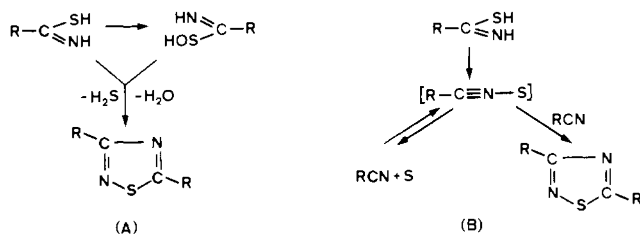

Figure 3. Possible modes of formation of the 3,5-diphenyl-1,2,4-thiadiazoles.

hitherto generally held opinion of the action of 2,6-dichlorothiobenzamide (**1e**) being due entirely to the formation of 2,6-dichlorobenzonitrile (**2e**) is only of limited validity.

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Studies on the Effect of Heat on the Dissociation, Denaturation, and Aggregation of Sesame α-Globulin

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The protein α-globulin, the major fraction of sesame seed (*Sesamum indicum* L.) proteins, coagulates on heating. Dissociation, denaturation, and aggregation of the protein upon heating have been studied by gel filtration, polyacrylamide gel electrophoresis, sedimentation velocity, pK_{app} of tyrosyl groups, and fluorescence measurements. The addition of β-mercaptoethanol does not reduce the extent of heat coagulation. The reassociation of the heat denatured subunits through hydrophobic interaction results in the formation of insoluble precipitate.

Sesame seed (*Sesamum indicum* L.) is a source of nutritionally important proteins due to their relatively high methionine content. The protein upon heating results in precipitation which restricts its use in certain food formulations, e.g., milk extender or beverage formulation. Recently, we have reported the association–dissociation and denaturation behavior of the major constituent (65–70%) α-globulin of sesame protein in different solutions (Prakash and Nandi, 1976, 1977a,b,c, 1978; Lakshmi and Nandi, 1977, 1978). In the present paper we report a study of the sequence and mechanism of heat aggregation of the protein.

MATERIALS AND METHODS

The protein α-globulin was isolated from sesame seeds (*Sesamum indicum* L., white variety) following the procedure developed in this laboratory (Prakash and Nandi, 1978). The total protein extract in 1 M NaCl obtained from defatted sesame flour was diluted 1:5.5 times with distilled water when α-globulin with some other protein fraction precipitated. The redissolution of the precipitate in 1 M NaCl, followed by dilution as above, yielded a protein which was found to be homogeneous (~95%) by gel electrophoresis, sedimentation analysis, and DEAE-cellulose chromatography (Prakash and Nandi, 1978). Phosphate buffer prepared from reagent grade chemicals and Tris (hydroxymethylaminomethane) obtained from Sigma were used in most of the experiments. Sepharose 6B-100 (Sigma) and urea (Sarabhai M. Chemicals) were used. $NaDodSO_4$ (Hindustan Levers) was crystallized twice from ethanol.

Heat coagulation experiments were carried out with protein solution in Tris–HCl buffer of pH 8.6 at 98 °C for 20 min. The absorbance of the supernatant was measured at 280 nm. The percentage of protein precipitated was determined by calculating the amount of protein present in the supernatant compared to the initial concentration of the protein solution. Protein concentration was calculated using $E_{1cm}^{1\%} = 10.8$.

A Sepharose 6B-100 column 46 × 2.5 cm (bed volume, $V_t \sim 200$ mL), was used for gel filtration experiments. The gel was equilibrated thrice the bed volume of the column with Tris–HCl buffer, 0.01 M, pH 8.6. The flow rate after loading the protein solution was adjusted to 18–20 mL/h and the protein concentration in the fractions was determined by measuring the absorbance at 280 nm.

Polyacrylamide gel electrophoresis (PAGE) was carried out in a Metrex gel electrophoresis unit using 0.02 M phosphate buffer at pH 7.5. A 10% gel in tubes having 7.5 × 0.5 cm dimensions was used. Protein samples (10 μg/μL) containing ~5% sucrose and 0.05% bromophenol blue (indicator dye) were used, and electrophoresis was carried out at a constant current of 3 mA/tube for 1 h and 40 min. The gels were stained for 45 min in 0.5% amido black in 7.5% (v/v) acetic acid, and destaining was carried out in 7.5% acetic acid solution.

