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Thermal properties of *Phaseolus angularis* (red bean) globulin

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Abstract

The thermal properties of *Phaseolus angularis* (red bean) globulin were studied by differential scanning calorimetry under various medium conditions. Red bean globulin (RGB) was fractionated by ion-exchange chromatography into a major fraction, with SDS–PAGE pattern corresponding to the 7S vicilin, and two minor fractions, probably representing residual vicilin and the 11S legumin, respectively. The thermogram of RBG showed a major endothermic peak at 86.4°C and a minor transition at 92.2°C. Vicilin exhibited two endothermic peaks (87.7 and 94.1°C), while legumin showed one transition at 89.5°C. The progressive increase in denaturation temperature (T_d) with increase in salt concentration, suggests a more compact conformation for RBG with higher thermal stability. Decreases in enthalpy and T_d were observed under the influence of highly acidic and alkaline pHs, chaotropic salts, and protein perturbants such as sodium dodecyl sulfate, urea and ethylene glycol, indicating partial denaturation and decrease in thermal stability. Dithiothreitol and N-ethylmaleimide have little effect on the thermal properties of RBG since the major protein component, vicilin, is devoid of disulfide bonds.

Keywords: *Phaseolus angularis*; Redbean; Differential scanning calorimetry; Globulin; Protein denaturation

1. Introduction

Phaseolus angularis (red bean or Adzuki bean) is an under-utilized legume indigenous to China. The flour and protein concentrate of red bean exhibited functional properties comparable with that of commercial soy protein products (Chau, 1997; Chau & Cheung, 1998). The salt-soluble globulin represents the major Osborne fraction of red bean proteins (Chau, Cheung & Wong, 1998). A better understanding of the physicochemical characteristics of red bean protein, particularly globulin, including its thermal properties, can enhance its potential utilization as a food ingredient.

Protein may undergo denaturation under various buffer conditions, attributed to the disruption of chemical forces that maintain the structural integrity of protein molecules. Solvent components can affect the conformational stability of protein by direct interaction with the protein and/or indirect modification of the solvent environment (Eagland, 1975). The changes may be endothermic, such as rupture of hydrogen bonds, or exothermic, such as breaking up of hydrophobic forces and aggregation of proteins (Arntfield & Murray, 1981; Jackson & Brandts, 1970; Privalov, 1982). Hence, thermoanalytical methods such as differential scanning calorimetry (DSC) are considered convenient and reliable for studying such changes. The conversion of protein from native to denatured state may be caused by disruption of various chemical forces which are thermally related and can be exhibited in the DSC thermograms (Wright, 1984). The peak transition or denaturation temperature, T_d, is a measure of thermal stability, and its determination under controlled conditions (scanning rate and protein concentration) can provide information on changes of protein thermal stability under the influence of various environmental conditions, such as pH and ionic strength. The enthalpy change (ΔH), measured as area under the endothermic peak, represents the proportion of undenatured protein in a sample (Arntfield & Murray, 1981), and is correlated with the extent of ordered structure of a protein (Koshiyama, Hamano & Fukushima, 1981).

Legumes and legume foods are routinely subjected to various thermal treatments during harvesting, processing, and preparation. These may include steaming, roasting, blanching and cooking. Legumes are known to contain anti-nutritional factors, e.g. trypsin inhibitors and hemagglutinins, that may be eliminated by high

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temperature processing. However, excessive heat treatment could cause undesirable changes in legume proteins, such as a lowering of their digestibility and bioavailability (Deshpande & Damodaran, 1989). Hence, thermal analysis, such as DSC, is particularly useful in evaluating effects of heat treatment on protein quality in foods such as legumes. It has been suggested that the less-than-desirable functional properties of legume and oilseed proteins may be related to their particular structural stability and hence high heat-stability (high T_d; Damodaran, 1997). Some functional properties of food proteins, e.g. heat-induced gelation, are directly affected by the thermal stability of the proteins. The rheological behaviour of soybean 7S globulin has been shown to relate closely with its DSC characteristics and is strongly influenced by the heating temperature (Nagano, Hirotsuka, Mori, Kohyama & Nishinari, 1992). DSC is therefore a valuable technique in studying the structure-function relation of legume proteins.

