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Thermal aggregation of globulin from an indigenous Chinese legume, *Phaseolus angularis* (red bean)

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Abstract

The thermal aggregation behavior of red bean (*Phaseolus angularis*) globulin (RBG) was studied at ≈1% (w/v) protein concentration in 0.01 M phosphate buffer, pH 7.4. The percentage of protein precipitated was affected by heating temperature, heating time and salt concentration. The influences of several salts of the chaotropic series and protein structure-modifying agents on thermal coagulation of RBG were also investigated. The effects of chaotropic salts did not follow the lyotropic series of anions. Sodium dodecyl sulfate caused a more pronounced reduction in heat-induced aggregation of RBG than did dithiothreitol, while *N*-ethylmaleimide did not affect aggregation until after a long heating period. Differential scanning calorimetric (DSC) data showed that heat aggregation of RBG was preceded by thermal denaturation. SDS-PAGE showed that heating led to the disappearance of some protein bands, and the basic polypeptide of 11S globulin (legumin) was not found in the buffer-soluble aggregates. Heating caused increases of surface hydrophobicity, again suggesting protein unfolding prior to aggregate formation. The buffer-insoluble aggregates did not show any DSC response, indicating extensive denaturation, and had a lower surface hydrophobicity and higher disulfide content than the buffer-soluble aggregates. The data suggest that electrostatic and hydrophobic interactions may play an important role in thermal aggregation of RBG, with disulfide bonds playing a limited role.

Keywords: *Phaseolus angularis*; Globulin; Protein; Thermal aggregation

1. Introduction

Thermal aggregation (or coagulation) and gelation are important functional properties of food proteins, contributing to the mouth feel and texture of many food systems. As defined by Hermansson (Hermansson, 1979), coagulation is the random interaction of protein molecules, leading to formation of aggregates that could be either soluble or insoluble.

For legumes, a rich source of proteins, thermal aggregation and gelation also play an important role in their utilization. For example, tofu, a popular traditional oriental food, is produced by the heat coagulation of soybean proteins. Moreover, recent studies provided evidence that insolubilization of legume proteins induced by heating had a detrimental effect on their digestibility either in vitro or in vivo (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997).

Soybean 11S globulin (glycinin) has been used as a model to study thermal aggregation of proteins (Yamagishi, Yamauchi, & Shibasaki, 1980, 1981a, 1981b), and sulfhydryl and disulfide residues were found to play a major role in aggregate formation. However, in soyfoods, 7S as well as 11S globulin contributed to aggregate formation by interacting with each other on heating. Yamagishi, Miyakawa, Noda, and Yamauchi (1983) reported that heating of 7S globulin (β-conglycinin) alone did not cause precipitation, but addition of glycinin did. They also showed that the basic subunits of glycinin interacted with the β-subunits of β-conglycinin through secondary forces, and the resulting aggregates were located in the precipitates, whereas the α and α′ subunits of β-conglycinin and the acidic subunit of glycinin interacted through disulfide bonds and were mainly located in the supernatant fraction. Utsumi, Damodaran, and Kinsella (1984) also demonstrated the marked affinity of the β-conglycinin β-subunit to the glycinin basic subunit, and that interactions between the two subunits were predominately electrostatic in nature.

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Yamauchi, Sato, and Yamagishi (1984) isolated and purified another 7S globulin, which was designated basic 7S globulin due to its isoelectric point in the pH range 9.05–9.26. It contains two types of subunits ($M_r \approx 26$ and 16 kDa) linked by disulfide bonds. Further studies showed that this basic 7S globulin is immunologically distinguishable from 11S and 7S globulins (Sato, Yamagishi, Kamata, & Yamauchi, 1987), and this sulfur-rich protein could also be heat-coagulated and subsequently form soluble and insoluble aggregates (Sathe, Mason, & weaver, 1989). This basic 7S subunit was also found in Adzuki bean or red bean, *Phaseolus angularis*, an indigenous Chinese legume (Hirano, Kagawa, & Okubo, 1992).

Thermal aggregation of proteins from other legumes has not been extensively studied. At appropriate protein concentrations, thermal aggregation was the initial stage for the formation of three-dimensional gel networks of soy glycinin and broad bean legumin, where hydrophobic interaction and disulfide bonding are the major molecular forces involved (Mori, Nakamura, & Utsumi, 1982, 1986; Nakamura, Utsumi, & Mori, 1984; Zheng, Matsumura, & Mori, 1991).

Various mechanisms for protein aggregation have been proposed. For monomeric proteins, such as ovalbumin and bovine serum albumin, thermal aggregation is normally preceded by denaturation, following the scheme: $N \leftrightharpoons D \rightarrow A$, where N denotes native protein, D: denatured protein and A: aggregated protein (Ferry, 1948). For oligomeric proteins with complex quarternary structures such as soy glycinin (11S globulin), heating may cause association/dissociation of the oligomer and disruption of the quarternary structure itself may result in aggregation (German, Damordaran, & Kinsella, 1982; Mori et al., 1982; Utsumi & Kinsella, 1985; Yamagishi et al., 1980; Yamagishi, Takahashi, & Yamauchi, 1987).

