Effect of Acid pH on the 11S Protein of Sunflower Seed

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Effect of acid pH on sunflower 11S protein has been monitored by the techniques of ultracentrifugation, polyacrylamide gel electrophoresis, viscosity, turbidity, difference ultraviolet spectra, fluorescence spectra, and near- and far-ultraviolet circular dichroism spectra. Ultracentrifugation and polyacrylamide gel electrophoresis suggest the dissociation of the protein up to pH ~3.0 and reaggregation below this place. Viscosity, turbidity, difference spectra, fluorescence spectra, and circular dichroism spectra suggest the denaturation of the protein up to pH ~3.0 and refolding of the protein molecule below this pH.

The 11S protein of sunflower seed constitutes nearly 70% of the total proteins. The physicochemical properties of the protein have been reported by several workers (Schwenke et al., 1975a,b, 1978, 1979; Rahma, 1979; Rahma and Narasinga Rao, 1981a). It is an oligomeric protein consisting of 10–12 subunits and has a molecular weight of 300 000 (Rahma, 1979). Studies have been made on the effect of denaturants such as urea, Gdn·HCl, SDS, and pH on the 11S protein (Schwenke et al., 1975b; Rahma and Narasinga Rao, 1981b). The interaction of CGA with 11S protein has been studied (Sastry, 1984). The protein binds CGA below its isoelectric point (pH 5.0). It is reported that 11S protein dissociates at acidic pH (Schwenke et al., 1975b). At acid pH values denaturation of proteins is known to occur (Lapanje, 1978). Denaturation causes changes in the structure and conformation, which in turn affects the physicochemical and functional properties of the protein. In this investigation the effect of acid pH on the 11S protein has been studied by a variety of techniques to determine the nature of changes that occur.

Most oilseed proteins show unusual behavior at acid pH. They dissociate and denature up to pH 3, below which reaggregation and refolding occur. In this laboratory we have been studying a number of oilseed proteins to understand the structural and molecular basis of this behavior. This paper is a part of this study.

EXPERIMENTAL SECTION

Materials. Sunflower seed (EC 68415), a Russian variety, grown in the State of Karnataka during the 1979–1980 season was purchased from Agro-Seed Corp., Mysore, India. It was stored in the cold (~4 °C). The sources of chemicals used were as follows: tris(hydroxymethyl)aminomethane (Tris), blue dextran, and Sepharose 6B-100, Sigma Chemical Co.; sodium nitrite, ammonium persulfate, and amido black, E. Merck; acrylamide and bisacrylamide, Koch-Light Laboratories; TEMED and β-mercaptoethanol, Fluka. All other reagents used were of analytical reagent grade.

Methods. *Isolation of Sunflower 11S Protein.* The procedure of Rahma and Narasinga Rao (1981a) was used. It was further purified by gel filtration on Sepharose 6B-100.

Protein Concentration. It was routinely determined from the absorbance of the solution at 280 nm with a value of 8.2 for $E^{1\%}_{1cm}$ (Rahma, 1979). All measurements were made with protein solution in water whose pH had been adjusted to the desired value by the addition of HCl. However, PAGE experiments were made in buffer solution.

Ultracentrifugation. This was performed with a Spinco Model E analytical ultracentrifuge equipped with Rotor Temperature Indicator and Control (RTIC) Unit and phase plate Schlieren optics using 1% protein solution. The runs were made at 60 000 rpm. Photographs were taken at different intervals of time and $S_{20,w}$ values calculated (Schachman, 1959).

Polyacrylamide Gel Electrophoresis (PAGE). It was carried out for 45 min at constant current of 3 mA/tube with use of 8% gels. Malachite green was used as a marker, and 90 μg of the protein was loaded. The gels were stained with 0.5% amido black in 7% acetic acid for 30 min and destained by diffusion with 7% acetic acid. The following buffers of 0.005 M were used: acetate–acetic acid (pH 4.2–3.6), glycine hydrochloride (pH 3.0–2.0), KCl–HCl (pH 1.5–1.0).

Viscosity. Measurements were made at 30 ± 0.1 °C on an Ostwald viscometer having a flow time of 174 s with distilled water. Protein solutions (0.5%) were used. Reduced viscosity, n_{red}, was calculated from the flow times of the solution and solvent.