In the present investigation, the effect of medium composition, such as pH, salt and protein structure perturbants, on the thermal properties of RBG will be studied by DSC. The role of covalent and non-covalent chemical forces in stabilizing the globulin conformation will be investigated. The mixed globulin will be fractionated by ion-exchange chromatography, and the DSC characteristics of the major fractions will be examined. The thermal properties of red bean globulin will be compared to those of other nutritionally important legumes.

2. Materials and methods

2.1. Extraction of RBG

P. angularis seeds were purchased from a local supplier. The seeds were soaked in distilled water for 12 h at 4°C and dehulled manually. The dehulled seeds were freeze-dried, ground and defatted by Soxhlet extraction with hexane. Globulin was extracted from the defatted flour with 1.0 M NaCl, following the procedure of Ma and Harwalkar (1984). The isolated globulin was freeze-dried, and then stored at −4°C.

2.2. Fractionation of RBG by ion-exchange chromatography

Ion-exchange chromatography was performed using a Pharmacia Fast Protein Liquid Chromatography (FPLC) system and a DEAE-Sepharose column (Phamacia Biotech AB, Uppsala, Sweden). Stepwise elution with 0.07 M and 0.25 M NaCl in phosphate–citric acid buffer, pH 7.0, was carried out according to Gueguen, Vu and Schaeffer (1984). The flow rate was 2.0 ml/min, and the eluant was monitored at 280 nm. The major fractions were collected, dialyzed and freeze-dried. The protein contents of the pooled fractions were determined by the method of Lowry, Rosenbrough, Farr and Randall (1951), using bovine serum albumin (BSA) as standard.

2.3. SDS–PAGE

The native globulin and three fractions from FPLC were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5% homogenous gels according to the method of Laemmli (1970). The standard markers were from a LMW Electrophoresis Calibration Kit (Pharmacia Biotech, USA). The molecular weights of protein bands were determined using a Phoretix densitometric image analysis system (Phoretix International, Newcastle Upon Tyne, UK).

2.4. Differential scanning calorimetry

The thermal properties of red bean globulin under various medium conditions were examined using a TA 2920 Modulated DSC thermal analyzer (TA Instruments, New Castle, DE). Approximately 1 mg of protein was weighed into the aluminium pan, and 10 μl of 0.01 M phosphate buffer, pH 7.4, was added. The pan was hermetically sealed and heated from 25 to 140°C at a rate of 10°C/min. A sealed empty pan was used as a reference. Onset temperature (T_m), peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from the thermograms by a Universal Analysis Program, Version 1.9D (TA Instruments). All experiments were conducted in triplicate and the coefficient of variation ranged from 0.3 to 0.6% for T_m and T_d, and 5 to 10% for ΔH. Some of the thermograms were comprised of large and small overlapping peaks, and only the ΔH of the combined transitions were measured, due to difficulties in accurately estimating the partial areas of the overlapping transitions.

3. Results and discussion

3.1. Fractionation of RBG by ion-exchange chromatography

RBG was fractionated into three fractions by ion-exchange FPLC using stepwise elution with NaCl (Fig. 1). Unbound protein was shown as a large peak (fraction A) when the sample was introduced to the column in buffer without NaCl. Both the second (B) and third (C) fraction show two unresolved peaks. When the fractions were pooled and the protein contents determined, the three fractions were found to represent 78, 10 and 12%, respectively, of total eluted protein. Total recovery of protein from the column was about 88%.

Fig. 2 shows the SDS–PAGE patterns of RBG and its FPLC column fractions, both in the absence (Fig. 2A)

and presence of β-mercaptoethanol (ME; Fig. 2B). The mixed red bean globulin showed a major band with estimated M_r of 54–67 kDa, and several minor bands with M_r ranging from 86–29 kDa (lane 2, Fig. 2A). In the presence of ME, the mobility of the major band was slightly increased, and an additional band at 22 kDa was observed (lane 2, Fig. 2B). Fraction A showed one major band with M_r of 54–65 kDa and several minor bands with M_r from 47 to 29 kDa (lane 3, Fig. 2A). The addition of ME again led to slight increase in mobility of the major band, and only two minor bands, with M_r of 33 and 29 kDa, respectively, were observed (lane 3, Fig. 2B). The SDS–PAGE pattern of fraction B (both of the two unresolved peaks) showed the major band of fraction A, and a minor band at 38 kDa (lane 4, Fig. 2A). The addition of ME did not change the PAGE pattern (lane 4, Fig. 2B). Fraction C showed a group of bands from 54 to 90 kDa under non-reduced conditions (lane 5, Fig. 2A). The addition of ME led to the dissociation of the higher molecular weight components, with the appearance of four major bands at 64, 58, 40, and 22 kDa and two minor bands at 53 and 27 kDa (lane 5, Fig. 2B). Some high molecular weight components were found as a faint band at the origin in this fraction, and were dissociated by adding ME.

