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MATERIALS AND METHODS

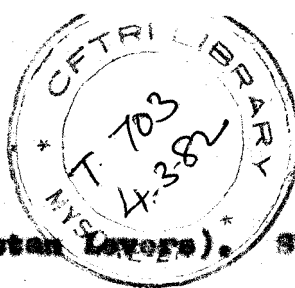
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## MATERIALS AND METHODS

### Materials:

White variety of sesame seeds were obtained from the local market. They were identified as Sesamum indicum L., during the flowering stage of the plant, by the Botany Department of the University of Mysore, Mysore.

The chemicals and reagents used are the following: The source of these materials are shown in the parenthesis. Sepharose 6B-100, Sephadex G-50, Sephadex G-75, DEAE-cellulose, bovine serum albumin, egg albumin, pepsin,  $\alpha$ -chymotrypsin, ribonuclease-A and  $\text{CuCl}_2$  (Sigma Chemicals); hemoglobin substrate (Worthington Biochemicals); sodium trichloroacetate and sodium perchlorate (E. Merck); NBS (L. Light and Co. Ltd.); bisacrylamide (Koch Light Laboratories); TEMED and  $\beta$ -mercaptoethanol (Fluka); Coomassie brilliant blue (Schwarz Mann); bromophenol blue, ammonium persulfate; methylene blue, sodium sulfate, sodium chloride, glucose and potassium hydrogen phosphate (BDH Chemicals); sodium bromide, sodium iodide, urea, trichloroacetic acid and TEA (Sarabhai M. Chemicals); tris (V. P. Chest Institute); sodium thiocyanate (Ajax Chemicals); perchloric acid (Riedel-



DC Haen, AG) and SDS (Hindustan Levers). SDS was re-crystallised twice from ethanol. The other reagents used were all of analytical grade.

### Methods:

The sesame seeds were soaked in water for 6 hr after which they were scrubbed to remove the hull. The dehulled and dried seeds were flaked followed by defatting by extraction with solvent n-hexane, with a solvent to flaked seed ratio as 1:1. The process of extraction was repeated at least six times and a meal containing less than 3% fat was obtained. This defatted meal was air dried in a cabinet drier at 45° for 6 hr after which it was powdered in a microatomiser and then passed through a 60 mesh sieve. The flour so obtained was further washed with n-hexane and a flour containing less than 1% fat was obtained. The defatted flour obtained was dried and used for the extraction of total protein.

### Extraction of total protein:

The protein in the defatted flour was extracted in solvent, 0.02M phosphate buffer, pH 7.5 containing 1M sodium chloride. The flour to solvent ratio was to 1:10. The slurry was stirred, not too vigorously, for ~ 1 hr and centrifuged at 4,000 rpm for 20 min. The supernatant obtained was dialysed against buffer system for ~ 24 hr in which subsequent studies were carried out.

### Isolation of $\alpha$ -globulin:

The protein  $\alpha$ -globulin was isolated by a minor modification of Nath and Giri's (1957b) procedure.

The supernatant obtained after centrifugation of the slurry was diluted with water 1:5.5 times instead of 1:10 times dilution done by Nath and Giri (1957b). The solution was centrifuged at 4000 rpm for 30 min. The precipitate was redissolved in the extraction solvent and the process of precipitation and centrifugation were repeated.  $\alpha$ -globulin thus obtained was dissolved in the extraction solvent and dialysed against the buffer system for  $\sim$  24 hr in which subsequent studies were carried out.

From a batch of 100 gm of the flour nearly 20-22 gm of  $\alpha$ -globulin was obtained, giving a percentage yield of  $\sim$  20%  $\alpha$ -globulin on the basis of flour weight.

### Protein concentration:

The concentration of  $\alpha$ -globulin was determined by macro Kjeldhal procedure (Official Methods of Analysis of AOAC, 1975). Nitrogen was estimated in a weighed amount of lyophilised  $\alpha$ -globulin and the nitrogen to protein conversion factor was determined as 6.25. A calibration curve relating the  $\mu$ g of nitrogen present in the protein sample with ultraviolet absorbance of the protein at 280 nm measured in a Carl Zeiss spectrophotometer was

was obtained for routine determination of protein concentration. The absorption coefficient,  $E$  of 1% protein solution at 280 nm i.e.  $E_{1\%}^{1\text{cm}}$  gave a value of 10.8 for  $\alpha$ -globulin. The corresponding value obtained for the total protein was 13.0.

