interest. The prominent band was observed at 1651 cm^{-1} with a maximum band intensity followed by the band intensities at 1644 and 1637 cm^{-1} (Fig. 1).

From the infrared spectra of the native protein, the secondary structure composition of both crude and purified rice globulin, estimated by integrating the major bands, was found to be predominantly β-sheets and turns (over 50%), followed by α-helices (about 30%) and random coils (about 20%). A previous FTIR study showed that globulin from cultivated and wild rice contains α-helix as the main ordered structure [35]. However, many seed globulins from monocotyledonous and dicotyledonous plants were found to possess low levels of α-helix and high levels of β-sheet secondary structure [36]. Other rice storage proteins, for example, rice albumin, have a predominant β-turns structure, whereas prolamin and glutelin exhibit α-helices and random coils as the major secondary structures [37]. Although both crude and purified rice globulin (mainly the α-globulin fraction) exhibited similar FTIR spectral characteristics, purified protein sample was used for subsequent experiments.

3.2. Effect of pH

Fig. 2 shows the infrared spectra of rice globulin dispersions at different pH and Fig. 3 shows the integrated intensity of several major bands of infrared spectra at each pH. At pH 3, the band attributed to β-sheets (1637 cm^{-1}) was shifted to lower wavenumber (1634 cm^{-1}) with a marked decrease in intensity. The intensity of the 1681 cm^{-1} band was increased with a shift to 1680 cm^{-1}. The results indicate a change in hydrogen bonding [38] or a change in conformation [21]. 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 [39]. The absence of an aggregation peak at 1618 cm^{-1} is an indication of the inflexible structure at acidic pH, inhibiting protein unfolding [39].

The IR spectra at pH 5 and 7 were similar to that of the control (in D_2O buffer without pH adjustment). Most proteins are stable near its isoelectric pH range where repulsive forces are low and the proteins remain in their native state. The isoelectric pH of mixed rice globulin and the major fraction were found to be 6.4 and 4.5, respectively [7,40]. Padhye and Salunkhe [41] also reported that the isoelectric point of milled rice globulins range from 5.85 to 7.27. Hence, the present data suggest that at the isoelectric pH range (pH 5–7), rice globulin exhibited native conformation.

At pH 9, the emergence of a 1622 cm^{-1} band may be associated with the formation of intermolecular hydrogen-bonded anti-parallel β-sheet structures [20,42]. A decrease in

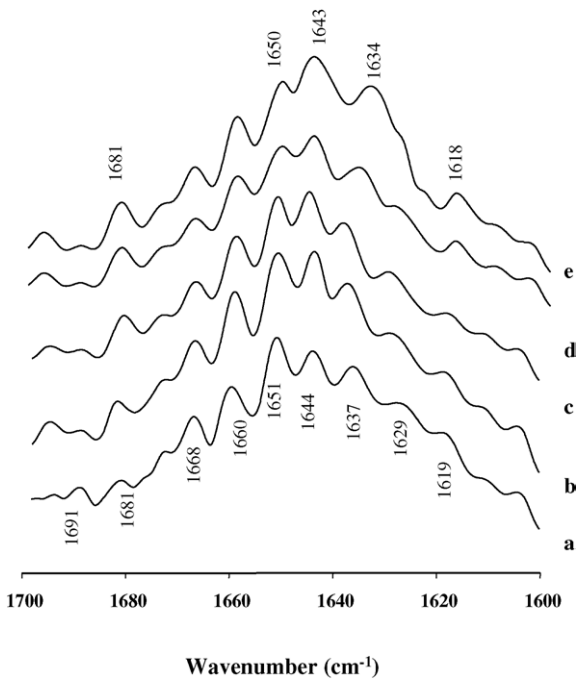

Fig. 4. Stacked plot of deconvoluted infrared spectra of rice globulin (10% in 0.01 M deuterated phosphate buffer, pD 7.4) in the presence of 1M chaotropic salts: (a) control (no added salt); (b) sodium chloride; (c) sodium bromide; (d) sodium iodide; (e) sodium thiocyanate.

the intensity of 1651 cm^{-1} band and increases in the intensities of other three bands at 1644, 1680 and 1622 cm^{-1} suggest transition from ordered conformation to random coils and aggregated strands. The shift in band position from 1637 to 1633 cm^{-1} indicates transition of β-sheets to β-strands. Previous studies have reported that the major subunit of rice globulin, α-globulin, undergoes a pH-dependent partial aggregation, which tends to forms pentamers [43]. Partial protein unfolding due to increases in pH may allow more solvent within the protein molecule, which could enhance hydrogen bonding [39].