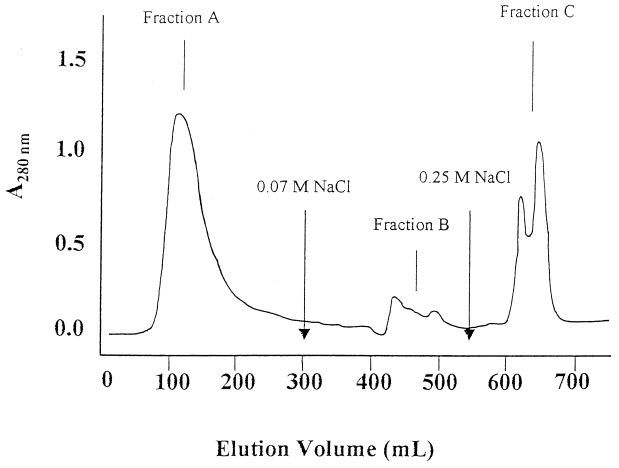

Fig. 1. Ion-exchange chromatography of red bean globulin by stepwise elution with NaCl.

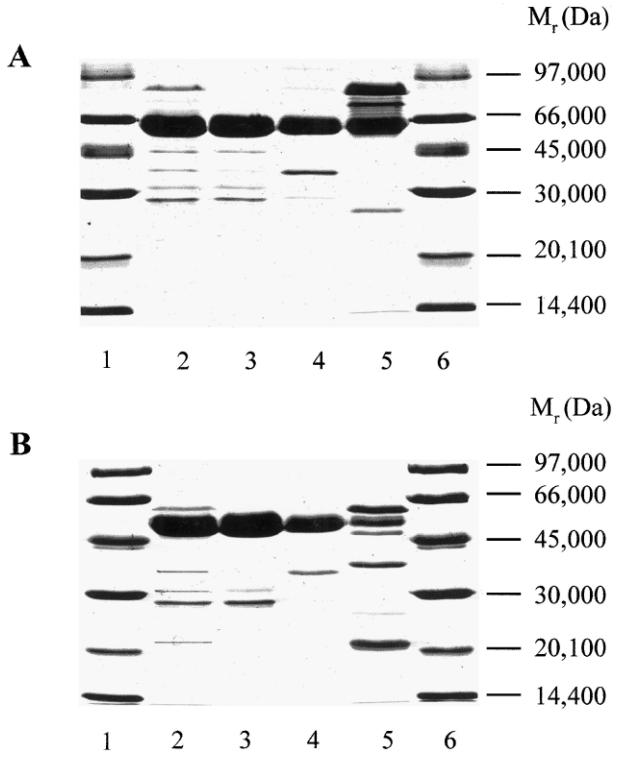

Fig. 2. SDS–PAGE of red bean globulin and its fractions from ion-exchange chromatography. A: without β-mercaptoethanol; B: with 5% β-mercaptoethanol; Lane 1 and 6: molecular weight markers; lane 2: red bean globulin; lane 3: fraction A; lane 4: fraction B; lane 5: fraction C.

The SDS–PAGE data show that mixed red bean globulin contains components from the three fractions, with the major component (51–62 kDa) from fraction A. Fraction B also contains the major band found in fraction A, but with a band (38 kDa) not found in other fractions. Neither fraction A nor B were dissociated by ME, suggesting that they do not contain subunits linked by disulfide bonds. Fraction C also contains the major band from fraction A, probably as cross-contaminant in column fractionation. The dissociation of high molecular weight components in fraction C into new bands, by a reducing agent, suggests the presence of subunit polypeptides linked by disulfide bonds.