Globulin has been isolated from red bean (*P. angularis*), and found to contain two fractions, a major 7S vicilin and a minor 11S legumin (Sakakibara, Aoki, & Noguchi, 1979). Our recent study (Meng & Ma, 2001b) also showed that vicilin is the major fraction in red bean seeds, representing about 80% of the total globulin. It is an oligomeric protein, containing three subunits, with molecular weights of 29, 33 and ≈60 kDa, respectively (Meng & Ma, 2001b). Red bean legumin, similar to other legume 11S globulins, is made up of six identical subunits. Each subunit is composed of an acidic ($M_r \approx 40$ kDa) and a basic ($M_r \approx 20$ kDa) polypeptide linked by disulfide bonds (Meng & Ma, 2001b). The six subunits are linked through non-covalent forces to form the hexamer with a M_r of 320–370 kDa (Derbyshire, Wright, & Boulter, 1976; Millerd, 1975; Nielsen, 1985).

In this study, factors affecting thermal aggregation of red bean globulin and the physicochemical properties of the heat-induced aggregates will be investigated. This will provide basic information on the thermal aggregation of legume proteins, in comparison to the extensively studied soybean proteins. Since red bean proteins are known to possess good nutritional properties (Chau, Cheung, & Wong, 1998), a study of the functional properties, such as heat aggregation and gelation, of the major protein fraction will enhance the utilization of red bean protein as a food ingredient.

2. Materials and methods

2.1. Preparation of red bean globulin

P. angularis seeds were purchased from a local seed 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. The protein content of RBG was 90.6±1.2%, as determined by a micro-Kjeldahl method (Concon & Soltess, 1973) using a nitrogen to protein conversion factor of 6.25.

2.2. Heat treatments

Red bean globulin (RBG) solutions (1.25%, w/v) were prepared in 0.01 M phosphate buffer, pH 7.4, containing 0.5 M NaCl. the insoluble materials were removed by centrifugation at 10,000 ***g*** for 10 min in a Beckman Avanti™ J-25 centrifuge (Palo Alto, CA). The final protein concentration was about 1%. Aliquots (about 2 ml) of RBG solution were pipetted into glass test tubes and each tube was stoppered with a glass marble to minimize water evaporation. The tubes were then heated at 80, 90 or 100 °C for various time periods in a temperature-controlled water bath. After heating, tubes were cooled by immersing in an ice bath for 5 min, and the solutions were centrifuged again at 10,000×***g*** for 10 min. Protein contents of the supernatant and unheated globulin solution were determined according to Lowry, Rosenbrough, Farr, and Randall (1951).

To study the effects of salts on thermal aggregation of RBG, sodium salts of the chaotropic series (NaCl, NaBr, NaI and NaSCN) were added as solids to RBG prepared in 0.01 M phosphate buffer (pH 7.4) to the desired concentration (1.0 M). Three protein structure perturbants, dithiothreitol (DTT), *N*-ethylmaleinide (NEM) and sodium dodecyl sulfate (SDS) were added as solids to RBG solutions in phosphate buffer containing 0.5 M NaCl. The concentrations of chaotropic salts and perturbants selected were based on previous studies (Meng & Ma, 2001a, 2001b) which showed significant conformational changes in RBG under these conditions.

To study some physicochemical properties of heat-aggregated RBG, including thermal characteristics,

subunit compositions, surface hydrophobicity, and SH and SS contents, aliquots (5 ml) of protein solutions (≈1%) in glass tubes stoppered with aluminium foil were heated in a temperature-controlled water bath at 90 °C for various time periods. The tubes were cooled rapidly by immersing in an ice bath. The heat-aggregated protein samples were dialyzed exhaustively against distilled water at 4 °C and freeze-dried.

2.3. Preparation of buffer-soluble and-insoluble aggregates

RBG solution (≈1% w/v) prepared in 0.01 M phosphate buffer, pH 7.4, containing 0.5 M NaCl was heated at 90 °C for 30 min in a temperature-controlled water bath, and then cooled by immersing in an ice bath for 5 min. The heated solution was centrifuged at 10,000 ***g*** for 10 min to separate into the buffer-soluble aggregates and buffer-insoluble aggregates. The soluble aggregates were dialyzed exhaustively against distilled water at 4 °C and recovered by freeze-drying. The insoluble aggregates were washed with distilled water and freeze-dried. Both soluble and insoluble aggregates were stored at −4 °C, similar to the RBG.

2.4. Differential scanning calorimetry (DSC)

The thermal properties of unheated and heat-aggregated RBG 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, were added. The pan was hermetically sealed and heated from 25 °C 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, New Castle, DE). All experiments were conducted in triplicate and the coefficient of variations ranged from 0.1 to 0.7% for T_m and T_d, and 5–10% for ΔH. Some of the thermograms comprised 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.

2.5. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE)

The subunit compositions of unheated and heat-coagulated RBG, and the buffer-soluble and insoluble aggregates were analyzed by SDS–PAGE in a Phast System™ (Pharmacia Biotech AB, Uppsala, Sweden) using 12.5% homogeneous gels according to the method of Laemmli (1970). Protein samples (≈5 mg/ml) were treated with sample buffer containing 10 mM Tris–HCl, 1 mM EDTA, 2.5% SDS and 0.01% bromophenol blue. The samples were heated for 5 min in a boiling water bath, cooled, and centrifuged at 15,000 ***g*** for 15 min. The amount of protein loaded was about 10–20 μg in 4 μl. Electrophoresis was carried out in the presence or absence of 5% β-mercaptoethanol. The standard protein markers were from a LMW Electrophoresis Calibration Kit (Pharmacia Biotech, Piscataway, NJ). The molecular weights of protein bands were determined using a Phoretix densitometric image analysis system (Phoretix International, Newcastle Upon Tyne, UK).

2.6. Surface hydrophobicity

The surface hydrophobicities of unheated and heat-coagulated RBG, and the heat-induced buffer-soluble and insoluble aggregates were determined by the fluorescence probe method (Hayakawa & Nakai, 1985; Kato & Nakai, 1980), using 1-anilino-8-naphthalene sulfonic acid (ANS) as the probe.

