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Isolation and characterization of the 7S protein from glanded cottonseed *(Gossypium herbacium)*

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Abstract. The major protein from glanded cottonseed has been isolated in a homogeneous form. Its $S_{20,w}$ value at 1% protein concentration is 6S in 1 M NaCl solution. It contains 1% carbohydrate and is free from phosphorus, gossypol (bound or free) and nucleic acid impurities. It consists of atleast seven non-identical subunits. The protein has an ultraviolet absorption maximum at 278 nm and fluorescence excitation and emission maxima at 280 nm and 325 nm respectively. Optical rotatory dispersion and circular dichroism measurements indicate that the protein consists predominantly of β-structure and random coil. The observed near-ultraviolet circular dichroic bands can be attributed to tyrosine, phenylalanine and tryptophan residues of the protein.

Keywords. Isolation; characterization; cottonseed protein; 7S protein; gossypol.

Introduction

Cottonseed proteins consist of three protein fractions, which are designated as 2S, 7S and 12S protein based on their sedimentation coefficient (Martinez *et al*., 1970). The 7S protein constitutes about 50% of the total proteins and is the major protein component (Martinez *et al*., 1970). Many methods have been reported for the isolation of the 7S protein (Karon *et al*., 1950; Rossi-Fanelli *et al*., 1964; Ibragimov *et al*., 1969; Phillips and Martinez, 1973; Youle and Huang, 1979), Some of its physico-chemical properties have also been reported (Rossi-Fanelli *et al*., 1964; Ibragimov *et al*., 1969; Phillips and Martinez, 1973; Youle and Huang, 1979; Zarins and Martinez, 1974). However, no information is available on the conformation of the protein. In this communication a method is described to isolate the 7S protein in a homogeneous form and the spectral and conformational properties of the protein are reported. This forms a part of our investigation on the conformation of isolated oilseed protein fractions and the effect of denaturants on them.

Abbreviations used: TEMED, N N N' N'— tetramethylethylene diamine; ORD, optical rotatory dispersion; CD, circular dichroism; SDS, sodium dodecyl sulfate; DEAE cellulose, diethyl aminoethyl cellulose; PAGE, polyacrylamide gel electrophoresis.

Materials and methods

Materials

Cottonseeds of the variety Jayadhar (*Gossypium herbacium*) were obtained from the Agricultural Research Station, University of Agricultural Sciences, Dharwar, Karnataka. Sepharose 6B-100 (40-210 μ) from Pharmacia Fine Chemicals (Sweden), DEAE-cellulose (coarse mesh of 0.80 meq/g exchange capacity), tetrasodium pyrophosphate, bovine serum albumin, egg albumin, pepsin, β-lacto-globulin and ribonuclease A from Sigma Chemical Co., St. Louis, Missouri, USA, Coomassie brilliant blue from Schwarz-Mann, New York, USA, acrylamide from E. Merck, Munich, West Germany, bis-acrylamide 'from Koch-Light Laboratories Ltd., Colnbrook, England, N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED) and β-mercaptoethanol from Fluka, Switzerland were used. All other chemicals used were of Reagent grade.

Isolation of the 7S protein

Cottonseeds were flaked in a Kvarnmaskiner (Type 5) flaking machine, dried in air, sieved to remove the hulls, defatted with n-hexane at room temperature (~30°C) and the solvent removed by aeration. The flakes free of the solvent were milled in an apex comminuting mill to a mesh size of 60 (B 3325). Moisture and protein content of the meal were 9.5% and 48%, respectively.

Ten grams of the defatted cottonseed flour were stirred with 100 ml of 1 M NaCl solution for 1 h. The insoluble residue was separated by centrifugation at 3330 *g* for 30 min and the clear supernatant dialysed against distilled water for 6 h. The water insoluble proteins in the dialysis tubing were separated by centrifugation at 2030 *g* for 30 min and the supernatant discarded. The precipitate was washed 2-3 times with small portions of distilled water, dissolved in 10-15 ml of 0.05 M tetrasodium pyrophosphate-HCl buffer of pH 7.8 (buffer A) and dialysed against the same buffer. The clear protein solution was loaded on DEAE-cellulose column (2.5×25 cm) equilibrated with buffer A. Protein and gossypol were monitored by measuring the absorbance at 280 nm and 358 nm respectively. The unadsorbed proteins were eluted with 300 ml of the same buffer and rejected. The adsorbed protein(s) was eluted with buffer containing 0.1 M NaCl. The peak fractions were pooled and the protein precipitated with 40% ammonium sulphate (w/v). The precipitate was dissolved in buffer A and dialysed against the same buffer. The dialysate was rechromatographed with a continuous gradient of NaCl (0 to 0.6 M) on DEAE-cellulose column (2×16 cm) equilibrated with buffer A. The unadsorbed protein was rejected. The peak fractions eluting at 0.1 M NaCl were pooled and precipitated with 40% ammonium sulphate (w/v). The precipitate separated by centrifugation was dissolved in the required solvent and dialysed against the same.