Osborne and Campbell (1896) proposed the distinction of two types of globulins in pea: legumin and vicilin; this was further extended to numerous legume species by Danielsson (1949). In the case of common beans, including *Phaseolus* species, a 7S vicilin fraction (also known as phaseolin, glycoprotein II or G1 globulin) was isolated which represents the major storage protein (Barker, Derbyshire, Yarwood & Boulter, 1976; Ericson & Chrispeels, 1973; Pernollet & Mosse, 1983). Vicilins are glycoproteins devoid of disulfide bonds and are frequently non-covalently associated in trimers or even hexamers. The sedimentation coefficient is 7S and the molecular weight is about 200±50 kDa. Other distinctive characteristics of vicilin are higher solubility in salt solution and lower temperature of coagulation than legumin (Pernollet & Mosse, 1983).

Sakakibara, Aokiand and Noguchi (1979) extracted proteins from *Phaseolus angularis* (Adzuki bean) with water and purified the major fraction, termed 7S protein-I, by chromatographic techniques. The fraction, shown to be a glycoprotein, was found to contain three subunits with M_r of 55, 28 and 25 kDa.

Our SDS–PAGE data suggest that fraction A, from ion-exchange chromatography contains mainly 7S vicilin, with three subunits having molecular weights slightly higher than those reported by Sakakibara et al. (1979). The fact that ME did not lead to dissociation of

the subunits further confirms that 7S vicilin is the major protein in fraction A. Fraction B probably represents residual vicilin bound to column matrix and eluted with low salt buffer. The 38 kDa band may represent a minor protein component in red bean, perhaps the 2S fraction reported by Sakakibara et al. (1979). Fraction C was eluted at a higher salt concentration, and the SDS–PAGE patterns suggest that the fraction contains mainly the 11S legumin. The quaternary structure of legumin in legume seeds has been well established, and shown to contain six monomers or subunits (M_r ≈60 kDa), with each subunit made up of an acidic (M_r ≈40 kDa) and a basic (M_r ≈20 kDa) polpeptide linked by disulfide bonds (Pernollet & Mosse, 1983). Our SDS–PAGE data indicate that the red bean legumin is made up of subunits with M_r of ≈64 kDa, which can be dissociated into an acidic (M_r ≈40 kDa) and basic (M_r ≈22 kDa) polypeptides. The estimated molecular weight of the hexamer, 384 kDa, is within the range for legumin. Definitive identification of these two fractions from RBG will require detailed structural analysis, including mass spectroscopy and N-terminal sequence analysis of individual subunits that are underway in our laboratory.

3.2. DSC thermograms of RBG and its column fractions

The DSC thermograms of RBG and the FPLC fractions are shown in Fig. 3. The mixed globulin shows a major endothermic peak at 86.4°C with a shoulder at 92.2°C (Fig. 3a). Fraction A exhibited two endothermic peaks at 87.7°C and 94.1°C, respectively (Fig. 3b), and fraction B showed a single peak at 88.6°C, close to that of the first peak in fraction A (Fig. 3c). Fraction C showed one endothermic transition at 89.5°C (Fig. 3d).

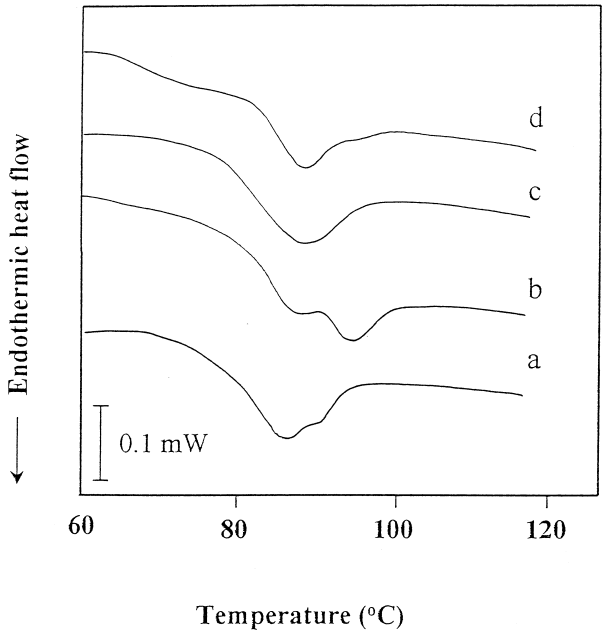

Fig. 3. DSC thermograms of red bean globulin and its fractions from ion-exchange chromatography. a: red bean globulin; b: fraction A; c: fraction B; d: fraction C.