Turbidity. This was obtained by absorbance measurements at 540 nm. Protein solution (0.183%) was used, and the solution at pH 7.0 was used as the reference. The percent turbidity was calculated from eq 1, where T_0 is the transmittance of the protein solution at pH 7.0 and T of the protein at different pH values.

$$\% \text{ turbidity} = (T_0 - T)/T_0 \times 100 \quad (1)$$

Difference Spectra. These were recorded in the range 330–240 nm on a Perkin-Elmer Model 124 double-beam recording spectrophotometer using 0.183% protein solution. The difference spectrum at acid pH values was recorded against the protein at pH 7.0.

Fluorescence Spectra. The fluorescence emission was measured in a Perkin-Elmer Model 203 spectrofluorimeter using 0.009% protein solution, in the range 300–400 nm. Excitation wavelength was 280 nm. Appropriate correction for the blank was applied.

Circular Dichroism (CD). Measurements were made at room temperature (~28 °C) on a Jasco 20C automatic spectropolarimeter equipped with Xenon arc lamp and calibrated with camphor-d_{10}-sulfonic acid.

Near-UV CD. The measurements were made in the wavelength range 330–250 nm with cells of 1-cm path length and protein solutions of 0.183% concentration.

Far-UV CD. The measurements were made in the range 190–250 nm with cells of 0.1 cm and protein solutions of 0.03%.

The mean residue ellipticity, $[\theta]_{MRW}$, was calculated from the measured ellipticity by eq 2, where θ is the measured ellipticity, d is the path length (dm), c is the concentration

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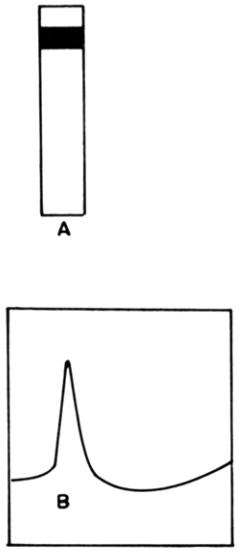

Figure 1. (A) Polyacrylamide gel electrophoresis pattern of sunflower 11S protein (0.05 M Tris–glycine buffer, pH 8.3). (B) Sedimentation velocity pattern of sunflower 11S protein (0.055 M Tris–glycine buffer, pH 8.3, containing 0.5 M NaCl).

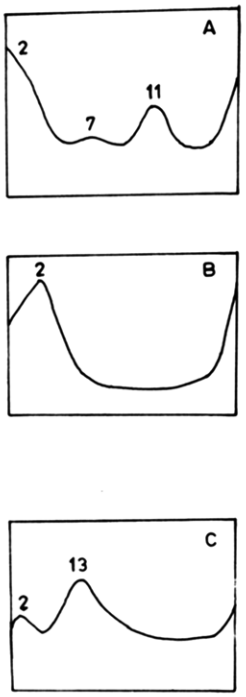

Figure 2. Sedimentation velocity pattern of sunflower 11S protein at different pH values: A, pH 4.0; B, pH 3.0; C, pH 2.0.

of protein solution (g/mL), and MRW is the mean residue weight. A value of 115, based on the amino acid composition, was used for MRW.

$$[\theta]_{MRW} = \theta MRW/dc \quad (2)$$

RESULTS AND DISCUSSION

The protein was found to be homogeneous by PAGE and ultracentrifugation (Figure 1A, B). It gave a single band in PAGE and a single, fairly symmetrical peak in ultracentrifugation with $S_{20,w}$ = 11.0 S.

Ultracentrifugation. Whereas the sedimentation velocity pattern at pH 8.3 consisted of a single peak of 11.0S, at lower pH values it consisted of several peaks (Figure 2). At pH 4.0, three peaks of 11S, 7S, and 2S were observed. However, at pH 2.0 the pattern consisted of two peaks, 2S and 13S. The latter may be an aggregate of dissociated proteins. These results suggest that dissociation of the protein occurred up to pH 3.0 and reaggregation below this pH. Such dissociation followed by reaggregation has been reported in a number of oilseed proteins (Prakash and Narasinga Rao, 1984).