Disc electrophoresis

Disc electrophoresis with polyacrylamide gels was carried out according to the method of Davis (1964), using 10% gels in 0.025 M tris-glycine buffer of pH 8.3. Gels were stained with 0.25% Coomassie brilliant blue and destained in water containing 7.5% acetic acid and 5% methanol (Weber and Osborn, 1968).

Sedimentation velocity: Sedimentation velocity measurements were made at 59,780 rpm at 20°C in a Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics and Rotor Temperature Indicator and Control (RTIC) unit. A 12-mm single sector duraluminum cell was used with 1% protein solution in 1 M NaCl. $S_{20,w}$ value was calculated by the standard procedure (Schachman, 1959).

Gel filtration: Sepharose 6B-100 equilibrated with 1 M NaCl solution was packed into a column, 2×100 cm. Elution of the protein was done with the same solvent. Fractions (3 ml) were collected and protein was monitored by measuring the absorbance at 280 nm. Absorbance at 358 nm, which is a measure of gossypol concentration (both bound and free) was also measured.

Protein concentration was estimated by measuring the absorbance of the solution at 280 nm. For the 7S protein a value of 6.0 for $E^{1\%}_{1cm}$ at 280 nm was used. This value was obtained by measuring the absorbance of a series of solutions whose concentrations were determined by the microKjeldahl procedure for the estimation of nitrogen. A value of 6.25 was used for converting nitrogen to protein.

Absorption spectrum: Ultraviolet (UV) absorption spectrum of protein in buffer A was recorded at room temperature (~30°C) in a Perkin-Elmer double beam recording spectrophotometer 124 in the range 240 to 370 nm.

Fluorescence spectrum of the protein was measured in a Perkin-Elmer fluorescence spectrophotometer, Model 203, at 25°C. The emission spectrum was measured in the range of 300-400 nm after excitation at 280 nm. The excitation spectrum was measured in the range of 240 to 300 nm after fixing the emission maximum at 325 nm. Protein solutions (0.01%) were used for these measurements.

Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra: These measurements were made on a JASCO-J20C automatic recording spectropolarimeter calibrated with d-10 camphor sulphonic acid. Quartz cells (1 cm) were used for measurements in the range 650 to 250 nm and 0.5 to 2 mm cells were used in the far UV region. All measurements were obtained with solutions having an absorbance of less than 2 at 280 nm and at 29°C unless otherwise mentioned. Slits were programmed to yield at 10^{o}_{A} (1 nm) band width at each wavelenth. Mean residue rotations $[\alpha]_{MRW}$ and mean residue ellipticities $[\theta]_{MRW}$(deg cm_2/Vd mole) were calculated by the standard procedure (Adler *et al*., 1973). A value of 115 for the mean residue weight (MRW) was assumed.

Carbohydrate content: Carbohydrate was estimated by the method of Montgomery (1961) using 2.5% protein solution in buffer A.

Phosphorus content: Phosphorus was estimated by the method of Taussky and Shorr (1953) using a 2.5% protein solution in 1 M NaCl.

Free and total gossypol content was estimated by the method of Pons and Guthrie (1949) and Pons *et al.* (1950) respectively using a 2.5% protein solution in 1 M NaCl.

Determination of subunits and: their molecular weight The number of subunits in the 7S protein and their molecular weight were determined by analytical SDS-polyacrylamide disc gel electrophoresis (Laemmli, 1970). The following proteins were used as molecular weight markers: bovine serum albumin (68,000), egg albumin (43,000), pepsin (35,000), β-lactoglobulin (18,400) and ribonuclease (13,600). Using 10% separating gel and 3% stacking gel, electrophoresis was carried out in 0.025M Tris-glycine buffer of pH 8.3, containing 0.1% SDS for 2 h at 3 mA/tube; 200-300 μg of the protein was loaded. Gels were stained with 0.25% Coomassie brilliant blue and destained in water containing 7.5% acetic acid and 5% methanol.