#### Gel filtration:

Gel filtration was carried out mostly in Sepharose 6B-100 for homogeneity test of  $\alpha$ -globulin and its dissociation behaviour in SDS solutions. The gel column used was 2x87 cms ( $V_t=265$  ml) in all these experiments. Sephadex G-50 having the above column dimension and bed volume was used for the study of dissociation of  $\alpha$ -globulin in acid solution. Sephadex G-75 column of the same dimension as above<sup>was</sup> used for the separation of protein fractions in urea solution.

The gel was equilibrated with approximately three times the bed volume of the column with buffer in which further experiment had to be carried out. Nearly 50 mg of the protein in buffer was loaded on the column. The flow rate was adjusted at 25-30 ml/hr and 2.5 ml fractions were collected in an Emenvee automatic fraction collector at room temperature ( $\sim 25^\circ$ ). The protein concentration in the fractions were determined by measuring the absorbance at 280 nm.

### Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis was carried out in a Metrex Gel electrophoresis unit identical with Acrylophor apparatus in 0.02M phosphate buffer pH 7.5. A 7.5% gel was used for determining the homogeneity of  $\alpha$ -globulin. For a typical run, 0.83 gm of acrylamide and 23 mg of methylene bisacrylamide were dissolved in 8 ml of the above buffer, filtered and the volume made upto 10 ml and kept in cold. To 9.5 ml of this cooled solution, 0.5 ml of freshly prepared ammonium persulfate solution (15 mg/ml) and 20  $\mu$ l of TEMED solution were added. The solution was polymerised in glass tubes (eight in number) of 7.5x0.5 cm dimension. Protein samples (10  $\mu$ gm/1  $\mu$ l) containing ~40% sucrose and 0.05% bromophenol blue (indicator/dye) were loaded on the gel and electrophoresis was performed at a constant current of 3 mA per gel for 70 min. The gels were stained for 1 hr in 0.5% amido black in 7.5% (v/v) acetic acid solution. Destaining was carried out in 7.5% acetic acid solution, till the gels were colourless and stored in 7.5% acetic acid solution.

In presence of SDS, the electrophoresis was carried out using 10% polyacrylamide gel because of relatively poor resolution of the bands in 7.5% gel. For a typical run in this solution, the protein, gel and the

running buffer contained same concentration of the detergent. A 10% gel was used for electrophoresis in 8M urea solution. In this experiment the protein and the gel contained 8M urea.

Determination of subunits and their molecular weight:

The determination of the number of subunits and their molecular weight was carried out by the method of Weber and Osborn (1969). The protein  $\alpha$ -globulin (0.6 mg/ml) and other standard proteins (1 mg/ml) viz., bovine serum albumin, egg albumin, pepsin,  $\alpha$ -chymotrypsin and ribonuclease-A were incubated at 37° for 2 hr in 0.01M sodium phosphate buffer pH 7.0, containing 1% SDS and 1%  $\beta$ -mercaptoethanol. After incubation, the protein solutions were dialysed against large volumes of 0.01M sodium phosphate buffer pH 7.0 containing 0.1% SDS and 0.1%  $\beta$ -mercaptoethanol and subjected to gel electrophoresis. Gel buffer contained 7.8 gm of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 38.6 gm of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  and 2 gm of SDS per litre. The gel buffer diluted 1:1 with water was used as running buffer in presence of 10% gels. For a typical run, 22 gm of acrylamide and 0.6 gm of methylene bisacrylamide were dissolved in water filtered and the volume made upto 100 ml and kept in cold. To 13.5 ml of this solution, 15 ml of buffer, 1.5 ml of freshly prepared ammonium persulfate (15 mg/ml) and 0.045 ml of TEMED

solution were added. The solution was polymerised in glass tubes of 10 cm x 0.6 cm dimension. About 60  $\mu$ l of protein sample containing ~40% sucrose and 0.05% bromophenol blue (indicator dye) were loaded on the gel and electrophoresis performed at a constant current of 6 mA per gel for 6 hr. The gels were stained in coomassie brilliant blue (1.25 gm of the dye in 454 ml of 50% methanol and 46 ml of glacial acetic acid) for 6 hr. Destaining was carried out in acetic acid:methanol:water (75:50:875) solution. The relative mobility of the protein band was calculated using equation (Weber and Osborn, 1969).

$$\text{Relative mobility} = \frac{\text{distance moved by the protein band from the top}}{\text{length of the gel after destaining}} \times \frac{\text{length of the gel before staining}}{\text{distance moved by the dye from the top}} \dots (9)$$

The relative mobilities were plotted against the logarithm of the molecular weight of the standard proteins used and the molecular weight of the subunits of the protein was determined.

