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Effect of alkaline pH on sunflower 11S protein

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Abstracts. The effect of alkaline pH on sunflower 11S protein has been monitored by the techniques of ultracentrifugation, polyacrylamide gel electrophoresis, turbidity, viscosity, ultraviolet absorption spectra and fluorescence spectra· Both ultracentrifugation and polyacrylamide gel electrophoresis show the dissociation of the protein with increase in pH. Turbidity values decrease with pH while viscosity increases· With increase in pH absorbance of the protein solution increases and there is a red shift in the absorption maximum. Fluorescence quenching and a red shift in the emission maximum are also observed. Both dissociation and denaturation of the protein occur. Analysis of turbidity, viscosity and fluorescence data suggests that apparently denaturation follows dissociation.

Keywords. Sunflower 11S protein; alkali; dissociation; denaturation.

Introduction

Sunflower 11S protein binds chlorogenic acid below its isoelectric point (pH 5·0); it binds very little at neutral pH (Sastry, 1984). The protein is dissociated at pH 4·0 (Schwenke *et al*., 1975). A study of the effect of low pH on 11 S protein, monitored by a variety of techniques, shows that the protein undergoes dissociation and denaturation upto ~ pH 3·0 and below this pH aggregation and renaturation occur. Therefore, it was of interest to study the effect of alkaline pH on the protein and compare it with that in acid pH·

Materials and methods

Materials

Sunflower seed (EC 68415) a Russian variety, grown in the State of Karnataka during the season, 1979–80, was obtained from Agro-Seed Corporation, Mysore. It was stored in the cold (~ 4°C)· The sources of the chemicals used were: Sepharose 6B-100 from Sigma Chemicals Co·, St. Louis, Missouri, USA; acrylamide, bisacrylamide from Koch-Light Laboratories, England; TEMED and *ß*-mercaptoethanol from Aluka, A. G. Switzerland and ammonium persulphate and amido black from E. Merck, Germany·

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Abbreviation used: PAGE, Polyacrylamide gel electrophoresis·

Methods

Sunflower 11S protein was isolated by the method of Rahma and Narasinga Rao (1981) and further purified by gel filtration on Sepharose 6B–100· It was found to be homogeneous by gel electrophoresis and ultracentrifugation (Sripad and Narasinga Rao, 1987).

The measurements (except electrophoresis) were performed in water whose pH had been adjusted to the desired value by the addition of alkali·

Ultracentrifugation: The experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator control unit and phase plate schlieren optics· A 1% protein solution was centrifuged at 60,000 rpm· Photographs were taken at different intervals of time and $S_{20,w}$ calculated (Schachman, 1959)·

Polyacrylamide gel electrophoresis (*PAGE*): This was done in the range pH 9·0–12·5 using 0·005 M glycine-NaOH buffers· Gels (8%) were used and electrophoresis was performed at a constant current of 3 mA/tube. About 90 μg of protein was loaded and bromophenol blue was used as indicator dye. The gels were stained for 30 min with 0·5% amido black in 7% acetic acid and destained by diffusion with 7% acetic acid·

Viscosity: The measurements were made at 30° ± 0·l°C using an Ostwald viscometer having a flow time of 173 s with distilled water· Protein (0·5%) solution adjusted to the desired pH value was equilibrated at 30°C for 30 min and the flow time recorded. Reduced viscosity was calculated from the flow time with the equation:

$$n_{\text{red}} = \frac{(t - t_o)}{t_o C},$$

where t is the flow time with the solution; t_o, of the solvent and C, the protein concentration in g/ml·

Turbidity: It was obtained by absorbance measurements at 540 nm· Protein solution (0·18%) was used and the solution at pH 7·0 was used as the reference. Percent turbidity was calculated with the equation:

$$\% \text{ Turbidity} = \frac{T_o - T}{T_o} \times 100,$$

where T_o is the transmittance of the protein solution at pH 7·0 and T of the protein at different pH values·

Absorption spectrum: It was recorded in Perkin–Elmer Model 124 recording spectrophotometer in the range 240–330 nm·

Fluorescence spectrum: The fluorescence emission was measured in a Perkin–Elmer

Model 203 spectrofluorimeter using 0·009% protein solution· The emission intensity was measured in the range 300–400 nm at room temperature (~28°C) after excitation at 280 nm·