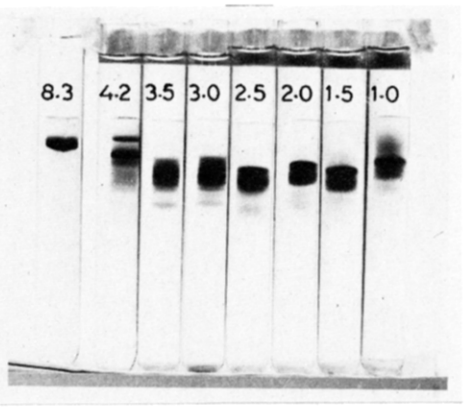

Figure 3. Effect of pH on the electrophoretic pattern of sunflower 11S protein: pH 4.2–3.5, 0.005 M acetate buffer; pH 3.0–2.5, 0.005 M glycine hydrochloride buffer; pH 1.5–1.0, 0.005 M KCl–HCl buffer. Number indicates the pH of measurement.

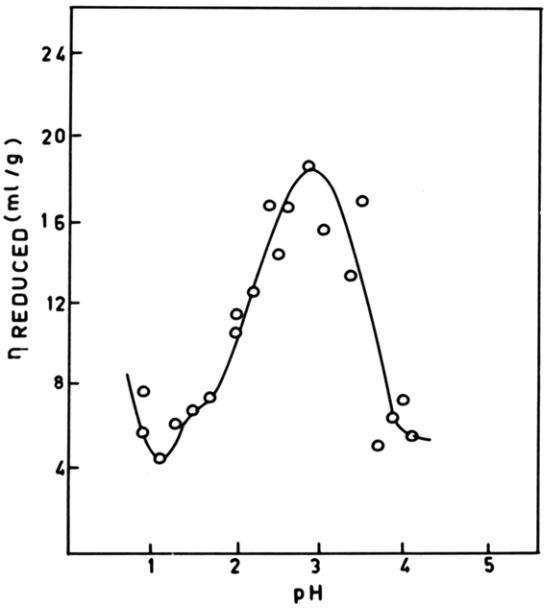

Figure 4. Effect of pH on the reduced viscosity of sunflower 11S protein.

Since the experiments were performed in salt-free solution, the decrease in $S_{20,w}$ value could be due to charge effect (Schachman, 1959). The sedimentation rate of ionized macromolecules in solution containing no added salt should be half the rate of the same macromolecules that either are uncharged or are in solutions containing 0.1–0.2 M salt. Since the observed decrease (from 11S to 2S) was much larger, dissociation of the protein was suggested.

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis indicated the dissociation of the protein at pH 4.2 (Figure 3). In addition to the dissociated proteins, an aggregate of low mobility was also observed. The aggregate disappeared at pH 3.5. At pH 3.0 nearly six to seven bands, all dissociated proteins, were observed. At this pH dissociation of the protein might not be complete, as the 11S protein consists of 10–12 subunits as suggested by SDS-PAGE. In the pH range 2.5–1.5, the number of bands decreased. This could be due to reaggregation of the dissociated protein molecules. At pH 1.0, a diffuse band of low mobility and another faint band were noticed.

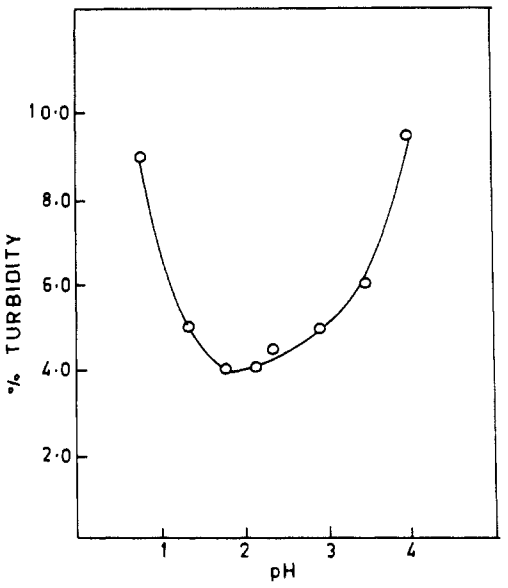

Figure 5. Effect of pH on the turbidity of sunflower 11S protein.