#### Ion-exchange chromatography:

DEAE-cellulose used in the present study was regenerated using 1N hydrochloric acid for the acid wash. The material was washed free of acid and then treated



with 1N sodium hydroxide for the alkali wash. The re-generated DEAE-cellulose was washed free of alkali with distilled water and was stored at 5° for further use. The material so obtained was equilibrated with 0.01M glycine sodium hydroxide buffer pH 9.0 and packed into a column (2.5x30 cm) by pressure. About 140 mg of the protein in 3 ml of the buffer was applied to the column and allowed to be absorbed. Protein was eluted with a linear gradient of 0-0.4M sodium chloride. 2.5 ml fractions were collected and the protein concentration determined by measuring the absorbance of the fractions at 280 nm. Sodium chloride was estimated in the fractions by Vohlards method (Vogel, 1961).

#### Ultracentrifugation

(1) Sedimentation velocity: Sedimentation velocity experiments were carried out at 25° (unless otherwise stated) in a Spinco Model E Analytical Ultracentrifuge equipped with a phase plate schlieren optics and a rotor temperature indicator and control (RTIC) unit. For a typical run a standard 12 mm duraluminium cell centerpiece and 1% protein solution were used at a speed of 59,780 rpm (unless otherwise stated). Plates were read on a Gaertner microcomparator and  $S_{20,w}$  values calculated by the standard procedure (Schachman, 1959) using equation 1 (see page No.36).

(ii) Molecular weight determination from  $S_{20,w}$  value: The molecular weight of  $\alpha$ -globulin from  $S_{20,w}$  value was calculated using equation 2 (see page No.37).

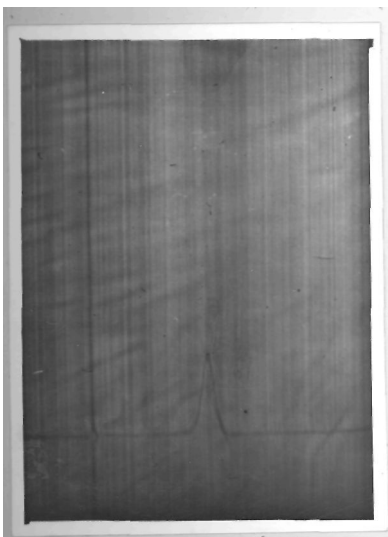
(iii) Molecular weight determination by Archibald method: In the first part of the experiment the synthetic boundary cell described by Klainer and Kegeles (1955) was used for the determination of  $C_0$ . The two side holes of the cell were filled with 0.07 ml of 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride and the centre of the cell with 0.16 ml of  $\alpha$ -globulin in the above buffer. The cell was centrifuged at 3948 rpm and the resulting boundary photographed immediately after the formation of peak (Fig. 2a).

In the second part of the experiment a standard 4° sector duraluminium cell was used. 0.7 ml of protein solution was filled into the cell, and was centrifuged at 8766 rpm and the schlieren patterns were photographed at intervals of 15 min (Fig. 2b). The temperature of the cell was measured both at the beginning and at the end of the run. From these photographic plates  $dc/dx$  values were read on a two dimensional microcomparator and the numerical value proportional to  $C_0$  was computed. With the other pattern (Fig. 2a)  $dc/dx$  values were read starting from the top meniscus to the 'plateau' region.