The two transition peaks observed in fraction A may be attributed to different vicilin subunits with different T_d. The fact that fraction B contained only the major SDS–PAGE band (54–65 kDa) found in fraction A suggests that the T_d of this vicilin subunit is near 88°C, while the T_d attributed to the other two subunits (33 and 29 kDa) is around 94°C. Alternatively, the lower T_d component may correspond to vicilin while the higher one may represent glycosylated subunits of vicilin. Sugars have been shown to have a protective effect on proteins against thermal denaturation (Back, Oakenfull & Smith, 1979; Grimaldi, Robbins & Edelhoch, 1985; Harwalkar & Ma, 1992). Kitabatake, Cuq and Cheftel (1985) found that a higher level of glycosylation has been shown to increase solubility and heat-stability of β-lactoglobulin. Differences in glycosylation of vicilin subunits were also found in mung bean (*Phaseolus aureus roxb.*; Ericson, 1974).

The thermogram of fraction C indicates that red bean legumin has a T_d intermediate between the two vicilin peaks, although legumins generally have higher thermal stability than vicilins (Arntfield, Murray & Ismond, 1986; Pernollet & Mosse, 1983). Since legumin only constituted 12% of total RBG, and its T_d was close to and intermediate between those of the vicilin peaks, the thermogram of RBG should represent mainly the transitions of vicilin. The slightly higher transition temperatures observed in the purified vicilin than the RBG might be due to the presence of non-protein components in RBG affecting the thermal stability of proteins.

The T_d values observed in the RBG were within the range of those reported in other nutritionally valuable legumes, such as soybeans (*Glycine max*), broad beans (*Vicia faba*), cowpeas (*Vigna unguiculata*) and dry beans (*Phaseolus vulgaris*). In general, the 11S globulins, such as those from soybeans (glycinin) and broad beans (legumin), have a T_d value in the range 102–105°C, considerably higher than those of the 7S globulins, including soybeans (89°C), cowpeas (94°C), dry beans (90.5°C), and broad beans (89°C; Wright & Boulter, 1980). Lower T_d values for soybean 11S (97–98.5°C) and 7S (77–78°C) globulins (Murray, Arntfield & Ismond, 1985; Sheard, Ledward & Mitchell, 1987), and broad bean vicilin (86°C) and legumin (94°C) have also been reported (Murray et al., 1985). When compared with other legumes, the red bean legumin seems to have relatively low T_d value.

The DSC data suggest that thermal treatments routinely employed to eliminate or reduce anti-nutritional factors in legumes, e.g. steaming and cooking, could lead to marked protein denaturation in red beans, since the T_d of the major protein fractions are below the boiling temperature of water. How such changes in protein conformation will affect the functional performance of RBG will need to be determined.

3.3. Effect of NaCl concentration

The effect of NaCl concentration on DSC characteristics of RBG is shown in Fig. 4. T_d of both the major and minor RBG components were progressively increased with increasing NaCl concentration, indicating the stabilizing effect of NaCl on RBG. Similar results were reported on oat (Harwalker & Ma, 1987) and fababean globulin (Arntfield et al., 1986). The stabilizing effect of NaCl solution at lower concentrations (<1.0 M) was attributed to the electrostatic response or induction of alteration of water structure around the protein, which enhanced the hydration of the protein molecules (Arakawa & Timasheff, 1982; von Hippel & Scheleich, 1969). At higher concentrations of NaCl (>1.0 M), the solubility of protein decreased, due to a "salting-out" phenomenon, which caused the aggregation or precipitation of protein molecules due to lack of water molecules in the competition between the protein and ions for water (Morrissey, Mulvihill & O'Neil, 1987). In this case, a more compact protein conformation was formed with increased thermal stability (higher T_d). There seemed to be no pronounced changes in enthalpy with changes in NaCl concentration (Fig. 4), indicating that the conformation was not influenced by NaCl.