2.7. Analysis of sulfhydryl and disulfide contents

The sulfhydryl (SH) and disulfide (SS) contents of unheated and heat-coagulated RBG and heat-induced buffer-soluble and insoluble aggregates were determined by the method of Beveridge, Toma, and Nakai (1974).

3. Results and discussion

3.1. Effects of heating time and temperature

When a RBG solution (≈1% in 0.01 M phosphate buffer, pH 7.4, containing 0.5 M NaCl) was heated at

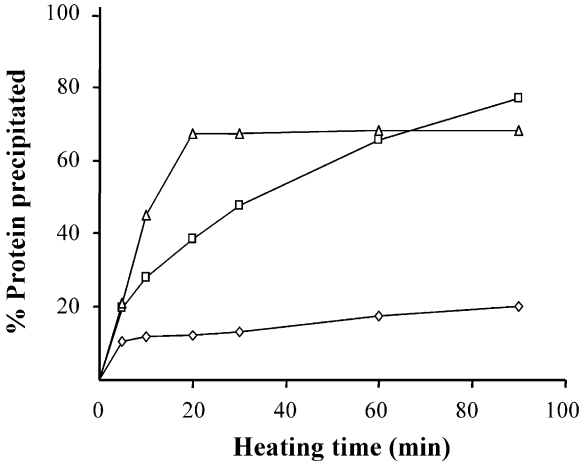

Fig. 1. Effect of heating time and temperature on the rate of thermal aggregation of red bean globulin. Protein solutions (≈1%) were heated at 80 (◇), 90 (□) and 100 °C (△).

80 °C, about 10% of total protein was aggregated after heating for 10 min (Fig. 1). Heating for longer time caused slight increases in protein precipitation, and about 19% protein was aggregated after 90 min. Heating at 90 °C led to a progressive increase in aggregate formation, at a rate much higher than at 80 °C, while at 100 °C, heat-induced aggregation increased rapidly and levelled off after 20 min (Fig. 1).

Upon heating, proteins may be reversibly, totally or partially denatured, or they may undergo aggregation, depending on the medium and treatment conditions (Damodaran, 1988). Heat treatments markedly decreased legume protein solubility, probably due to protein denaturation and subsequent aggregation (Carbonaro et al., 1997; Dench, 1982; Gujska & Khan, 1991). Our previous DSC study show that the denaturation temperature (T_d) of RBG was 95.2 °C in 0.5 M NaCl (Meng & Ma, 2001b). The present results indicate that RBG could be heat-coagulated well below its T_d. The two major protein fractions of RBG, vicilin and legumin, are both oligomeric proteins, and heat treatments may disrupt the quarternary structure, resulting in aggregation without extensive denaturation of the proteins (German et al., 1982).

When heated at 100 °C, RBG aggregated rapidly. Since heating above T_d could disrupt the oligmeric structure and denature the monomeric proteins, further rearrangement and/or aggregation can be initiated, leading to more extensive protein coagulation. Studies on legume globulins (Carbonaro et al., 1997; Derbyshire et al., 1976; Utsumi et al., 1984) have shown that both vicilin and legumin were dissociated into their constituent subunits upon thermal treatment, with re-association of monomers and formation of either soluble or insoluble complexes.

3.2. Effects of salt concentration

Fig. 2 shows the effects of salt concentration on thermal aggregation of RBG. The amount of protein precipitated was found to increase progressively with increasing NaCl concentration, levelling off at 0.5 M. When RBG was heated at 100 °C in buffer without added salt, less than 10% protein could form aggregates (Fig. 2). When RBG solution was heated at 90 °C in the absence of salt, almost no protein was precipitated (data not shown).

It has been suggested that one of the characteristics of legume seed globulins is their capacity to show an association/dissociation behaviour, particularly under conditions of varying ionic strength or pH (Derbyshire et al., 1976; Duranti, Guerrieri, Takahashi, & Cerletti, 1988). Our results indicate that salt was an essential factor for the thermal coagulation of RBG, as few aggregates were formed when the protein was heated in buffer without added NaCl.

Salts profoundly influence protein conformation and function by affecting the physical state of water, breaking the hydrogen-bonded structure of water and weakening intra-molecular hydrophobic interactions of proteins (Kinsella, 1982; von Hippel & Schleich, 1969). The salt-dissociated proteins easily formed aggregates upon heating due to protein-protein interactions. As an electrolyte, the effect of a given salt on the stability of protein structures depends on the concentration and/or ionic strength. At lower salt concentrations ($\mu < 0.5$), the stabilizing effect has been attributed to an electrostatic response (von Hippel & Schleich, 1969). Therefore, the stabilization of protein in this ionic strength range should be independent of salt type. At $\mu > 0.5$, the ability of salts to stabilize protein structures has been related to the preferential hydration of the protein molecule as a result of a salt-induced alteration of water structure in the vicinity of the protein (Arakawa & Timasheff, 1982).