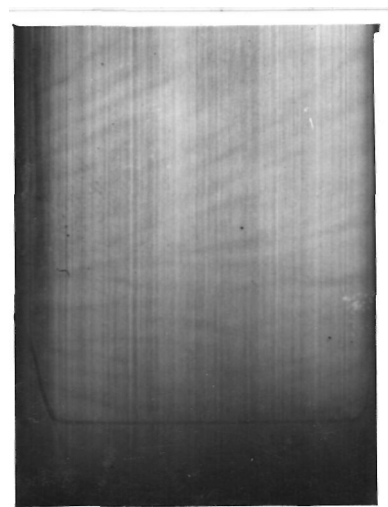
**Fig. 2** Molecular weight determination by Archibald method in 0.02M phosphate buffer pH 7.5 containing 1M sodium chloride.

- (a) Synthetic boundary pattern and
- (b) Archibald run pattern.

Fig. 2



a



b

$dc/dx$  at  $X_0$  was calculated by extrapolation of the above  $dc/dx$  values. Using these data the molecular weight  $M$  from equation 3 (see page No. 38) was calculated.

### Viscosity:

Viscosity measurements were made at  $23 \pm 0.1^\circ$  with an Ostwald viscometer having a flow time of 345 sec with distilled water. Viscosity was calculated from the equation

$$\eta = \rho \left( At - \frac{B}{t} \right) \quad \dots (10)$$

where  $\eta$  is the viscosity,  $\rho$  the density,  $t$  the flow time in sec,  $A$  and  $B$  the constants of the viscometer.  $A$  and  $B$  were determined by measuring the viscosity of water at different temperatures and a plot of  $(\eta/\rho)t$  against  $t^2$  was obtained. The slope of the graph gives  $A$  and the intercept on  $Y$ -axis gives the value of  $B$ . The value of  $B$  was found to be zero. Protein concentration (from 1%-6%) was used for the determination of intrinsic viscosity using equation 4 (see page No. 38). Reduced viscosities were determined using 1% protein solution.

### Optical rotation:

Optical rotation was measured at 578 nm in a Carl-Zeiss spectropolarimeter using a 1 dm tube at  $23^\circ$ .

Protein solution (0.75%) was used. Specific rotation  $[\alpha]_d$ , was calculated from equation (Adler *et al.*, 1973).

$$[\alpha]_d = \frac{\alpha}{\lambda C} \quad \dots \quad (11)$$

where  $\alpha$  is the observed rotation (degrees),  $\lambda$  is the length of the sample (dm) and C is the concentration of protein (g/l).

#### Ultraviolet spectra and difference spectra:

The spectrum and the difference spectrum of the protein were recorded in a Perkin-Elmer 124 double beam spectrophotometer. Protein solution of 0.86 OD/ml (0.08%) was used for the determination of spectrum. For the difference spectrum 2 OD/ml (0.18%) protein solution in presence of SDS, 1.8 OD/ml (0.166%) in presence of urea and GuHCl, 0.76 OD/ml (0.07%) in acidic solutions and 0.73 OD/ml (0.067%) for the spectrum in alkaline solutions were used. Instead of using tandem cells, matched 1 cm cells were used for difference spectra measurements (Donovan, 1969; Donovan *et al.*, 1969). By subtracting the absorbance of the perturbant, in the range of measurements, from the recorded difference spectrum, actual difference spectrum of the protein was determined. The change in the molar extinction coefficient,  $\Delta\epsilon$  of the protein was calculated taking  $2.5 \times 10^5$  daltons as the molecular weight of  $\alpha$ -globulin.

### Fluorescence:

Fluorescence measurements were made in a Perkin-Elmer Hitachi Fluorescence Spectrophotometer at 28°. Protein solution having an absorbance of 0.04 (0.004%) at 280 nm was used. The excitation wavelength was 280 nm. The fluorescence emission was measured after 10 sec. when the fluorescence intensity attained constancy. The fluorescence of blanks were measured and used for obtaining fluorescence values due to the protein molecules.

### Equilibrium dialysis:

The binding study of the detergent SDS with the protein  $\alpha$ -globulin was carried out by equilibrium dialysis. 2.5 ml of 1% protein solution in the dialysis bag was equilibrated at 30° in a Brunswick incubator shaker with 10 ml of the detergent solution, having different concentrations. Attainment of equilibration was checked by taking out aliquots of the external solution at regular intervals of time and measuring the detergent concentration. The concentration of the detergent in the external solution attained a constant value within 36 hr and all the experimental solutions were equilibrated for this period of time. From the estimation of the amount of detergent bound to the protein molecule, (mole to mole basis) a binding isotherm was constructed.