Viscosity. The value of n_{red} for 11S protein at pH 4.2 was 5.2 mL/g (Figure 4), which is slightly higher than the values reported for globular proteins (Tanford, 1961). As the pH was reduced, n_{red} value increased to 18 mL/g at pH 2.75, and below this pH, it decreased to a value of 4.2 mL/g at pH 1.2. The increase in viscosity upto pH 2.75 may be due to unfolding of the dissociated molecules. At low pH values, the positive charge on the protein molecule increases due to protonation of carboxyl groups, and due to electrostatic repulsion, expansion of the molecule occurs. The molecules are not in a completely unfolded state since the value of 18 mL/g is lower than that for a completely unfolded molecule, ~29 mL/g (Wang and Hamlin, 1974).

The decrease in n_{red} below pH 2.75 could be due to refolding/reduction in expansion of the protein molecules as a result of decrease in repulsion between them. Around pH 2.75, the positive charge on the protein molecule would be maximum as all carboxyl groups would have been protonated; pK_{int} of carboxyl (aspartic acid) is 4.0 and of carboxyl (glutamic acid) is 4.5 (Tanford, 1962; Nozaki and Tanford, 1967). A further decrease in pH would serve merely to increase the ionic strength of medium, which would have a charge-shielding effect. Similar observations have been reported by Yang and Foster (1954) in the case of bovine serum albumin and by Kishore Kumar Murthy and Narasinga Rao (1984) in the case of mustard 12S protein.

The increase in viscosity below pH 1.2 may be due to aggregation of the unfolded molecules as suggested by sedimentation velocity measurements. Around pH 0.5, the protein precipitated completely.

Turbidity. The turbidity of the solution at different pH values is given in Figure 5. It decreased from pH 4.0 to 2.0. Since turbidity is dependent on size and shape of protein molecule, the decrease could be due to dissociation of the protein into low molecular weight polypeptides and subunits. When the pH was decreased further, the turbidity increased up to pH 0.8, suggesting formation of aggregates.

Fluorescence Spectra. Sunflower 11S protein gave an emission maximum at 325 nm at pH 7.0 in water when the excitation wavelength was 280 nm (Figure 6). As the pH was decreased from 7.0 to 1.0, fluorescence quenching and a red shift in λ_{max} were observed. This may be attributed to the dissociation and denaturation of the proteins. Due to denaturation, the fluorophores would be exposed from

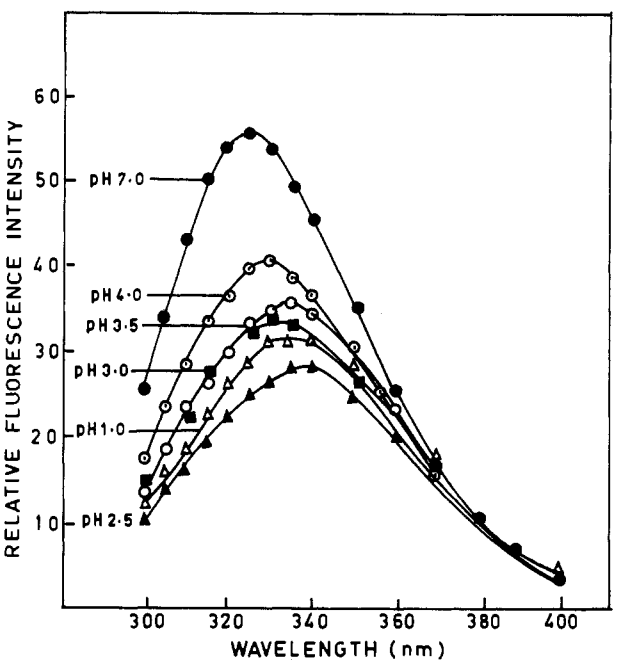

Figure 6. Effect of pH on the fluorescence spectrum of sunflower 11S protein.

the interior, nonpolar environment of the molecule with a dielectric constant of 4.0 to the polar environment of dielectric constant 80 (Schultz and Shrimer, 1979). Studies with tryptophan have shown that increase in dielectric constant of the medium causes both quenching and red shift in the λ_{max} (Teale, 1960). Shifrin et al. (1971) reported that tryptophan has its emission maximum ~345 nm in aqueous medium. The red shift in the emission maximum suggested that tryptophan moieties of the 11S protein were exposed to aqueous medium after denaturation. If these moieties were in the contact area between the subunits of the protein, dissociation of the protein would also lead to the same effect.