With increasing NaCl concentration, the resolutions of the two overlapping endotherms were enhanced (Fig. 4). Although the thermal stability of both vicilin components was increased by salt addition, the extent of increase may be different for the two proteins, leading to the separation of endotherms. The separation of legumin and vicilin by increasing salt concentration was observed in fababean in which legumin was the major protein fraction (Arntfield et al., 1986).

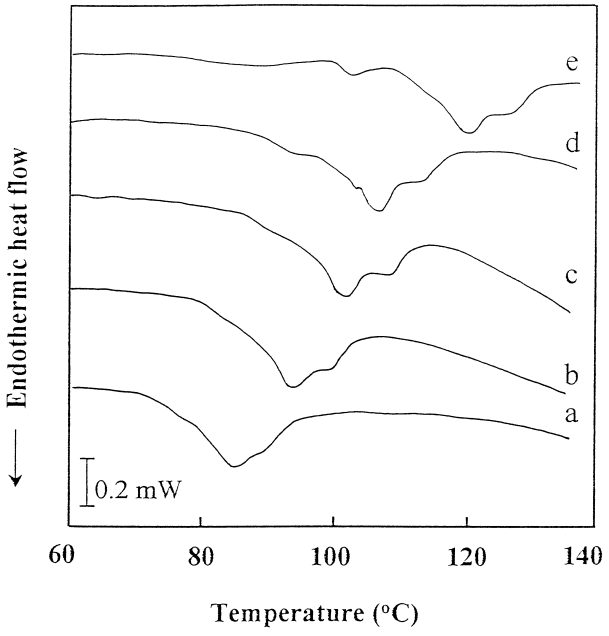

Fig. 4. Effect of salt concentration on DSC thermograms of red bean globulin. a: control (no salt); b: 0.5 M NaCl; c: 1.0 M NaCl; d: 2.0 M NaCl; e: 4.0 M NaCl.

3.4. Effect of chaotropic salts

The effects of some chaotropic salts on the thermal properties of RBG were also studied. The T_d of both transition peaks and ΔH of the combined endotherm decreased progressively when the anion was changed from Cl^- to Br^-, I^- and SCN^- (Table 1), following the lyotropic series of anions (Hatefi & Hanstein, 1969). In this series, Cl^- and Br^- can promote salting-out and aggregation due to higher molar surface tension, which may stabilize protein conformation. I^- and SCN^-, on the other hand, are destabilizing anions because of their higher hydration energy and steric hindrance, which promote unfolding, dissociation and salting-in of proteins (Boye, Ma & Harwalkar, 1997). However, the T_d of RBG in the presence of 1.0 M NaI was slightly higher than that of the control (no added salt), which may be because of insufficiently high I^- concentration. As in fababean, there was no destabilizing effect of iodide until the concentration exceeded 1.0 M, following an equation of $y=86.4+7.4x-5.2x^2$, where y stands for T_d, and x represents ionic strength of the medium (Arntfiled et al., 1986).

3.5. Effect of pH

The effect of pH on the thermal characteristics of RBG is shown in Fig. 5. At pH 3, 5 and 7, a major peak with a shoulder similar to the control (Fig. 3a) were observed, whereas one peak was detected at pH 9 and 11 (data not shown). The results show that both T_d (of the major peak) and ΔH of RBG decreased at alkaline or acidic pH, with highest T_d at pH 5 and highest ΔH at

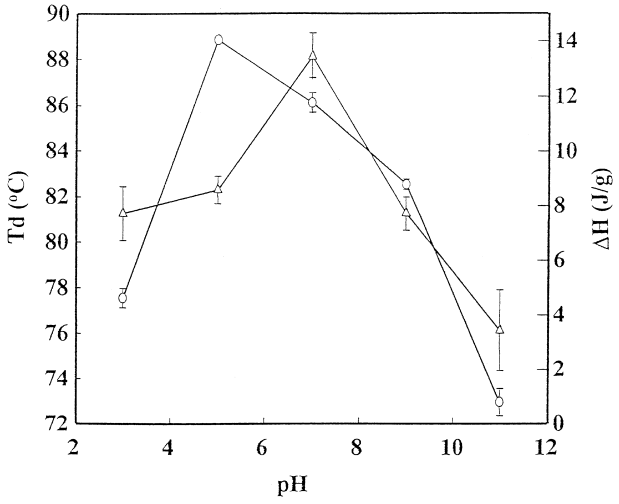

Fig. 5. Effect of pH on thermal characteristics of red bean globulin. The bars represent standard deviations of triplicate determinations. ○: T_d; △: ΔH.

pH 7. Most proteins are stable over a specific pH range, normally near the isoelectric pH, when the repulsive forces are quite low and therefore the proteins remain in a native state. At high or low pH, large net charges are induced and repulsive forces increase, resulting in unfolding of proteins (Morrissey et al., 1987).