### Estimation of detergent:

The amount of detergent in the solution was estimated by mixing an aliquot of the external solution with  $1 \times 10^{-4}$  M methylene blue in 0.01N hydrochloric acid and extracting the dye-detergent complex in chloroform and measuring the colour intensity at 650 nm in a Carl-Zeiss Spectrophotometer. A standard curve was obtained by using known concentration of SDS, from which, SDS concentration of the external solution in the experimental solutions were determined.

### Turbidity measurements:

The turbid protein solution in presence of different concentrations of urea indicated minimum transmittance at 540 nm. The percent transmittance of 0.1% protein solution was determined at this wavelength in presence of different concentrations of urea and at various temperatures viz. 15°, 25° and 45° (Jamieson et al., 1972).

### Precipitation reactions:

Precipitation experiments in the presence of low concentration of urea and  $\text{CaHCl}$  were carried out by equilibrating, 0.1% protein solution, in varying concentration of these reagents at 30° in a Brunswick incubator shaker for 24 hr. The incubated solutions were



centrifuged at 20,000 x g. The supernatant of protein solutions in different concentrations of urea and GuHCl were measured in 6M urea and 4M GuHCl solution respectively against appropriate blanks. This was done in order to avoid any other effect which might contribute to the absorbance of protein due to the presence of different concentrations of the reagents present in the supernatants. Corrections for the absorbance by blanks were also made. The percentage of protein precipitated was determined by calculating the amount of protein present in the supernatant at each concentration of the denaturants as compared to the initial concentration of the protein solution.

#### Amino acid analysis:

The amino acid analysis was carried out in a Hitachi KLA-3B amino acid analyser. 2.5 mg of the protein in 1 ml of hydrochloric acid (6N) was frozen in a bath of liquid N<sub>2</sub>, and evacuated with an oil pump and sealed. The hydrolysis was carried out in an oven at 110° for 24 hr, and after hydrolysis the hydrochloric acid was removed rapidly at 40° under reduced pressure. The residue was dissolved in 2.25 ml of 0.2N sodium citrate buffer pH 2.2. 0.5 ml of the buffer solution containing amino acid mixture was loaded on the column. Micromoles of each amino acid in the sample was calculated by the

height-width method using similarly eluted standard amino acids (Spackman et al., 1958).

Determination of tryptophan

(i) NBS method: In this method, 2 ml of protein solution having an absorbance of 0.5 (0.046%) at 280 nm in 0.05M acetate buffer pH 4.0 containing 8M urea was pipetted out into a 2.5 ml cuvette. After noting the initial absorbance at 280 nm against buffer containing 8M urea, a 9mM aqueous solution of NBS was added in stepwise of 10  $\mu$ l with a micro-syringe and stirred well. The decrease in absorbance was noted 5 min after each addition of NBS. Stepwise addition of NBS continued till no further decrease in absorbance at 280 nm is observed. The minimum absorbance was noted and a correction for the increase in volume was applied. The tryptophan content was calculated using equation 7 (see page No. 45).

(ii) Edelhoch's method: In this method, 0.8 OD/ml (0.075%) protein solution in 6M GuHCl in 0.02M phosphate buffer pH 6.5, was used for the determination of the spectra. The spectra was obtained in a Perkin-Elmer 124 double beam spectrophotometer with 6M GuHCl as blank. The extinction coefficients at 280 and 288 nm were calculated on the basis of  $2.3 \times 10^5$  daltons for the molecular

weight of the protein and molar concentration of tryptophan  $M_{\text{Try}}$  was calculated from equation 8 (see page No.45).

(iii) Microbiological method: Tryptophan in the sample was estimated by microbiological method using Lactobacillus arabinosus. 50 mg of the protein was hydrolysed in vacuum in presence of 50 ml of sodium hydroxide (6N) at 110° in an oven for 18 hr. The pH of the hydrolysate was adjusted to 6.8 and was taken in graded amounts along with the inoculated organism and the medium. The organisms were allowed to grow for 72 hr at 37° and the acid produced titrated against 0.1N sodium hydroxide using bromothymol blue as the indicator. The percentage of each of the amino acid was calculated by comparison with standard curve (Barton-Wright, 1952).