At highly alkaline pH (pH 11), changes in spectral characteristics were more pronounced. The 1637 cm^{-1} band was shifted to lower wavenumber (1634 cm^{-1}) and the band intensity was increased. There was a marked increase in the 1644 cm^{-1} band intensity and decrease in the 1652 cm^{-1} band intensity, suggesting extensive protein denaturation. The appearance of two prominent bands at 1622 and 1680 cm^{-1} can be attributed to the formation of anti-parallel aggregated strands [20,42]. Highly alkaline pH could lead to ionization of the Asp carboxyl groups, which would tend to increase self-association and thus increases in β-strands content [39].

3.3. Effect of chaotropic salts

In the presence of 1.0 M sodium salts, the 1651 cm^{-1} (α-helix) and 1637 cm^{-1} (β-sheet) bands were progressively shifted to lower wavenumbers, 1650 and 1633 cm^{-1} respectively (Fig. 4), following the order of the lyotropic series of anions, $Cl^- > Br^- > I^- > SCN^-$ [44]. Progressive reduction in α-helical band intensity was accompanied by intensity increases of random coil (1644 cm^{-1}) and β-strand (1634–1633 cm^{-1}) bands (Fig. 5). A gradual disappearance

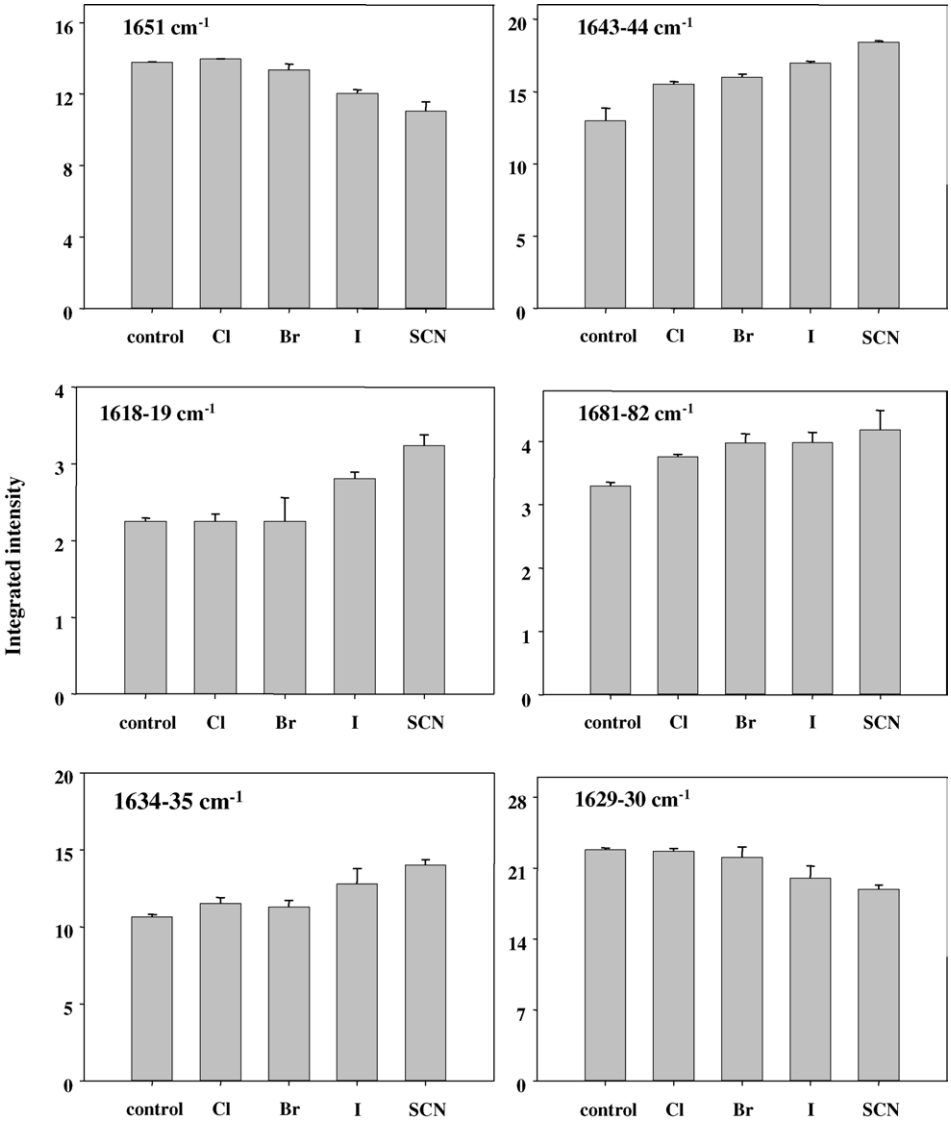

Fig. 5. Plots of integrated intensity of several infrared spectral bands of rice globulin (10% in 0.01 M deuterated phosphate buffer, pD 7.4) in the presence of 1 M chaotropic salts: control (no added salt); Cl: chloride; Br: bromide; I: iodide; SCN: thiocyanate. Error bars represent standard deviations of the means.

of the 1629 cm^{-1} band was observed with a shift in band position. There were also progressive intensity increases in the 1618 and 1682 cm^{-1} bands associated with the formation of aggregated strands. Hence, the results show progressive conformational changes in rice globulin along with protein aggregation, following the order of the lyotropic series of anions.

Salts could significantly perturb protein conformation by affecting electrostatic and hydrophobic interactions via a modification of water structure [45,46]. The extent of water structure modification is dependent on the nature of anions, following the lyotropic series [44]. Anions (I^- and SCN^-) higher in the series could reduce the free energy required to transfer the non-polar groups into water. They may also weaken intramolecular hydrophobic