Results and discussion

Ultracentrifugation

The sedimentation velocity pattern of the 11S protein at pH 8·3 consisted of a single symmetrical peak with $S_{20,w}$ value of 11·0 (figure l). At pH 9·5, it consisted of 3 peaks with $S_{20,w}$ values of 2·0, 7·0 and 11·0. At pH 10·5 also it consisted of 3 peaks· But the proportion of the 7S protein had increased while that of the 1lS protein decreased. There was no change in the proportion of the 2S protein. At pH 11·5, the pattern consisted of only two peaks with $S_{20,w}$ values of 0·7 and 3·5. Thus with increase in pH, dissociation of the protein to low molecular weight proteins occurred·

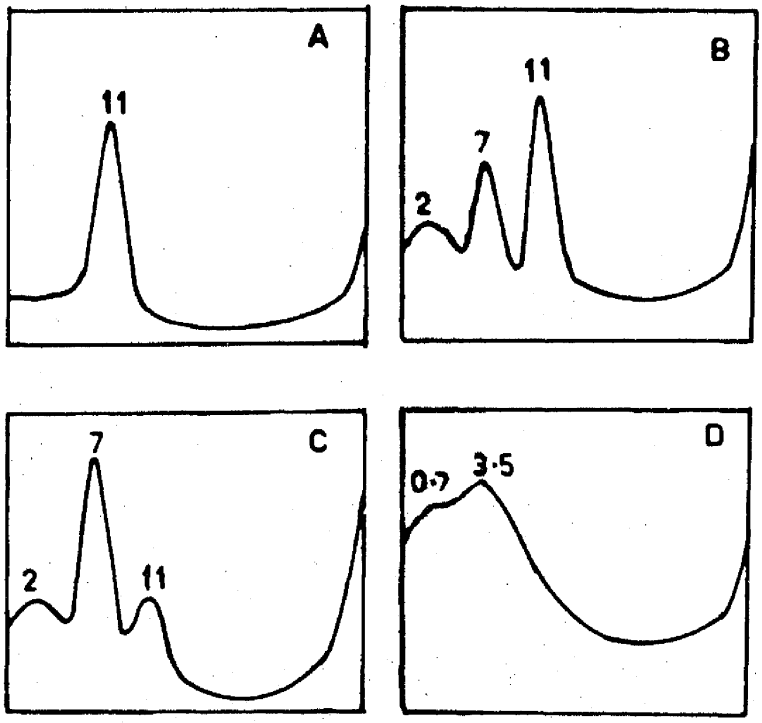

Figure 1. Effects of pH on the sedimentation velocity pattern of sunflower 11S protein (A), pH 8·3; (B), pH 9·5; (C), pH 10·5; (D), pH 11·5 (numbers indicate $S_{20,w}$ values).

Kelley and Pressy (1966) have reported that at alkaline pH glycinin (of soybean) dissociates and Prakash and Nandi (1977) have reported that α-globulin of sesame also dissociates at alkaline pH·

Electrophoresis

Electrophoresis was performed in the pH range 9·0 and 12·6 and the PAGE patterns are given in figure 2. At pH 8·3 and 9·0, the 11S protein gave a single band with low mobility and no other bands were observed. At pH 9·8 a faint fast moving band was observed in addition to the original band. The intensity of this band increased at pH 10·6. At pH 11·2, its intensity increased further with a concomittant decrease in that of the original band; in addition a faster moving band was also observed. The diffuse nature of the band suggested that it was polydisperse in nature· At pH 11·8, the band due to 11S protein almost disappeared with a decrease in the intensity of the second band also. There was an increase in the intensity of the diffuse fast moving band. At pH 12·6, only the diffuse, fast moving band was observed· Taken in conjunction with the sedimentation velocity data, PAGE experiments also indicated the dissociation of the 11S protein at higher pH values·