Sedimentation velocity values were measured in a Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics. A standard 12-mm duraluminum cell centerpiece was used. Plates were read on a Gaertner microcomparator and $s_{20,w}$ values calculated

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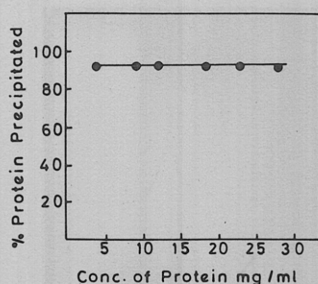

Figure 1. Effect of temperature on the percentage of protein precipitation using varying concentrations of protein in the solution. Temperature, 98 °C; time of heating, 20 min.

(Schachman, 1959). The percentage of the different components was estimated from enlarged tracings of sedimentation velocity patterns.

Spectrophotometric titration of the phenolic groups in the protein was carried out by measuring the absorbance at 295 nm as a function of pH (Donovan, 1973; Mihalyi, 1968). A protein solution of 0.05% (0.45 OD at 280 nm) and the supernatant of the heated protein solution in 0.1 M Tris of pH 8.6 were used for the titration. The pH was adjusted by adding 10% NaOH to the protein which was initially present in Tris buffer of pH 8.6. Possible turbidity corrections were made by subtracting absorbance at 330 nm from 295 nm absorbance (Mihalyi, 1968).

Fluorescence was measured in a Perkin-Elmer Hitachi fluorescence spectrophotometer using 0.005% protein solution in 0.1 M Tris–HCl of pH 8.6. The excitation wavelength was 280 nm.

The number of SH groups in α-globulin was estimated using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid), Beveridge et al., (1974)]. An aliquot (0.2 mL) of 1% protein solution was added to 2.5 mL of buffer or 8 M urea and the color was developed by addition of 0.02 mL of Ellman's reagent. The absorbance was measured at 412 nm in a colorimeter and the moles of SH/mole of protein was calculated from

$$[1/(1.36 \times 10^4)]A_{412}(D/C)$$

where 1.36×10^4 is the molar absorptivity of the complex, A_{412} is the absorbance at 412 nm, D is the dilution factor, and C is the sample concentration in milligrams/milliliter. The number of SH groups in the absence and presence of urea were 1 and 7 mol of SH groups/mole of protein, respectively.

RESULTS AND DISCUSSION

The protein solution when heated at 98 °C for 20 min results in 92% precipitation (Figure 1) and the amount of precipitation was independent of the initial concentration of the protein within the range studied (4.6–27.8 mg/mL).

The protein in 0.1 M Tris–HCl buffer of pH 8.6 in the Sepharose 6B-100 column elutes near the void volume of the gel (~68 mL). Supernatant of the heated protein solution shows (Figure 2) four components eluting at ~25, ~50, ~90 , and ~150 mL in addition to the peak appearing near 68 mL. The results indicate that in the heated protein solution both aggregated and dissociated products are present.

The supernatant protein solution (98 °C, 10 min) subjected to PAGE showed (Figure 3) six major and one minor bands; the presence of a high polymer and several dissociated components could be observed. The solution on prolonged heating (98 °C, 30 min) indicated a decrease in the amount of the polymeric form. The mobility of the dissociated products was nearly the same in the two samples.

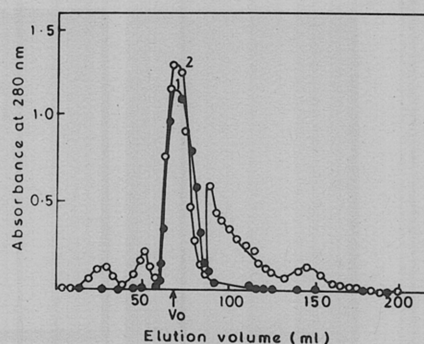

Figure 2. Gel filtration pattern of (1) control α-globulin and (2) heated protein in Sepharose 6B-100 gel column, dimension 46 × 2.5 cm; bed volume (V_t), 200 mL in 0.1 M Tris–HCl buffer of pH 8.6.

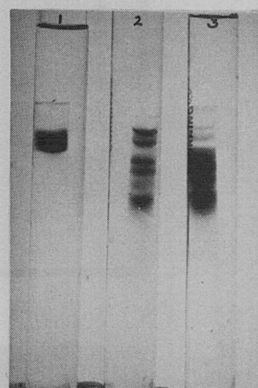

Figure 3. PAGE pattern of (1) control α-globulin, (2) α-globulin heated for 10 min, and (3) α-globulin heated for 30 min in 0.01 M phosphate buffer of pH 7.5.