interactions and enhance the unfolding tendency of proteins [47]. I^- and SCN^- are destabilizing agents due to their high hydration energy and steric hindrance which promote unfolding, dissociation and salting-in of proteins [39]. In contrast, Cl^- and Br^- could promote salting-out and aggregation due to their higher hydration molar surface tension and could stabilize protein conformation.

3.4. Effect of protein structure perturbants

The effect of some protein structure perturbants on the IR spectral characteristics of rice globulin is demonstrated in Fig. 6. In the presence of 40 mM SDS, loss of the 1637 cm^{-1} band (β-sheets) and broadening of the 1644 cm^{-1} (random coils) band were observed, with a shift in band position to 1641 cm^{-1}. The appearance of a minor 1612 cm^{-1} transition (side chain vibrations) was also observed. Pronounced increase in the 1680 cm^{-1} band intensity was also observed (Fig. 7). Based on these observations, a significant proportion of β-sheet structures was converted to random coils, indicating protein denaturation. SDS is an anionic detergent which can bind to proteins by non-covalent forces, and the polypeptides could unfold due to increased negative charge and ionic repulsion [48].

Addition of 30 mM DTT caused the disappearance of the 1637 cm^{-1} band β-sheet and broadening of the 1643 cm^{-1} band (random coil) (Fig. 6). There was a shift in the 1629 cm^{-1} band (β-strand) to 1632 cm^{-1} with an increase in intensity. Pronounced increases in the 1681 and 1618 cm^{-1} band intensities were also observed (Fig. 7), suggesting the formation of hydrogen-bonded β-sheet structures associated with aggregate formation. The disappearance of the 1690 cm^{-1} band further suggests protein unfolding. DTT is a reducing agent which can break up disulfide bonds and dissociate oligomeric proteins into subunits, thus, causing major structural changes and promoting aggregation.

Under the influence of 10 mM NEM, only minor reduction in the intensity of 1666 and 1660 cm^{-1} bands (β-turns) was observed (Fig. 6). The results imply that NEM could protect the native protein structure without causing marked conformational changes. NEM is a sulfhydryl-blocking agent which could shift the conformation of protein molecules to a state that contributes much less to the unfolding transition [27].