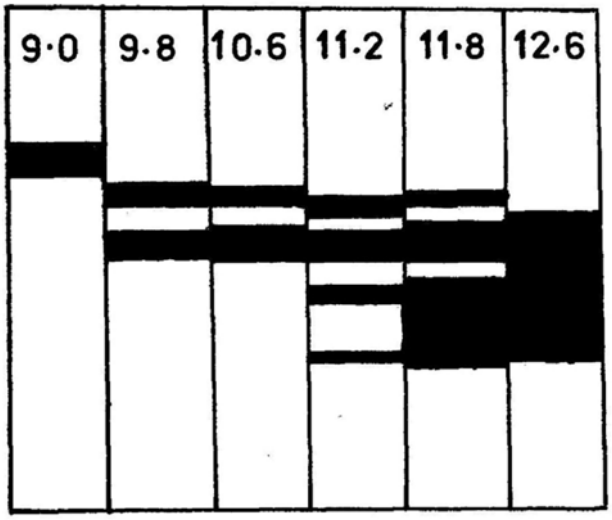

Figure 2. Effect of pH on the electrophoretic pattern of sunflower 11S protein (0·005 M glycine-NaOH buffers). Numbers refer to pH of measurement·

Viscosity

The effect of alkaline pH on the reduced viscosity (n_{red}) is shown in figure 3· Upto pH 10·2, n_{red} remained constant at the value of 4·2 ml/g. As the pH increased above pH 10·2, n_{red} increased and again a constant value of 10 ml/g was observed in the pH range 12·0–12·5. The midpoint of transition in the curve was around pH 11·5. The increase in n_{red} value indicated unfolding/expansion of the protein molecule (Joly, 1965). The unfolding appeared to be less at alkaline pH than at acid pH (Sripad and Narasinga Rao, 1987). The value of 10 ml/g shows that the protein was not in a completely unfolded state because the value for a completely unfolded protein is around 29 ml/g (Tanford, 1968; Wang and Hamlin, 1974).

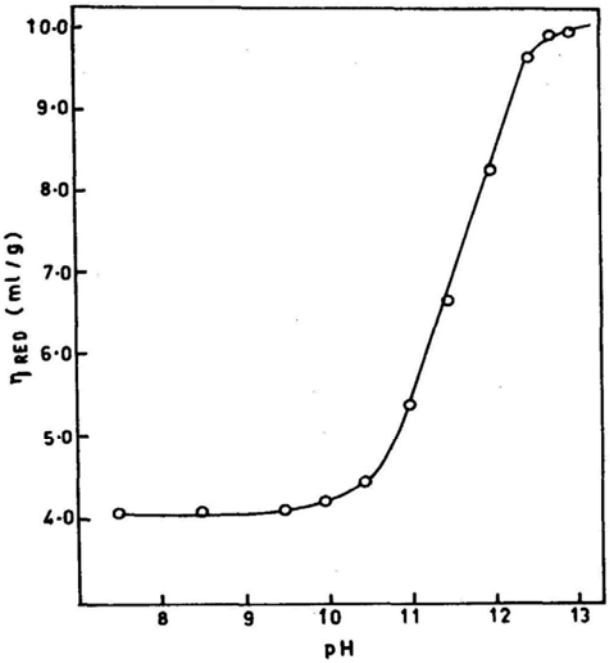

Figure 3. Effect of pH on the reduced viscosity of sunflower 11S protein·

Turbidity

There was no change in the turbidity of the protein solution in the pH range 7·0–8·5 (figure 4). It decreased sharply upto pH 10·0 and then decreased gradually upto pH 12·8. Eventhough there was no dissociation in the region pH 8·5–9·0 the turbidity decreased. The decrease in turbidity above pH 9·0 could be due to the dissociation of the protein·

Absorption spectra

The spectra recorded in the range 330–240 nm of the protein solution at different pH values showed considerable difference (figure 5). The spectrum in the pH range 7·4–11·0 consisted of a single maximum at 280 nm· A minimum in 250–270 nm region was also observed· However, as the pH increased the absorbance at the minimum increased and it showed a red shift also· Above pH 11·3 two maxima, one at 280 nm and the other at 288 nm, were observed· Evidently, the second maximum was due to the ionized tyrosine groups (Donovan, 1973). Upon ionization of phenolic group, the absorbance in the 280–290 nm region increases and the maximum shows a red shift. Thus the change in the shape of the protein spectrum at alkaline pH could be due to both tyrosyl ionization and conformational changes·