The protein α-globulin has a sedimentation coefficient of 13S in Tris–HCl buffer, 0.1 M, pH 7.0 (Lakshmi and Nandi, 1977). At pH 8.6, Tris–HCl, the protein dissociates (~20%) to an 8S component (Figure 4a,b) at 28 °C. The protein solution at 40 °C indicated a decrease in the concentration (10%) of the 8S component with a concomitant increase (90%) of 13S component. Upon cooling the solution to 28 °C, the same sedimentation pattern as observed with the unheated protein solution was observed. This indicated that association–dissociation of α-globulin up to 40 °C was reversible. The protein heated to 50 °C showed two components sedimenting with 15S and 8S (Figure 4c) value. The pattern did not change up to 60 °C (Figure 4d). The protein solution heated at 80 °C for 5 min showed a higher percentage (95%) of the 15S component. In addition, the presence of a dissociated fraction having 3S value was observed (Figure 4e). The same solution heated at 80 °C for 10 min showed four components with sedimentation values of 17, 15, 10 and 3S (Figure 4f). The solution heated at 80 °C for 15 min showed a fast moving component sedimenting with 77S value (Figure 4g) together with a lower aggregate of 26S value and the dissociated products of 6 and 3S (Figure 4h). Heating for 30 min at 80 °C resulted in the disappearance of the fast moving components. The solution showed a component of 15S, constituting ~80% of the total, and two other components, 8 and 3S (10% each) (Figure 4i). The supernatant of the protein solution heated to 98 °C for 20 min showed the same pattern as that of the protein heated at 80 °C for 30 min (Figure 4j).

The apparent pK_{int} value of tyrosyl groups of sesame α-globulin in 0.1 M Tris is 11.0 which indicates that tyrosyl groups are abnormal and are not in contact with the aqueous environment (Donovan, 1973). The supernatant

Figure 4. Effect of temperature on the sedimentation velocity pattern of α-globulin in 0.1 M Tris–HCl buffer, pH 8.6. (a) α-Globulin in 0.05 M Tris–HCl buffer of pH 7.2, α-globulin at pH 8.6 (b) at 28 °C, (c) at 50 °C for 20 min, (d) at 60 °C for 20 min, (e) at 80 °C for 5 min, (f) at 80 °C for 10 min, (g) at 80 °C for 15 min (27 150 rpm), (h) at 80 °C for 15 min, (i) at 80 °C for 30 min, (j) at 98 °C for 20 min. Sedimentation proceeds from left to right. A speed of 59 780 rpm was used unless otherwise indicated. Temperature, 27 °C.

of the heated protein solution (98 °C, 30 min) showed a pK_{int} value of 10.6 (Figure 5). This suggested that the tyrosyl groups are experiencing a more polar environment as a consequence of denaturation upon heating.

The emission maximum of α-globulin when excited by 280-nm light is at 325–328 nm. This arises from the tryptophan groups embedded in the nonpolar environment of the protein and, the tyrosyl groups do not contribute to the observed fluorescence (Lakshmi and Nandi, 1977). The fluorescence spectrum of the protein solution heated at temperatures up to 50 °C showed neither a change in the emission maximum nor intensity. The solution at 57 °C, however, showed considerable decrease in intensity with a slight red shift to 333 nm. Heating at 80 °C results in further decrease in the intensity and a shift in the maximum to 340 nm. The samples heated at 88 and 97 °C showed further decrease in the intensity and shift in the emission maximum to 345 nm (Figure 6). This shift of the emission maximum toward red arises from the presence of the tryptophan groups in a polar aqueous

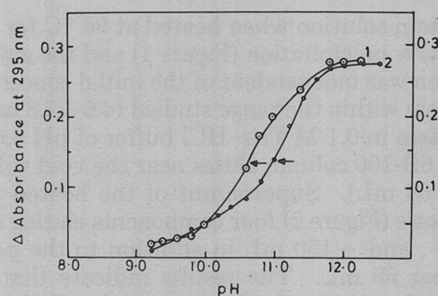

Figure 5. Spectrophotometric titration to determine the apparent pK_{int} value of the tyrosyl groups of α-globulin at room temperature (28 °C) and supernatant of the heated protein solution at 98 °C. (1) pK_{int} value of heated α-globulin, 10.6; (2) pK_{int} value of control α-globulin, 11.0.

environment. This has resulted from the unfolding, i.e., denaturation of the protein molecule at high temperature.