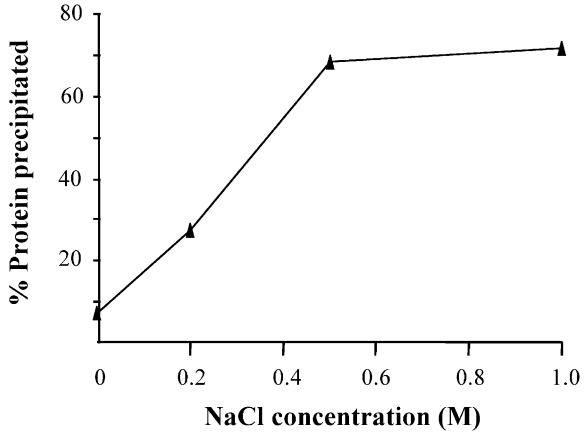

Fig. 2. Effect of salt concentration on the rate of thermal aggregation of red bean globulin. Protein solutions (≈1%) were heated at 100 °C for 60 min.

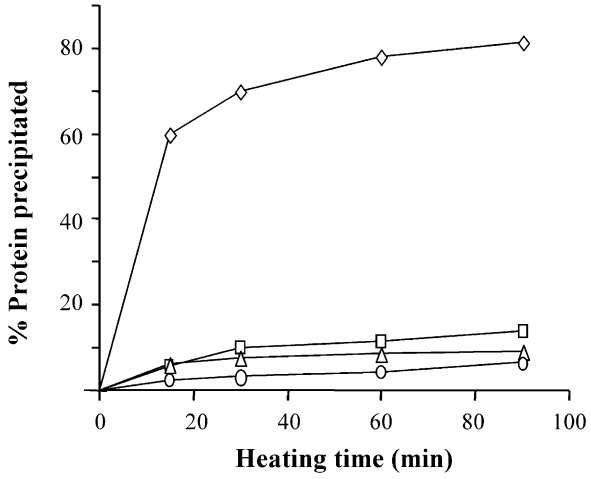

Fig. 3. Effect of salts of chaotropic series (1.0 M) on the rate of thermal aggregation of red bean globulin. Protein solutions (≈1%) were heated at 100 °C. ◇, Cl^-; □, Br^-; Δ, I^-; ○, SCN^-.

3.3. Effects of chaotropic salts

The effects of several salts of the chaotropic series on heat-induced aggregation of RBG are shown in Fig. 3. When RBG solution in 0.01 M phosphate buffer (pH 7.4) containing 1.0 M NaCl was heated at 100 °C, aggregates formed rapidly and levelled off after 30 min, with over 65% protein precipitated. As the anion was changed from Cl^- to Br^-, I^- or SCN^-, the% protein precipitated decreased markedly. Extended heating after 15 min did not cause further aggregation (Fig. 3). Heating at lower tempera tures, e.g. 90 °C, failed to show clear differences among the salts (data not shown).

The effects of anions and cations on protein conformation have been shown to follow the lyotropic series (Hatefi & Hanstein, 1969). Anions or cations higher in the series have a greater tendency to denature protein by reducing the free energy required to transfer polar groups to water, thus decreasing thermal stability (von Hippel & Wong, 1964). In a previous study (Meng & Ma, 2001b), anions higher in the lyotropic series were found to cause more extensive denaturation and decreases in thermal stability of RBG. Similar results were reported in oat globulin where decreases in heat stability and partial denaturation under the influence of chaotropic salts were found to lead to increases in heat-induced aggregation, and the extent of increases followed the lyotropic series of anions (Ma & Harwalkar, 1987). The influence of anions on thermal aggregation of RBG, however, did not follow such a trend. The "non-ideal" effect of anions was also observed in the heat-coagulation of almond protein isolate, and has been attributed to changes in pH of the heating medium since a buffer was not used (Sathe & Sze, 1996). Since a buffer was used in this study, the "non-ideal" behaviour cannot be attributed to differences in pH in various salt media.

3.4. Effects of protein perturbants

The effects of some protein structure perturbants on thermal aggregation of RBG at 90 °C are presented in Fig. 4. In the presence of 10 mM DTT, the rate of RBG precipitation was slightly decreased. Addition of 10 mM NEM caused no pronounced change in the amount of protein precipitated at the initial heating period, followed by a decrease after 30 min. SDS (10 mM) markedly inhibited thermal aggregation of RBG (Fig. 4).

The data show that thermal aggregation of RBG was affected by its conformation. DTT is a reducing agent, which can break the disulfide bonds in proteins. It was reported that DTT increased the aggregation of oat globulin (Ma & Harwalkar, 1987) and soy glycinin (Wolf & Tamura, 1969), as the oligomeric structures of these two proteins were stabilized by disulfide bridges between acidic and basic polypeptides. However, RBG is composed mainly of vicilin with a few disulfide bonds (Meng & Ma, 2001b). Therefore, DTT did not cause marked changes in the aggregation of RBG. NEM is a sulfhydryl-blocking reagent, which can hinder the SH–SS interchange in protein interactions. The results suggest that thiol-disulfide exchange played a limited role in RBG aggregation.

As an anionic detergent, SDS can bind to proteins by non-covalent forces, causing ionic repulsion and unfolding of polypeptides (Steinhardt, 1975). Increased negative charge on proteins could hinder protein–protein interactions and prevent the formation of aggregates. A similar influence of SDS on thermal aggregation was observed in oat globulin (Ma & Harwalkar, 1987) and conalbumin (Hegg, 1978). The results, together with the effects of NaCl, suggest that thermal aggregation of RBG depends on a critical balance of electrostatic attraction and repulsion of the protein molecules.