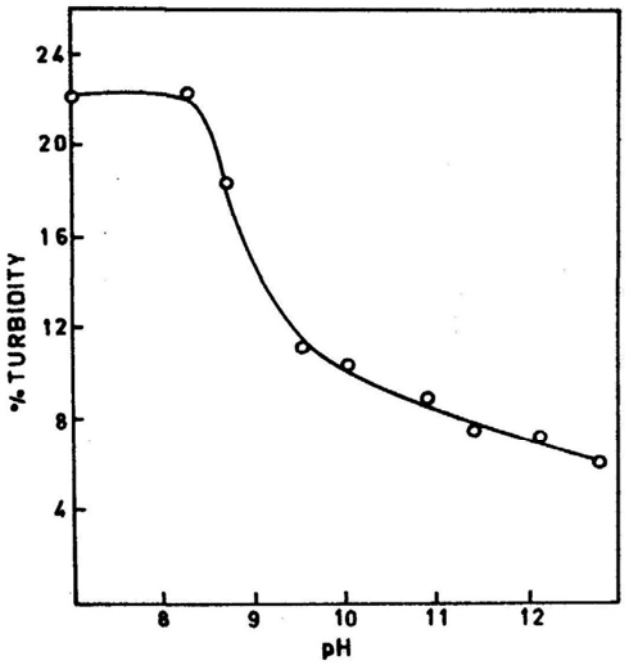

Figure 4. Effect of pH on the turbidity of sunflower 11S protein·

Fluorescence spectra

At pH 8·l, the 11S protein gave a fluorescence emission spectrum with a maximum at 325 nm (figure 6). Quenching of fluorescence intensity and a red shift in the maximum occurred as the pH was increased. The effects were pronounced in the pH range 10·9–11·7. The maximum shifted from 325 nm at pH 8·l to 340 nm at pH 12·6. The quenching of fluorescence intensity could be due to the exposure of tryptophan residues to the polar environment from the interior hydrophobic environment (Teale, 1960). The quenching could also result from the transfer of resonance energy from indole groups of tryptophan residues to ionized phenol groups of tyrosine residues (Brand and Withold, 1967). The observed red shift in the emission maximum would suggest denaturation of the protein (Teale, 1960)·

Turbidity and fluorescence intensity decreased in the pH range 7·0–9·0, eventhough there was no dissociation of the protein in this pH range. Both ultracentrifugation and PAGE experiments showed the dissociation of the 11S protein at alkaline pH. Decrease in turbidity with increase in pH could also possibly be due to dissociation of the protein. Increase in viscosity (fluorescence quenching and the red shift in emission maximum) could be taken as evidence of denaturation of the protein· Thus at alkaline pH, as in acid pH, sunflower 11 S protein dissociated and denatured·