The red shift was observed up to pH 2.5; below this, a decrease in red shift and an increase in fluorescence intensity were observed (Figure 6). Possibly, association and refolding of the dissociated and unfolded molecules occurred as a result of which the fluorophors were buried once again in the nonpolar environment. Steiner and Edelhoch (1962) reported that acid quenching of lysozyme was reduced in the pressure of KCl because of ionic strength effect. At pH values below 2.5, chloride ions would serve to increase the ionic strength.

UV Difference Spectra. The difference spectra of the protein obtained at different pH values with reference to protein at pH 7.0 showed a blue shift with three prominent minima at 279, 286, and 292 nm (Figure 7). These can be attributed to tyrosine and tryptophan residues (Wetlaufer, 1962; Donovan, 1969; Kronman and Robbins, 1970). The appearance of blue shift could be due to the exposure of chromophores present at the interface of the subunits or from the interior of the molecule due to dissociation and denaturation (Herskovits, 1967; Donovan, 1973; Imoto et al., 1975).

An increase in the intensity of the minima was observed upto pH 2.5, and below this pH there was a decrease. The decrease in intensity could be due to the reaggregation of the dissociated subunits or refolding of the denatured molecules. Similar observations have been reported for single-chain and oligomeric proteins, human carbonic anhdyrase B (Flanagan and Hasketh, 1974), glycinin (Catsimpoolas et al., 1970; Koshiyama, 1972), sesame α-globulin (Prakash and Nandi, 1977), arachin (Navin Kumar, 1982),

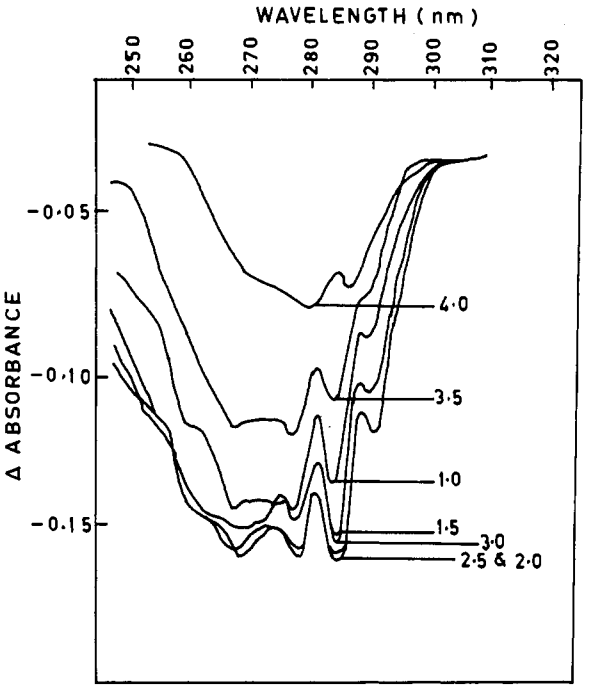

Figure 7. Effect of pH on the UV difference spectrum of sunflower 11S protein. Number indicates the pH of measurements.

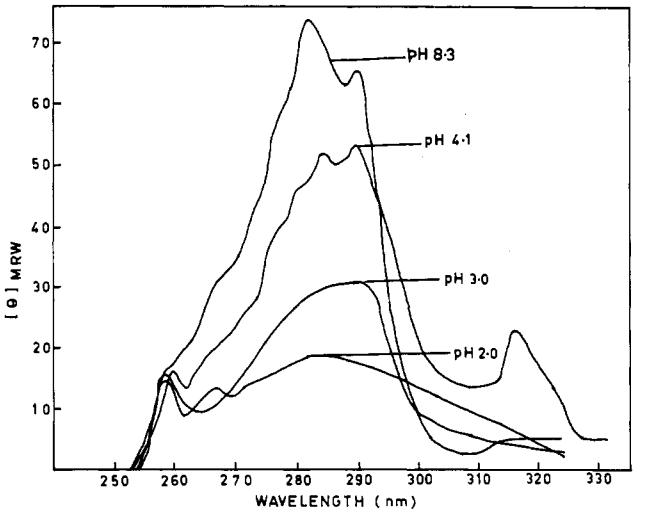

Figure 8. Effect of pH on the near-UV CD spectrum of sunflower 11S protein.

and mustard 12S protein (Kishore Kumar Murthy and Narasinga Rao, 1984).