Spectrophotometric titration of tyrosine:

Spectrophotometric titration of phenolic groups in the protein was carried out by increasing the pH of the protein solution and measuring the absorbance change at 295 nm resulting from tyrosyl ionisation (Dosevan 1973b; Mihyali, 1968). 0.55 OD/ml (0.051%) protein solution in 0.5M KCl was used. The protein solution was adjusted to different pH's with 1M Analar sodium hydroxide. The adjustment of pH was also carried out by

various buffers e.g. sodium phosphate, tris, glycine, carbonate-bicarbonate in their respective buffering ranges. Extinction changes were calculated from the change in absorbance at 295 nm. A Radiometer Titrator TTT 2 (Copenhagen) was used for the measurement of pH. The Radiometer Titrator was calibrated with 0.05M potassium acid phthalate (pH 4.01 at 25°), standard phosphate buffer (pH 6.5 at 25°) and 0.05M borax solution (pH 9.13 at 25°), prepared in deionised water.

#### Carbohydrate estimation:

The carbohydrate content of total protein and  $\alpha$ -globulin was estimated by the method of Montgomery (1961). 0.2 ml of 1% protein solution in 1M sodium chloride solution was mixed with 0.1 ml of 80% phenol and 5 ml of concentrated sulphuric acid. The solution was shaken well and was allowed to stand for 30 min at 27°. The absorbance of the developed colour was measured at 490 nm. A standard curve was obtained using glucose instead of the protein sample. The carbohydrate content of the protein was expressed as glucose equivalents.

#### Phosphorus estimation:

The phosphorus content of total protein and  $\alpha$ -globulin was estimated by the method of Taussky and Sherr (1953). To 0.5 ml of 0.5% protein solution was

added 0.5 ml of 10N sulphuric acid and was digested on a sand bath till the solution became dark brown in colour. The solution was cooled and a few drops of 60% perchloric acid ( $\text{HClO}_4$ ) added to it and the digestion was continued till the solution became colourless. The volume was made upto 2 ml with glass distilled water. To this was added 2 ml of ammonium molybdate reagent and the solution allowed to stand for 30 min at room temperature. The developed colour was read at 660 nm in a Klett-Summerson colorimeter. A standard curve was obtained using 0-16  $\mu\text{g/ml}$  of potassium hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ).

#### Proteolytic activity:

The proteolytic activity of  $\alpha$ -globulin was determined using denatured hemoglobin as substrate. The rate of hydrolysis was carried out in 0.1M borate buffer, pH 7.8. A 2% solution of hemoglobin and a 1% solution of  $\alpha$ -globulin in the above buffer were used. The substrate to  $\alpha$ -globulin ratio was 1:1. 2 ml aliquots of the solution were pipetted out at regular intervals of time till a period of 24 hr and the enzymatic activity (if any) inhibited by adding 2 ml of 10% trichloroacetic acid. The solutions were kept at 4° for 2 hr and then centrifuged at 4000 rpm for 20 min.

The absorbance of the supernatants at 280 nm was measured. A blank of both the substrate and  $\alpha$ -globulin was carried out (Greenberg, 1955).

Preparation of protein solutions in different reagents:

For the measurements in acid solution, the protein was dialysed against 0.3M citrate-phosphate buffer, pH 3.0 and 0.1M acetate buffer pH 4.1 for 24 hr. The pH of the solution below pH 4.1 was adjusted by the addition of 1M hydrochloric acid to pH 4.1 solution. For the measurements in alkaline solutions upto pH 10, the extracted protein was dialysed against 0.05M Tris-HCl buffer of different pH's. The pH of the solution, above pH 10 was adjusted by the addition of 1M sodium hydroxide to pH 10 solution.

Due to the limitation of protein solubility and appearance of turbidity near neutral pH, the experiments in presence of electrolytes, urea,  $\text{CuHCl}$  and SDS were carried out at pH 9.0. The protein solution was dialysed against 0.05M Tris-HCl buffer, pH 9.0, for studying the effect of electrolytes. The protein in 0.05M Tris-HCl buffer, pH 9.0, was used for the measurements in urea,  $\text{CuHCl}$  and SDS solutions. In all these experiments equal volumes of protein and reagent solutions were added to get the desired concentrations of both the components.