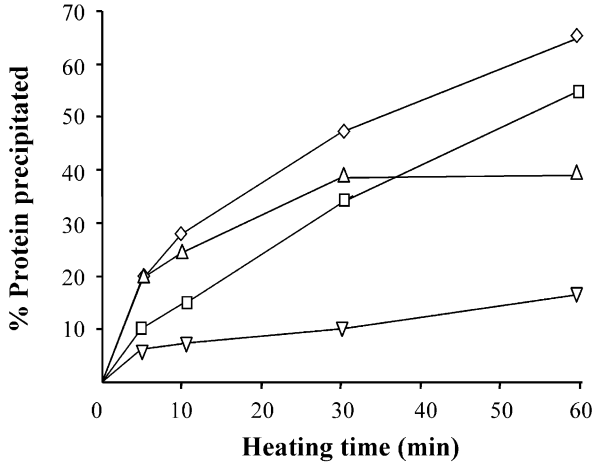

Fig. 4. Effect of protein structure perturbants on the rate of thermal aggregation of red bean globulin. Protein solutions (≈1%) were heated at 90 °C. ◇, Control (no additive); □, 10 mM dithiothreitol; Δ, 10 mM *N*-ethylmaleimide; ∇, 10 mM sodium dodecyl sulfate.

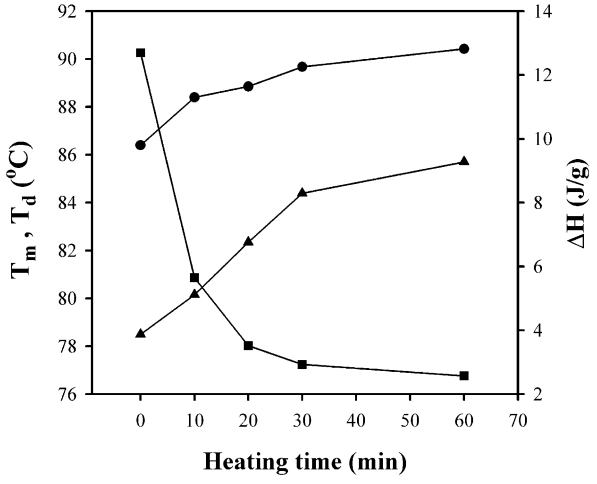

Fig. 5. Effect of heat treatments on thermal transition properties of red bean globulin. Protein solutions (≈1%) were heated at 90 °C for various time periods, cooled, dialysed, and freeze-dried. ▲, T_m; ●, T_d; ■, ΔH.

3.5. DSC

Fig. 5 shows the progressive increases of both the onset (T_m) and denaturation (T_d) temperatures when RBG solutions were heated under conditions that promoted aggregation. There was a concomitant rapid decrease in enthalpy, levelling off at about 30 min (Fig. 5). The decreases in enthalpy values suggest thermal denaturation of RBG, since partially unfolded proteins would require less heat energy (lower enthalpy) to denature. The increases in transition temperatures suggest the formation of macrocomplexes with more compact structures and hence higher thermal stability. Similar results were observed during heat aggregation of oat globulin (Ma & Harwalkar, 1988) which also showed a dramatic decrease in width at half-peak height, indicating that the heat-treated oat globulin would denature in a highly cooperative fashion (Privalov, Khechinashvili, & Atanssaov, 1971). In this study, the presence of multi-peak transitions made it difficult to measure the half-peak width accurately.

The relationship between thermal denaturation (as measured from the enthalpy values of the pre-heated RBG by DSC) and aggregation at 90 °C is demonstrated in Fig. 6. There were concurrent increases in the amounts of protein precipitated and the level of denaturation. Similar to oat globulin (Ma & Harwalkar, 1988), the percent of denaturation was higher than the percent of precipitation at any time. Although DSC cannot provide a very accurate measure of level of protein denaturation, as other endothermic and exothermic events may contribute to the enthalpy value, it can give an estimate of proportion of undenatured protein in a sample (Arntfield & Murray, 1981).

The thermal behaviour of the heat-induced buffer-soluble (SA) and insoluble (ISA) aggregates of RBG was also studied by DSC (Fig. 7). Results showed that SA (Fig. 7b) had markedly lower enthalpy value than the unheated control (Fig. 7a), suggesting significant denaturation of RBG during the formation of SA. The thermogram of the ISA showed no endothermic response, indicating complete denaturation of RBG. The data indicate that buffer-soluble aggregates of RBG were formed from extensively denatured proteins. In contrast, soluble aggregates of oat globulin were shown to contain native proteins, even when the aggregates were formed at a temperature close to the T_d, and under conditions that led to marked protein denaturation (Ma & Harwalkar, 1988). Insoluble aggregates of oat globulin, on the other hand, did not exhibit any DSC response, indicating complete denaturation (Ma & Harwalkar, 1988). Such re-distribution of native and denatured proteins into buffer-soluble and insoluble aggregates, respectively, was not clearly demonstrated in RBG. It should be noted that the observed decreases in enthalpy value in the SA and ISA fractions could also be attributed to exothermic events, such as protein aggregation and the breakup of hydrophobic interactions (Arntfield & Murray, 1981; Jackson & Brandts, 1970; Privalov, 1982). Hence, RBG may not require extensive heat denaturation, as suggested by the marked decrease in ΔH, to form soluble aggregates.