Near-UV CD. The spectrum in the region 330–250 nm consisted of two peaks at 282 and 288 nm with two shoulders at 258 and 266 nm (Figure 8). Two humps were also observed at 273 and 277 nm. The bands at 282 and 288 nm may be attributed to tryptophan residues, the shoulders at 258 and 266 nm to phenylalanine residues, and the two humps at 273 and 277 nm to tyrosine residues (Strickland, 1976).

As the pH was decreased to 4.1, a red shift and a decrease in intensity of the bands were observed. Also a new band at 316 nm appeared; this may be due to tryptophan residues. As the pH was reduced further, the bands disappeared, indicating a gross conformational change. The ellipticity of the 282-nm band reduced from 74 to 20°, indicating denaturation of the protein (Yang and Doty, 1957). The conformational change causes a change in the asymmetric environment of the aromatic residue in the molecule, which results in the loss of tertiary structure and reduced ellipticity (Wang and Hamlin, 1974). Prakash et

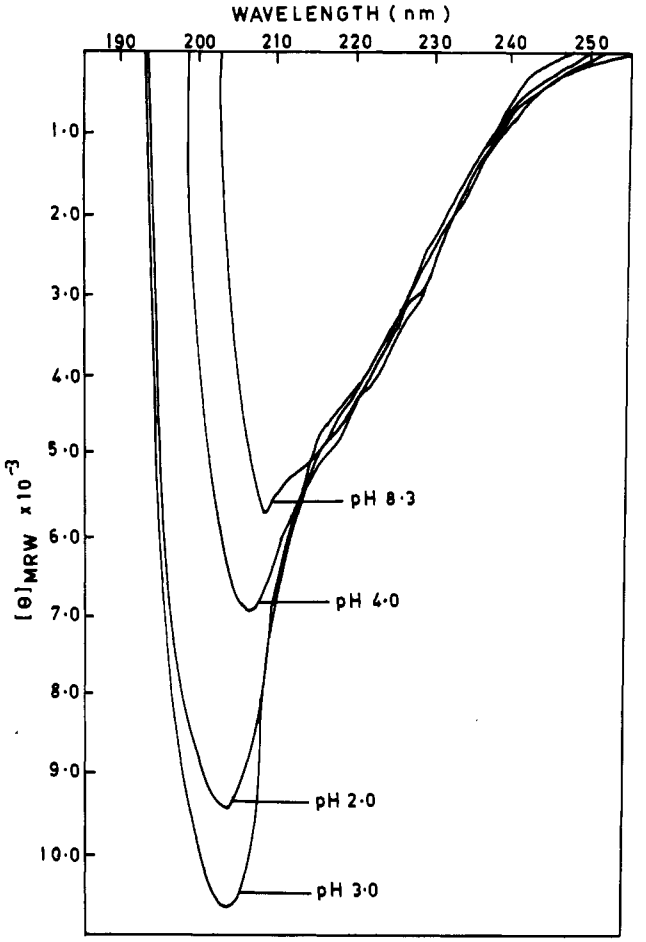

Figure 9. Effect of pH on the far-UV CD spectrum of sunflower 11S protein.

al. (1980) reported that the near-UV CD spectrum of sesame α-globulin at low pH was different from that at neutral pH. A similar result has been reported by Kishore Kumar Murthy and Narasinga Rao (1984).