3.6. Effect of protein structure perturbants

The thermal transition characteristics of RBG in the presence of several protein structure perturbing agents are listed in Table 2. Two transition peaks were observed in the presence of these perturbants, except for SDS, when one peak was found. With the addition of 10–40 mM sodium dodecyl sulfate (SDS), there were progressive decreases in T_d and ΔH. SDS is an anionic detergent which can bind to protein by non-covalent forces, causing ionic repulsion and unfolding of polypeptide (Steinhardt, 1975), with decrease in thermal stability and lowering in enthalpy. Dithiothreitol (DTT) is a reducing agent and can break disulfide bonds in the protein, causing destabilization, with decreases in both T_d and ΔH. However, the present data show no marked changes in DSC characteristics of RBG by adding DTT, and the thermal properties, in the presence of both DTT and SDS, were similar to those observed in the presence of SDS alone (Table 2). Our data are in contrast to other oligomeric proteins such as soy glycinin and oat globulin (Brinegar & Peterson, 1982; Wolf & Tarmura, 1969) in which the breaking of disulfide linkages by DTT led to dissociation of the oligomers and marked decreases in thermal stability and enthalpy. However, the data further confirm that RBG contains mainly the 7S vicilin which is devoid of disulfide linkages, and its conformation would not be significantly influenced by reducing agents. Similarly, N-ethylmaleimide, a sulfhydryl-blocking reagent, did not change the DSC characteristics of RBG, indicating that SS–SH interchange may not play a major role in stabilizing the conformation of the protein molecules.

The effect of urea on the thermal characteristics of RBG was also studied. With increasing concentration of urea, both T_d and ΔH were progressively decreased (Fig. 6). At 8 M urea, no discernible endothermic response can be observed (Fig. 6d), indicating extensive protein denaturation. Urea can denature proteins due to its destabilizing effect on hydrogen bonding and hydrophobic interactions (Kinsella, 1982). Apart from decreases in T_d and ΔH, urea caused a progressive broadening in the peak width ($\Delta T_{1/2}$), indicating loss of cooperativity in the thermal denaturation process (Privalov, 1982).

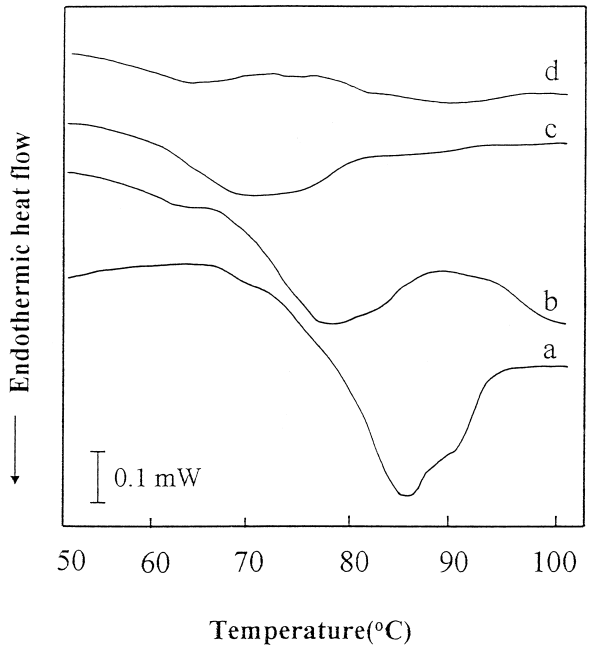

Fig. 6. Effect of urea on DSC thermograms of red bean globulin. a: control (no urea); b: 3 M urea; c: 6 M urea; d: 8 M urea.