When viscosity, turbidity or fluorescence intensity at 325 nm was plotted as a

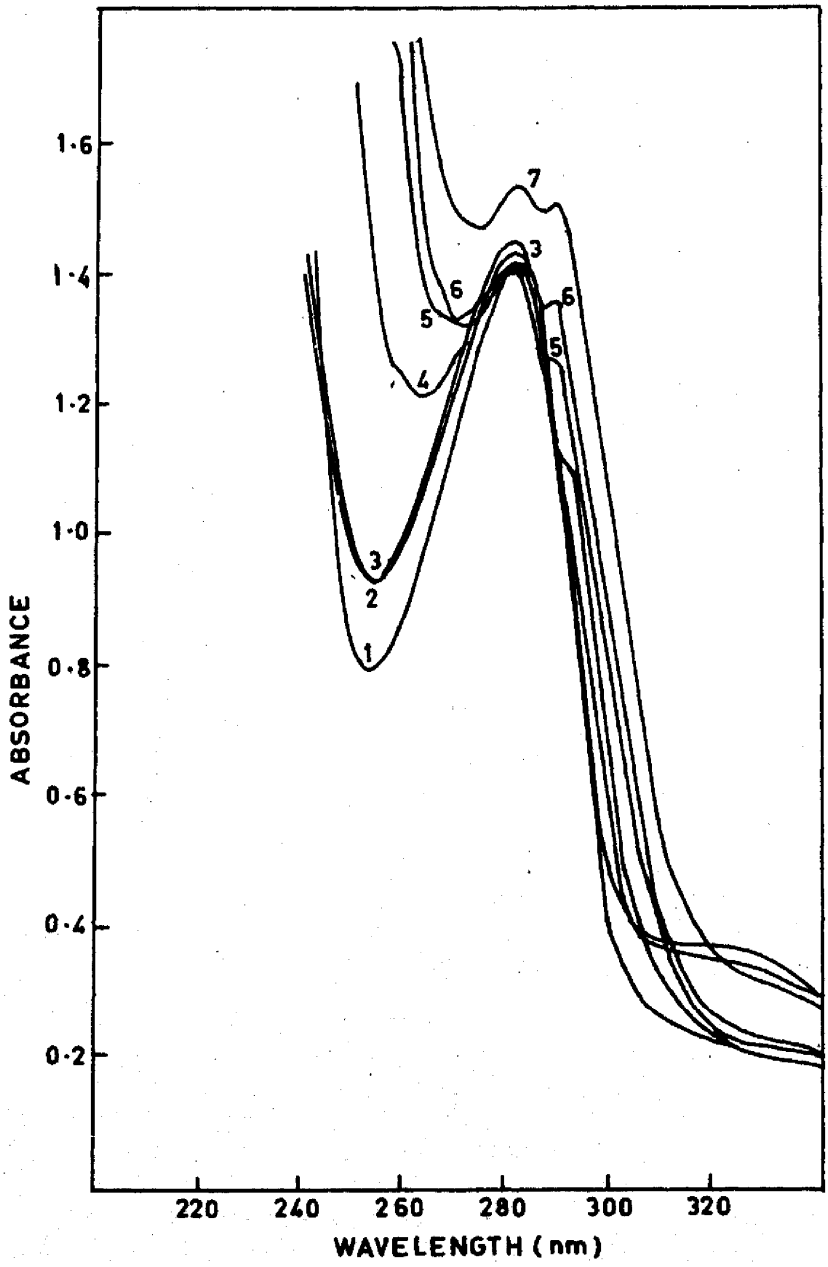

Figure 5. Effect of pH on the absorption spectrum of sunflower 11 S protein· (1), pH 7·4; (2), pH 8·0; (3), pH 9·3; (4), pH 10·5; (5), pH 11·3; (6), pH 12·2; (7), pH 12·8.

function of pH, a sigmoidal curve was obtained in each case. Therefore, it was possible to calculate from these data the fraction of the protein dissociated/ denatured as a function of pH (Tanford, 1968). The following equation was used:

$$Y=Y_N(1-\alpha)+aY_D,$$

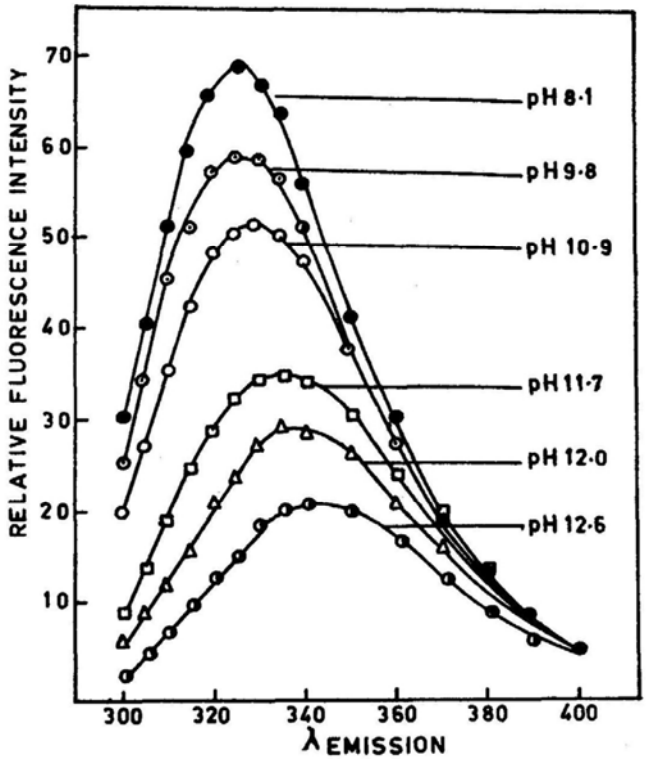

Figure 6. Effect of pH on the fluorescence emission spectrum of sunflower 11S protein. Numbers refer to pH of measurement·

where Y is the measured value of the parameter (viscosity, turbidity, fluorescence intensity) at a particular pH; Y_N, the value of the native protein; Y_D, that of the dissociated/denatured protein and α, the fraction of the protein dissociated/denatured. The values characteristic of the native protein were obtained from the plateau region values between pH 7·0 and 8·0, and those of the dissociated/denatured protein from the plateau region values between pH 12·0 and 13·0.