Far-UV CD. The spectrum of the 11S protein at pH 8.3 showed a minimum at 208 nm with $[\theta]_{MRW}$ of −5750° cm²/dmol. A broad shoulder between 210 and 220 nm was also observed (Figure 9). At pH 4.0 the value was −6900 with a blue shift in the minimum to 206 nm. At pH 3.0, the corresponding values were −10 700 and 202 nm. However, at pH 2.0 the $[\theta]_{MRW}$ value was −9400.

The CD spectra of unordered polypeptide chains in solution can vary in position, intensity, and sign; hence, quantitative estimation of the conformation of proteins on such basis is unreliable (Fasman et al., 1970). However, it could be concluded qualitatively that the protein became more unordered up to pH 3.0 and below this pH a somewhat ordered structure was formed. The original structure was obviously not regained since the spectrum at pH 2.0 was not the same as that at pH 8.3.

The results of physical and optical measurements indicated that there were two distinct regions of pH where changes occurred in the molecular properties of the protein: (1) pH 4.0–2.8 where dissociation and denaturation of the protein occurred; (2) pH 2.7–1.0 where aggregation and refolding of the dissociated protein molecules occurred. Dissociation and reaggregation were indicated by ultracentrifugation, PAGE, and turbidity measurements whereas denaturation and refolding were indicated by viscosity, difference spectra, fluorescence, and CD spectra.

Many oligomeric proteins of plant origin appear to possess the property of dissociation/denaturation and reaggregation/refolding at low pH values: glycinin (Cat-

simpoolas et al., 1970), sesame α-globulin (Prakash and Nandi, 1977), mustard 12S protein (Kishore Kumar Murthy and Narasinga Rao, 1984), and arachin (Navin Kumar, 1982). Catsimpoolas et al. (1970) reported that reassociation of glycinin at low pH was due to hydrogen bonding between the uncharged carboxyl groups of unfolded polypeptide chains. Prakash and Nandi (1977) reported that reaggregation of sesame α-globulin was due to hydrophobic bonding since hydrogen bonding does not have significant stability in aqueous solution (Klotz and Franzen, 1962). Hjerten et al. (1974) reported that hydrophobic interaction increases with an increase in ionic strength of the medium while the opposite is true of hydrogen bonding based interactions. At low pH values ionic strength increases, and this could strengthen reaggregation based on hydrophobic bonding.

ABBREVIATIONS USED

Gdn·HCl, guanidinium hydrochloride; SDS, sodium dodecyl sulfate; CGA, chlorogenic acid; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism.