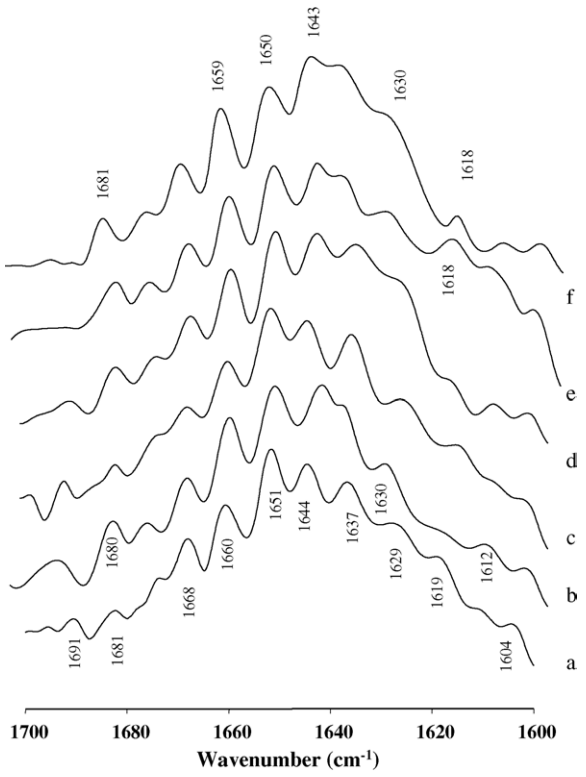

Fig. 6. Stacked plot of deconvoluted infrared spectra of rice globulin (10% in 0.01 M deuterated phosphate buffer, pD 7.4) in the presence of protein perturbants: (a) control (no additive); (b) 40 mM sodium dodecyl sulfate; (c) 50 mM *N*-ethylmaleimide; (d) 40% ethylene glycol; (e) 40 mM dithiothreitol; (f) 6 M urea.

In the presence of 40% (v/v) EG, the intensities of the 1637 cm^{-1} (β-sheet) and 1630 cm^{-1} (β-strand) bands were decreased markedly (Fig. 7), suggesting loss of ordered structures. It was reported that organic solvents such as EG could destabilize protein conformation. They can lower the dielectric constant of the medium and weaken the hydrophobic effect, thus, enhancing hydrogen bond formation and electrostatic interactions [46,49]. The perturbation promotes protein unfolding and refolding into an α-helical conformation [50]. Moreover, it has been revealed that EG has a high enough dipole moment to disrupt intramolecular hydrogen bonds and has a low enough dielectric constant to reduce the hydrophobic effect to induce formation of α-helices in soybean glycinin [50].

Addition of 6 M urea caused marked increases in band intensity at 1618, 1682 and 1643 cm^{-1} and decreases at 1637, 1651 and 1690 cm^{-1}. The results show enhanced formation of aggregated strands and random coil structure at the expense of β-sheets and α-helices. Urea markedly disrupts the hydrogen-bonded structure of water and facilitates protein unfolding by weakening hydrophobic interactions [51]. It can also increase the "permitivity" of water of the apolar residues, causing loss of protein structure and heat stability [52].