The α values calculated thus are given in figure 7 as a function of pH. The values obtained from the 3 different techniques did not fit the same curve. In protein denaturation studies it is normally assumed that if data from different techniques fit the same curve of α *vs* pH, it is indicative of a two-state process (Tanford, 1968). In such a process the native protein is directly converted into the denatured product without any intermediate species·

From figure 7, it may be seen that turbidity decrease (indicative of dissociation of the protein) occurred at a lower pH value than that at which fluorescence intensity and viscosity changes occurred. If viscosity change can be taken as indicative of protein denaturation, it occurred at a much higher pH value. For instance from turbidity data α had a value of 0·9 at pH 11·5. At the same pH, α from viscosity data

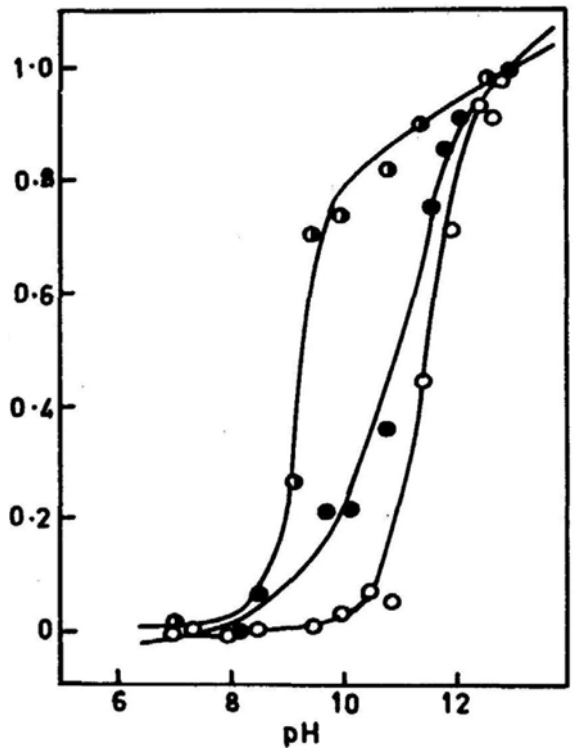

Figure 7. Fraction (α) of sunflower 11S protein dissociated/denatured as a function of pH. (O), From n_{red} ; (◑), from turbidity; (●), from fulorescence intensity at 325 nm

had a value of 0·5 (figure 7). One explanation could be dissociation occurred first and then denaturation later; the two phenomena do not occur simultaneously.

It is not surprising that the data from fluorescence spectra and viscosity do not fit the same curve. Fluorescence quenching is not due to denaturation alone. The (dissociated) phenolic groups also contribute to quenching.

A comparison of the effect of low pH and high pH on the sunflower 11S indicates the following: (i) dissociation and denaturation of the protein occur at both low and high pH; (ii) when the measured property is plotted as a function of pH, a sigmoidal curve is obtained in the alkaline pH range whereas a minimum (or a maximum) is observed in the acid pH range and (iii) in the alkaline pH dissociation/denaturation of the protein goes to completion as the pH is increased and it does not go to completion in acid pH. Below pH 2·5 reaggregation/refolding of the protein occurs.

References

Brand, L. and Withold, B.·(1967) in *Methods Enzymol*., **2,** 831·
Donovan, J. W. (1973) in *Methods Enzymol*., **27,** 525·
Joly, M. (1965) *A physico-chemical approach to the denaturation of proteins* (London: Academic Press).
Kelley, J.·J. and Pressey, R. (1966) *Cereal Chem.*, **43,** 195.
Prakash, V. and Nandi, P. K. (1977) *Int. J. Peptide Protein Res*., **15,** 305·
Rahma, E. H. and Narasinga Rao, M. S. (1981) *J. Agri Food Chem*., **29,** 518.

Sastry, M. C. S. (1985) *Interactions of Sunflower Proteins,* Ph. D. thesis, University of Mysore, Mysore.
Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry* (New York: Academic Press)
Schwenke, K. D., Schultz, M. and Linow, K. J. (1975) *Die Nahrung,* **19,** 817.
Sripad, G. and Narasinga Rao, M. S. (1987) *J. Agri. Food Chem*·, (in press).
Tanford, C. (1968) *Adv Protein Chem.,* **23,** 121·
Teale, F. W. J. (1960) *Biochem. J*., **76,** 381·
Wang, K. L. and Hamlin, L. M. (1974) *Biochemistry*, **13,** 2578.