3.6. SDS–PAGE

Fig. 8 shows the SDS–PAGE patterns of RBG heated at 90 °C for 0, 10, and 60 min. In the absence of β-mercaptoethanol (Fig. 8a), the unheated control (lane 2) showed a large band with M_r of (63 kDa, and several bands with M_r ranging from 29 to over 100 kDa. Heating caused the disappearance of two bands at M_r of 44 and 82 kDa, respectively (Fig. 8a). The addition of β-mercaptoethanol led to the dissociation of these two bands in the control, and the appearance of a new band

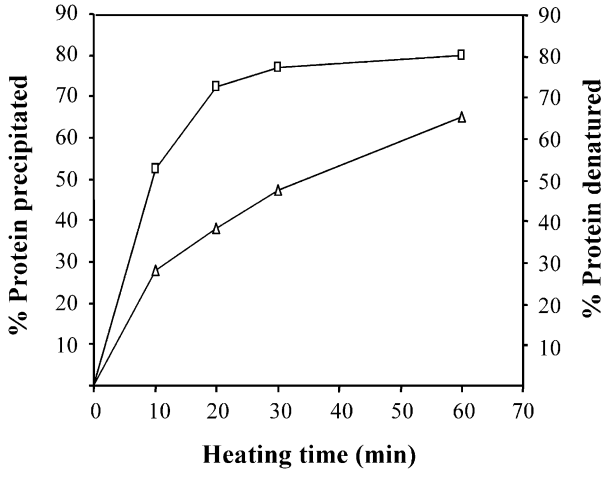

Fig. 6. Relationship between heat-induced denaturation and aggregation of red bean globulin at 90 °C. □, % Protein denatured; Δ, % protein precipitated.

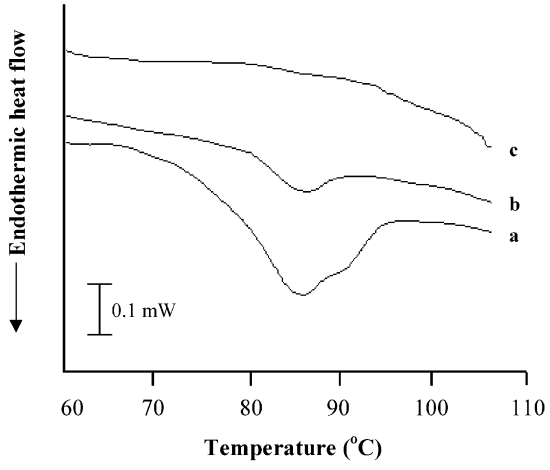

Fig. 7. DSC thermograms of red bean globulin and the heat-induced buffer-soluble and insoluble aggregates. a, Red bean globulin; b, buffer-soluble aggregates; c, buffer-insoluble aggregates.

at 22 kDa (lane 2, Fig. 8b). The intensity of a high M_r band (107 kDa) increased in the presence of ME. Heat treatment did not lead to marked changes in the electrophoretic pattern, except for the disappearance of a minor band at 64 kDa (Fig. 8b).

Fig. 9 shows the SDS–PAGE patterns of RBG and the heat-induced buffer-soluble (SA) and insoluble aggregates (ISA), both in the absence (Fig. 9a) and presence of reducing agent (Fig. 9b). Two bands (M_r 44 and 82 kDa) found in the unheated RBG (lane 2) were not found in either the SA (lane 3) or ISA (lane 4). In the presence of the reducing agent (Fig. 9b), the 22 kDa band was found in the unheated control (lane 2) and ISA (lane 4), but not in SA (lane 3).

The SDS–PAGE pattern of the unheated RBG was similar to that reported previously (Meng & Ma, 2001b), although a different electrophoretic system was used in that study. As shown in the previous report, RBG, the salt-soluble globulin extracted from red bean seeds by the Osborne fractionation procedure (Ma & Harwalkar, 1984), is a mixture of two globulins, the 7S vicilin and the 11S legumin. The mixed red bean globulin can be fractionated into the 7S and 11S globulins by ion-exchange chromatography, using stepwise elution with buffers containing different NaCl concentrations. The 7S globulin, constituting about 80% of protein in the mixed globulin, was eluted by buffer containing no salt, and SDS–PAGE showed a major subunit with $M_r \approx 60$ kDa and two minor bands of 29 and 33 kDa, respectively. These subunits were not dissociated by reducing agent, suggesting that they were not linked by disulfide bonds, like other legume vicilins (Pernollet & Mosse, 1983). The legumin fraction, constituting about 12% of total RBG protein and eluted by buffer containing 0.25 M NaCl, contains several subunits having M_r ranging from 60 to 90 kDa in the absence of β-mercaptoethanol. In the presence of the reductant, four major bands with M_r of 22, 40, 58 and 64 kDa, were observed (Meng & Ma, 2001b). It is suggested that the 22 and 40 kDa components represent the basic polypeptide and acidic polypeptide, respectively, similar to other 11S seed globulins (Derbyshire et al., 1976; Neilsen, 1985; Pernollet & Mosse, 1983). In the present study, all the three 7S globulin bands and some of the 11S globulin bands were observed. However, the basic polypeptide band of legumin was not found in the buffer-soluble fraction.

The disappearance of the 82 and 44 kDa bands when RBG was heated (Fig. 8a), and their absence in the aggregates (Fig. 9a) may be due to the formation of high molecular weight components which cannot enter the separating gels. The fact that these two protein bands were also dissociated by reducing agent suggests that they contain disulfide bond-linked polypeptides, most likely the legumin components. The minor 64 kDa band, presumably the legumin monomer similar to other legumes (Pernollet & Mosse, 1983), was also found to disappear in the heated RBG (Fig. 8) and aggregates (Fig. 9).