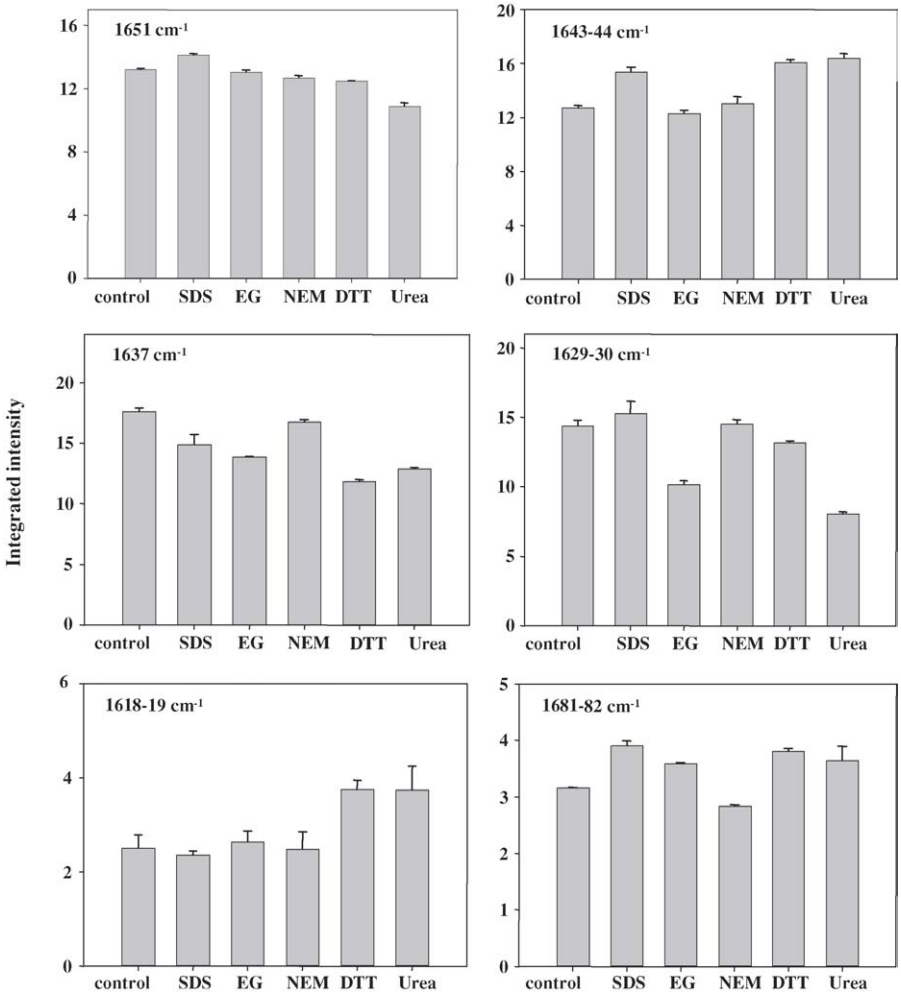

Fig. 7. Plots of integrated intensity of several infrared spectral bands of rice globulin (10% in deuterated phosphate buffer, pD 7.4) in the presence of some protein structure perturbants: control (no additive); SDS: 40 mM sodium dodecyl sulfate; EG: 40% ethylene glycol; NEM: 50 mM *N*-ethylmaleimide; DTT: 40 mM dithiothreitol; urea: 6 M. Error bars represent standard deviations of the means.

3.5. *Effect of heat treatments*

Fig. 8 shows the FTIR spectra of rice globulin dispersions heated from 40 to 110 °C. Major changes observed were band intensity decreases at 1651 and 1691 cm^{-1} and increases at 1682, 1620, 1634, 1644 and 1614 cm^{-1}. Progressive decreases in the intensity of the α-helical band and concomitant increases in the random coil band suggest the transition from ordered to unordered structures [53]. Increase of the 1634 cm^{-1} band intensity could be associated with the formation of β-strands from β-sheet structures. There was a general increase in band intensities in the amide II′ region (1600–1500 cm^{-1}) (Fig. 8), indicating changes in secondary structure [34,53].

Heating also led to progressive intensity increases in bands associated with the formation of intermolecular anti-parallel β-sheet structures and aggregated strands (1618–1620 and 1682 cm^{-1}) (Fig. 9). A progressive decrease in 1690 cm^{-1} (β-type structure) band intensity has been associated with the onset of protein unfolding [20]. The appearance of a

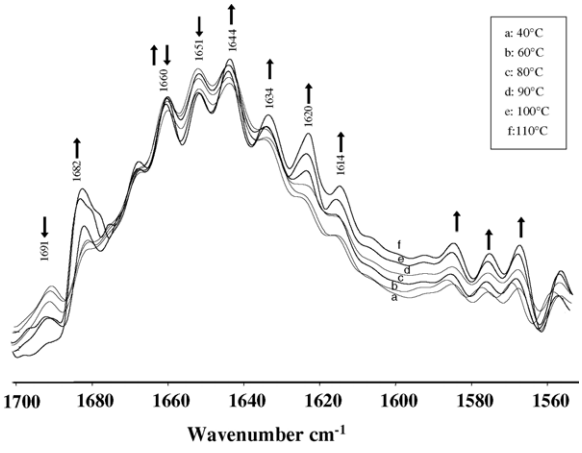

Fig. 8. Stacked plot of deconvoluted infrared spectra of rice globulin (10% in 0.01 M deuterated phosphate buffer, pD 7.4) continuously heated from 40 to 110 °C: (a) 40 °C; (b) 60 °C; (c) 80 °C; (d) 90 °C; (e) 100 °C; (f) 110 °C. The arrows indicate either increase or decrease of band intensities with increasing heating temperature.

band at 1614 cm^{-1} indicates the presence of intermolecular β-sheet networks, which could occur during aggregation and gel formation [26,42]. The intensity of the 1668 cm^{-1} band (β-turns) was changed during heating. Turns can be a product of unfolding of any higher order structures whereas anti-parallel β-strands can be formed in aggregated protein molecules [32,54].