Table 1
Effects of anions on thermal transition characteristics of red bean globulin[a]

Anions	T_m (°C)[b]	T_d (°C)[c]	ΔH (J/g)[d]
Cl^-	96.4±0.41	103.1±0.22 109.8±0.27[e]	14.9±1.64
Br^-	90.8±0.18	96.9±0.17 103.7±0.16[e]	14.4±1.36
I^-	83.0±0.39	89.7±0.19 97.9±0.11[e]	13.9±1.16
SCN^-	67.9±1.46	75.1±1.36 85.5±0.83[e]	9.1±0.88

[a] Averages and deviations of triplicates determined. Cl^-, Br^-, I^- and SCN^- were used in 1.0 M concentration.
[b] On-set temperature.
[c] Denaturation temperature.
[d] Enthalpy of the combined endotherm.
[e] Second transition peak.

Table 2
Effects of some perturbants on thermal transition characteristics of red bean globulin[a]

Perturbants	T_m (°C)[b]	T_d (°C)[c]	ΔH (J/g)[d]
Control (No additive)	78.0±0.55	86.4±0.35 92.2±0.68[e]	12.7±0.54
10 mM SDS	72.0±0.46	85.3±0.73	9.5±0.76
20 mM SDS	70.3±0.66	83.0±0.35	8.7±1.31
40 mM SDS	69.6±0.57	82.4±1.38	5.3±0.42
10 mM DTT	76.2±0.45	86.2±0.18 88.3±0.59[e]	13.1±1.36
10 mM NEM	74.4±0.22	85.6±0.24 91.4±0.14[e]	12.2±0.67

[a] Averages and standard deviations of triplicate determinations.
[b] On-set temperature.
[c] Denaturation temperature.
[d] Enthalpy of the combined endotherm.
[e] Second transition peak.

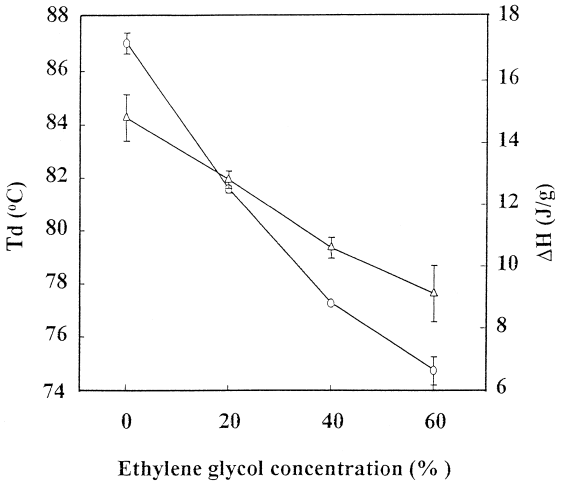

Fig. 7. Effect of ethylene glycol on thermal characteristics of red bean globulin. The bars represent standard deviations of triplicate determinations. ○: T_d; Δ: ΔH.

The effect of ethylene glycol on T_d and ΔH of RBG is shown in Fig. 7. At ethylene glycol concentration higher than 20%, only one endothermic peak (corresponding to the major peak in the control) was observed. Unlike other polyols or sugars which can protect proteins from thermal denaturation (Back et al., 1979; Gerlsma & Stuur, 1972; Harwalkar & Ma, 1992), ethylene glycol decreased both T_d and ΔH of RBG, similarly to β-lactoglobulin (Harwalkar & Ma, 1992), lysozyme and ribonuclease (Gerlsma & Stuur, 1972). Ethylene glycol, a water-miscible solvent, could lower the dielectric constant of the medium, waken the non-polar interactions between protein molecules, and enhance hydrogen-bonding and electrostatic interactions (Damodaran & Kinsella, 1982; Tanford, 1962). There was a progressive sharpening of the endothermic peak with increasing ethylene glycol concentration (not shown), indicating a highly cooperative transition.

4. Conclusion

The present data show that vicilin is the major protein in red bean globulin; it exhibits two endothermic transitions, probably representing the non-glycoslated and glycoslated vicilin. The DSC characteristics of RBG were influenced by environmental factors, including ionic strength, pH, chaotropic salts and protein structure perturbants. The results suggest that hydrophobic interactions, hydrogen bonds and ionic interactions play an important role in stabilizing the conformation of the protein molecule. Disulfide linkages and SS–SH interchanges did not seem to make a significant contribution to protein stability, since vicilin, the major RBG component, is devoid of disulfide bonds.

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