Despite variation in the ratios of vicilin and legumin, and their subunit composition among legume species (Shewry, Napier, & Tatham, 1995; Wright, 1987), both globulins were shown to undergo dissociation into their constituent subunits by heat treatment, with re-association of monomers and formation of either soluble or insoluble complexes (Carbonaro et al., 1997; Derbyshire et al., 1976; Utsumi et al., 1984). Mori et al. (1982) studied the heat coagulation behaviour of soy 11S glycinin, in which thermal changes included formation of soluble aggregates, dissociation of the soluble aggregates into acidic and basic polypeptides and finally association of basic polypeptides to insoluble aggregates, whereas the acidic polypeptides remain soluble. More detailed research revealed that among the subunits of 7S globulin, the β-subunit exhibits marked affinity for the basic subunits of 11S globulin and the interaction between them led to formation of highly heterogeneous soluble macrocomplexes with a minimum molecular weight of about one million (Utsumi et al., 1984).

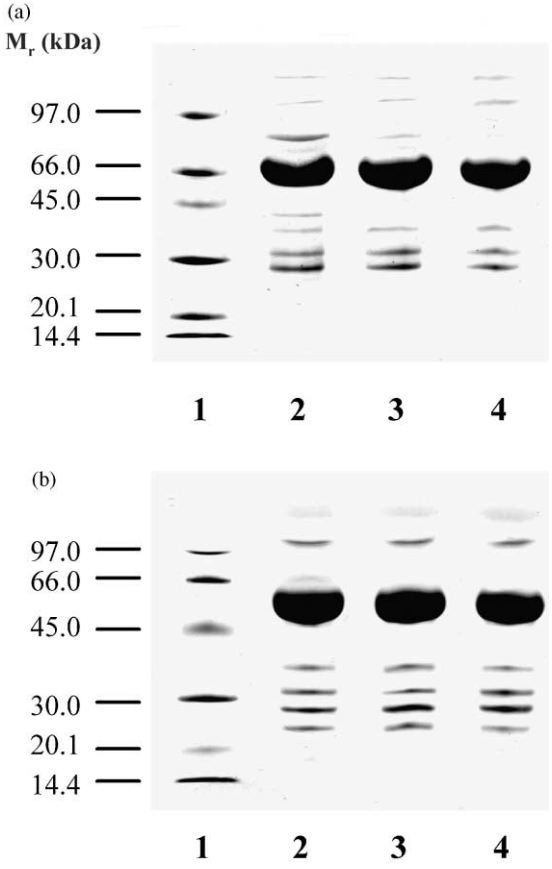

Fig. 8. SDS–PAGE patterns of red bean globulin heated at 90 °C. (a), Without β-mercaptoethanol; (b), with β-mercaptoethanol. Lane 1, molecular weight markers; lane 2, red bean globulin control; lane 3, heated for 10 min; lane 4, heated for 60 min.

In the present study, heat treatments did not clearly lead to the dissociation of vicilin and legumin into their subunits, despite the disappearance of a few protein bands (Fig. 8). The only conclusive evidence seems to be the formation of insoluble aggregates from the basic polypeptides of legumin. Since the acidic polypeptide ($M_r \approx 40$ kDa) was not clearly shown in the SDS–PAGE patterns, it was not known whether it remained in the soluble aggregates, as in soy glycinin. A detailed study of the dissociation/association behaviour of RBG vicilin and legumin, and the mechanism of thermal aggregation will require the use of the purified 7S and 11S globulins.

3.7. Surface hydrophobicity

Table 1 shows the changes in surface hydrophobicity of RBG during heating under aggregating conditions. The surface hydrophobicities of the buffer-soluble and insoluble aggregates were also measured (Table 1). Heating led to rapid increases in surface hydrophobicity of RBG, reaching a maximum at 20 min, and it then decreased gradually at 30 and 60 min. The unheated control had a relatively low surface hydrophobicity value when compared with the buffer-soluble and insoluble aggregates, and the soluble aggregates had much higher hydrophobicities than the insoluble aggregates (Table 1).

During thermal denaturation of globular proteins, buried hydrophobic residues were exposed, leading to increases of surface hydrophobicity (Kato & Nakai, 1980). With extended heating, the exposed hydrophobic groups may be involved in hydrophobic interactions during aggregate formation, resulting in a decrease in surface hydrophobicity. Extensive hydrophobic interactions during insoluble aggregate formation may lead to lower surface hydrophobicity values than soluble aggregates. The high hydrophobicity values in both types of aggregates agreed with the DSC data (Fig. 7) which indicate extensive protein denaturation in these fractions.

The results suggest that hydrophobic interactions are important driving forces for the formation of RBG aggregates. Studies with other globular proteins showed variable results. Heating caused an increase in surface hydrophobicity for ovalbumin (Galazka, Smith, Ledward, & Dickinson, 1999; Hayakawa, Kajihara, Morikawa, Oda, & Fujio, 1992; Kato, Osako, Matsudomi, & Kobayashi, 1983), but a decrease for bovine serum albumin and β-lactoglobulin (Kato et al., 1983). A recent study on β-lactoglobulin showed that changes of surface hydrophobicity depended on heating temperature (Relkin, 1998). The binding properties, to ANS probe, did not change significantly when heating was carried out at a temperature below the T_{max} (equivalent to T_d). When the heating temperature was higher than the T_{max}, the surface hydrophobicity increased (Relkin, 1998).