The results obtained from the above experiments suggest that α-globulin upon heating undergoes dissociation,

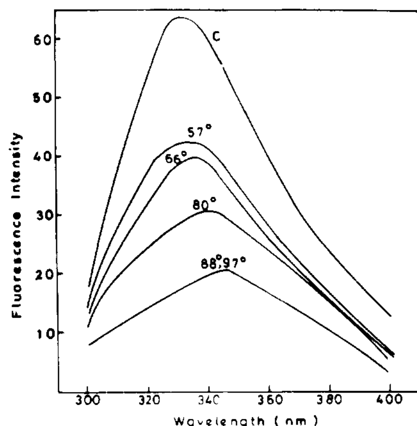

Figure 6. Fluorescence spectra of α-globulin at room temperature (28 °C) and protein solution heated at different temperatures. Time of heating, 20 min; excitation, 280 nm.

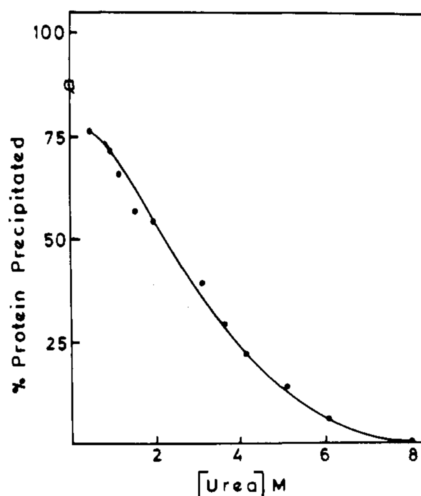

Figure 7. Effect of increasing concentrations of urea on the percent precipitation of protein upon heating at 98 °C for 20 min.

denaturation, and aggregation. The formation of higher aggregates is endothermic in nature. This would suggest that, upon heat denaturation, the newly exposed nonpolar groups of the protein due to their unfavorable interaction with the solvent water tend to come together, leading to aggregation. In addition, the polymerization process may also result from the formation of S–S linkages between the polypeptide chains as a consequence of the oxidation of the newly exposed SH groups (see Materials and Methods section) of the heat denatured protein (Tanrord, 1968, 1970). However, the addition of β-mercaptoethanol at room temperature precipitated α-globulin and there was no prevention of heat coagulation by the reagent. Catsimpoolas et al. (1970) observed that this reagent also did not prevent the heat coagulation of the soybean glycinin and concluded that polymerization of the heat denatured protein does not take place through S–S linkage. The prevention of heat coagulation of α-globulin in urea, viz. 100% in 8 M urea (Figure 7) and anionic detergent sodium dodecyl sulfate above 2×10^{-3} M (Figure 8), would suggest that the aggregation process of heat denatured sesame α-globulin takes place predominantly by the hydrophobic interaction of the newly exposed nonpolar groups.

The amount of charge and number of polar groups are the same in both the native and denatured state of the

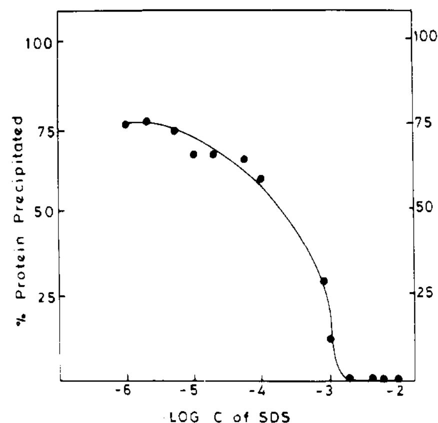

Figure 8. Effect of increasing concentration of $NaDodSO_4$ the heat coagulation pattern of α-globulin.

protein. In the denatured state, the favorable interaction of the charged and polar groups with water (resulting from charge solvation and hydrogen bonding ability with water, respectively) would not be able to overcome the unfavorable interaction of the nonpolar groups with water which leads to the aggregation of the heat denatured protein. Further, the electrostatic repulsion between the different protein subunits as they carry overall negative charges at the experimental pH 8.6 is not sufficient to prevent aggregation of the denatured sesame α-globulin upon heating.

The present results show that sesame α-globulin undergoes dissociation, denaturation, and aggregation upon heating. The aggregate does not dissolve upon cooling. This apparently "irreversible" aggregation is always potentially reversible since no covalent bond formation has taken place in the process. But the activation enthalpy barrier for dissociation of the aggregate is high, so that with the decrease in temperature, refolding into original protein will be a very slow process. This is the reason why, even upon cooling, the aggregate cannot dissolve but can be dissociated by urea, amides (Lumry and Biltonen, 1969), and sodium dodecyl sulfate solution.

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