LITERATURE CITED

Catsimpoolas, N.; Wang, J.; Berg, T. *Int. J. Protein Res.* **1970**, *2*, 277.
Donovan, J. W. In *Physical Principles and Techniques of Protein Chemistry*; Leach, S. J., Ed.; Academic: New York, 1969; Part A, p 101.
Donovan, J. W. *Methods Enzymol.* **1973**, *XXVII*, 497.
Fasman, G. D.; Hoving, H.; Timasheff, S. N. *Biochemistry* **1970**, *9*, 3316.
Flanagan, M. T.; Hesketh, T. R. *Eur. J. Biochem.* **1974**, *44*, 251.
Herskovite, T. T. *Methods Enzymol.* **1967**, *XI*, 748.
Hjerten, S.; Rosengren, J.; Pahlnan, S. *J. Chromatogr.* **1974**, *101*, 281.
Imoto, T.; Andrews, L. J.; Banerjee, S. K.; Sharke, A.; Forester, L. S.; Rupley, J. A. *J. Biol. Chem.* **1975**, *250*, 8275.
Kishore Kumar Murthy, N. V.; Narasinga Rao, M. S. *Int. J. Peptide Protein Res.* **1984**, *23*, 94.
Klotz, I. M.; Franazen, J. S. *J. Am. Chem. Soc.* **1962**, *84*, 3461.
Koshiyama, I. *J. Sci. Food. Agric.* **1972**, *23*, 853.
Kronman, M. J.; Robbins, F. M. In *Fine Structure of Proteins and Nucleic Acids*; Fasman, G. D., Timasheff, S. N., Eds.; Marcel Dekker: New York, 1970; p 271.
Lapanje, S. *Physico-chemical Aspects of Protein Denaturation*; Wiley: New York, 1978.
Navin Kumar, K. D. Ph.D. Dissertation, University of Mysore at Mysore, 1982.
Nozaki, Y.; Tanford, C. *J. Biol. Chem.* **1967**, *242*, 4731.
Prakash, V.; Nandi, P. K. *Int. J. Peptide Protein Res.* **1977**, *9*, 319.
Prakash, V.; Narasinga Rao, M. S. *Proc.—Indian Acad. Sci., Chem. Sci.* **1984**, *93*, 1205.
Prakash, V.; Nandi, P. K.; Jirgensons, B. *Int. J. Peptide Protein Res.* **1980**, *15*, 305.
Rahma, E. H. Ph.D. Dissertation, University of Mysore at Mysore, 1979.
Rahma, E. H.; Narasinga Rao, M. S. *J. Agric. Food Chem.* **1981a**, *29*, 518.
Rahma, E. H.; Narasinga Rao, M. S. *J. Biosci.* **1981b**, *3*, 407.
Sastry, M. C. S. Ph.D. Dissertation, University of Mysore at Mysore, 1984.
Schachman, H. K. *Ultracentrifugation in Biochemistry*; Academic: New York, 1959.
Schultz, G. E.; Shrimer, R. H. In *Principles of Protein Structure*; Springer-Verlag: New York, 1979; p 27.
Schwenke, K. D.; Schultz, M.; Linew, K. J. *Die Nahrung* **1975a**, *19*, 425.
Schwenke, K. D.; Schultz, M.; Linew, K. J. *Die Nahrung* **1975b**, *19*, 817.
Schwenke, K. D.; Sologub, L. P.; Brando, E. E.; Tolstogrzov, W. B. *Die Nahrung* **1978**, *22*, 491.
Schwenke, K. D.; Pahtz, W.; Linew, K. J.; Raab, B.; Schultz, M. *Die Nahrung* **1979**, *23*, 241.
Shifrin, S.; Luborsky, S. W.; Grochowski, B. J. *J. Biol. Chem.* **1971**, *246*, 7708.
Steiner, R. F.; Edelhoch, H. *Nature* (London) **1962**, *193*, 375.
Strickland, E. H. In *CRC Handbook of Biochemistry and Molecular Biology—Proteins*; Fasman, G. D., Ed.; CRC: Boca Raton, FL, 1976; Vol. 3, p 141.
Tanford, C. *Physical Chemistry of Macromolecules*; Wiley: New York, 1961; p 390.
Tanford, C. *Adv. Protein Chem.* **1962**, *17*, 80.
Teale, F. W. J. *Biochem. J.* **1960**, *76*, 881.
Wang, K. P.; Hamlin, L. W. *Biochemistry* **1974**, *13*, 2578.
Wetlaufer, D. B. *Adv. Protein Chem.* **1962**, *17*, 303.
Yang, J. T.; Foster, J. F. *J. Am. Chem. Soc.* **1954**, *76*, 1580.
Yang, J. T.; Doty, P. *J. Am. Chem. Soc.* **1957**, *79*, 761.

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SDS-Catalyzed Deamidation of Oilseed Proteins

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The effects of sodium dodecyl sulfate (SDS) on the acid hydrolysis of cottonseed and other oilseed proteins have been investigated. Under relatively mild acid (0.2 N HCl) and temperature (70 °C) conditions, small amounts of SDS (0.02–0.06 M) catalyzed the hydrolysis of amide groups (deamidation) in cottonseed protein in preference to the hydrolysis of peptide bonds. High degrees of deamidation could be achieved with minimal peptide bond degradation, and functional properties of the protein such as solubility, water binding, emulsion capacity, and whippability were improved. The SDS-catalyzed deamidation of soybean and peanut proteins has also been investigated.

Oilseeds are a good source of low-cost protein, and there have been efforts to enhance the functional properties of oilseed proteins to make them more suitable for food uses. Enzyme treatments of oilseed proteins have been reported to improve the solubility but sometimes destroy the emulsifying capacity and foam stability (Sekul et al., 1978; Beuchat et al., 1975, Arzu et al., 1972). Chemical modifications normally improve functionality of the oilseed proteins using techniques that include sulfonation (Sair, 1961), succinylation (Franzen and Kinsella, 1976), phos-

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