3.8. SH and SS contents

Fig. 10 shows the changes in SH and SS contents of RBG during heat treatments. No marked changes in SH

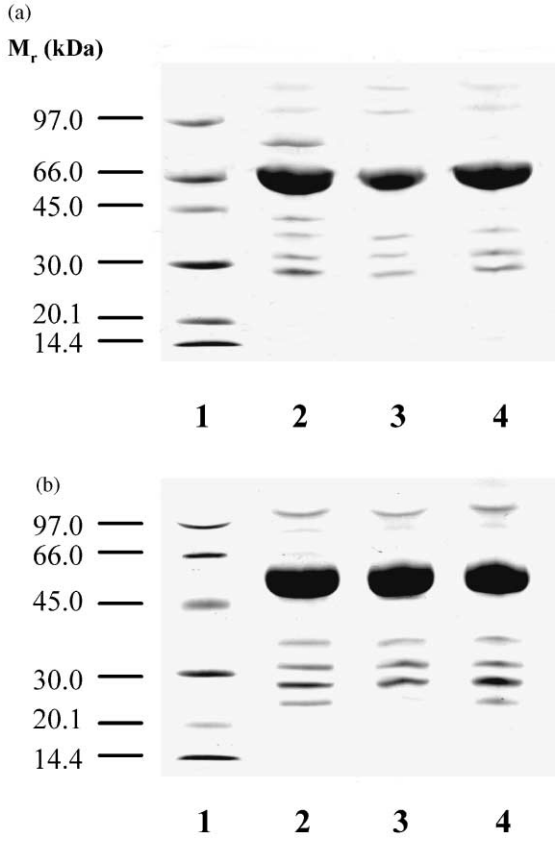

Fig. 9. SDS–PAGE patterns of heat-induced buffer-soluble and insoluble aggregates of red bean globulin. (a), Without β-mercaptoethanol; (b), with β-mercaptoethanol. Lane 1, molecular weight markers; lane 2, red bean globulin control; lane 3, buffer-soluble aggregates; lane 4, buffer-insoluble aggregates.

Table 1
Surface hydrophobicity of heat-aggregated red bean globulin[a]

	Surface hydrophobicity
Unheated	202[b]
Heated for 10 min	992
Heated for 20 min	1360
Heated for 30 min	1190
Heated for 60 min	947
Soluble aggregates	1255
Insoluble aggregates	818

[a] Protein solutions (≈1%) were heated at 90 °C for various time periods, and surface hydrophobicities of the cooled and diluted samples were determined. Buffer-soluble and insoluble aggregates were prepared as described in Section 2.

[b] Averages of duplicate determinations.

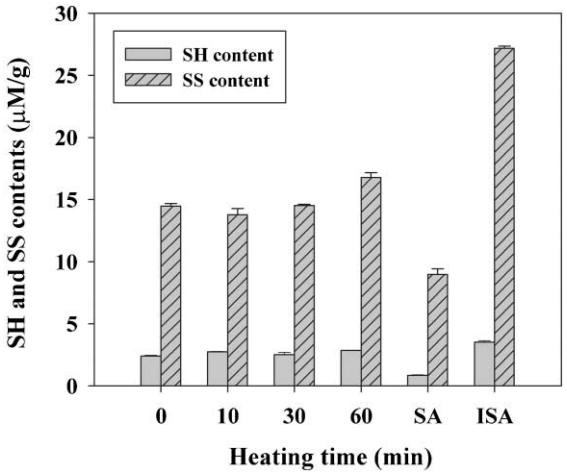

Fig. 10. SH and SS contents of heat-aggregated red bean globulin. SA, buffer-soluble aggregates; ISA, buffer-insoluble aggregates. The error bars represent standard deviations of the means.

contents were observed, and the SS contents only increased slightly after the protein was heated for more than 30 min. The SH and SS contents ofthe heat-induced buffer-soluble and insoluble aggregates were also determined. The results show that soluble aggregates have lower sulfhydryl and disulfide contents than the unheated control, while insoluble aggregates have the highest SH and SS contents.

Disulfide bonds have been shown to be involved in the cross-linking of denatured protein molecules to form aggregates (Catsimpoolas & Meyer, 1970; Morrissey, Mulvihill, & O'Weill, 1987). It has been suggested that the ability of some proteins to form intermolecular disulfide bonds during thermal treatment is a prerequisite for their coagulation and gelation (Utsumi & Kinsella, 1985). Heat treatments can lead to cleavage of existing disulfide bonds or "activation" of buried sulfhydryl groups through protein unfolding. These newly formed or activated SH groups can form new intermolecular SS bonds, which are essential for aggregate formation. The role of SH–SS interchange reactions in the cross-linking of protein gels and aggregates has been demonstrated (Wang & Damodaran, 1990). The present data show that heat treatments did not lead to marked changes in SH and SS contents in RBG. This is consistent with the fact that the major protein fraction in RBG, vicilin, is devoid of SS bonds. The minor change in SS content, after extended heating, may be attributed to the disulfide-linked legumin which may also contain free SH groups (Pernollet & Mosse, 1983).

The changes in SH and SS contents in SA and ISA suggest their participation in the formation of aggregates. The increases in both SS and free SH contents in the ISA suggest both the breaking of disulfide bonds and "activation" of buried sulfhydryl groups. The difference in SH and SS contents between the two fractions indicates that the buffer-insoluble aggregates may contain more legumin than the soluble aggregates. This is consistent with the SDS-PAGE data which showed the presence of the legumin basic polypeptides only in the ISA fraction. The exact role played by SH and SS groups in the thermal coagulation of RBG and the formation of soluble and insoluble aggregates will require more detailed studies using purified protein fractions.

4. Conclusion

The present data show that RBG is a heat-sensitive protein, which could form aggregates at temperatures well below its T_d. Both salts and SDS have great influence on thermal aggregation of RBG, suggesting the importance of electrostatic interactions in the aggregation process. Hydrophobic interactions also seem to play a major role, while disulfide or SH–SS exchange reactions may not be critical, probably due to the lack of disulfide bonds in the major protein fraction, vicilin.

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