The present data reveal marked changes in protein conformation when rice globulin was heated at around 90–100 °C, and the appearance of bands associated with the formation of anti-parallel β-strands indicates the onset of aggregation at this temperature range. FTIR and DSC studies of β-lactoglobulin showed that the DSC onset temperature (T_m) rather than the peak temperature (T_d) was related with the denaturation temperature based on the amide I′ band of FTIR spectra, and that T_d seems to be more indicative of aggregation [39]. In rice globulin, the DSC onset temperature (89.5 °C) [55] is also close to the temperature (90 °C) at which denaturation was observed by FTIR, while the DSC peak temperature (98.5 °C) [55] is again close to the temperature (100 °C) at which distinct aggregation bands were observed in the FTIR spectra.

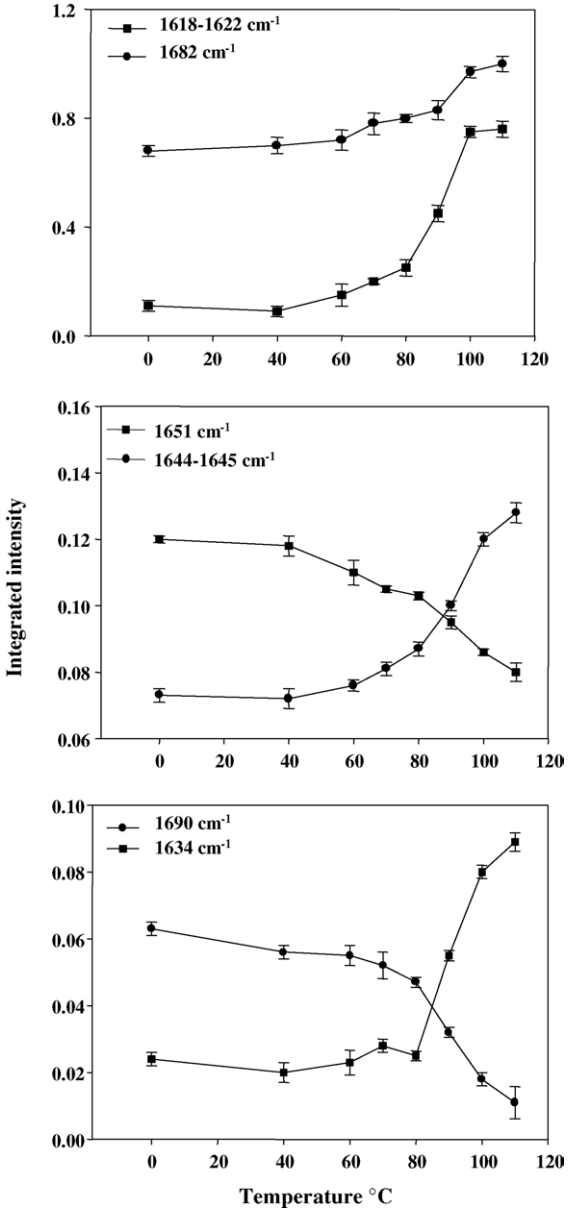

Fig. 9. Plots of peak areas (integrated intensities) of several infrared spectral bands of rice globulin (10% in deuterated phosphate buffer, pD 7.4) heated at different temperatures in the range of 40–110 °C. Error bars represent standard deviations of the means.

4. Conclusions

The present study demonstrates that conformation of rice globulin was influenced by various buffer environments and heat treatments. The data can provide some insight into the relative importance of different chemical forces (non-covalent and disulfide bonds) in maintaining the conformation of rice globulin. The present study shows that FTIR spectroscopy is appropriate technique to study plant proteins with low solubility, such as rice globulin. Moreover, it can also be used to monitor heat-induced protein aggregation in situ.

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