Results

Homogeneity of the 7S protein

The homogeneity of the 7S protein was determined by gel electrophoresis, gel filtration and ultracentrifugation. The disc gel electrophoresis pattern of the protein at pH 8.3 showed a single sharp band indicating its homogeneity (figure 1). The protein eluted as a single symmetrical peak in gel filtration (figure 2) with an elution volume of 188 ml. This corresponds to peak II in the gel filtration pattern of cottonseed total protein (figure 3).

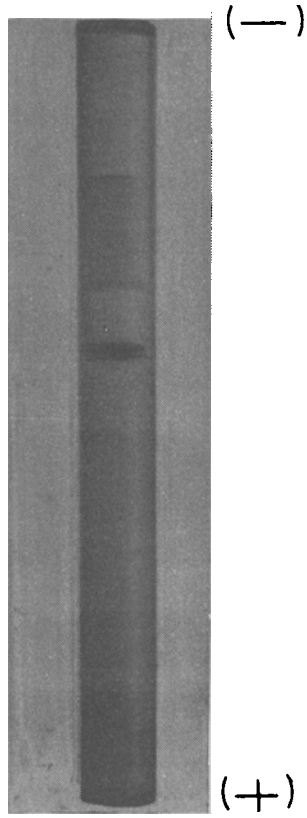

Figure 1. Disc electrophoretic pattern of 7S protein (0.025 M tris-glycine buffer, pH 8.3).

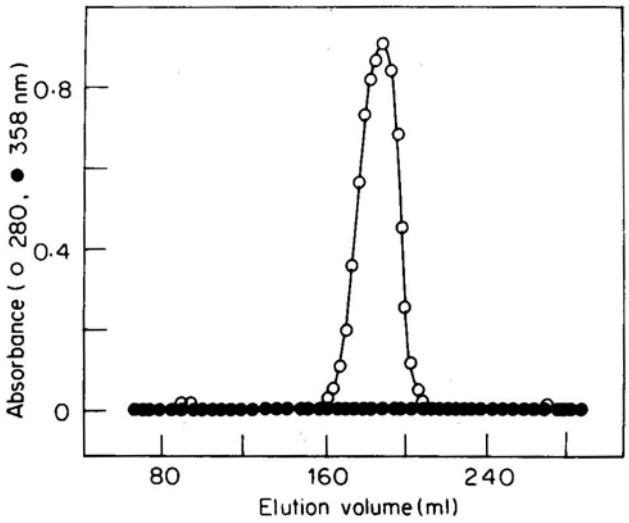

Figure 2. Gel filtration pattern of 7S protein in 1 M NaCl solution on Sepharose 6B-100.

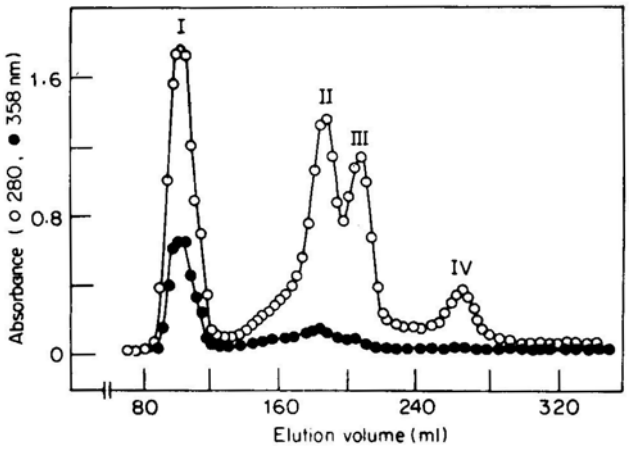

Figure 3. Gel filtration pattern of cottonseed total proteins in 1M NaCl solution on Sepharose 6B-100.

The sedimentation velocity pattern of the protein in 1 M NaCl consisted of a symmetrical major peak with an $S_{20,w}$ value of 6S and a faster moving component (9.6S) which formed less than 5% of the total (figure 4). Both disc gel electrophoresis and gel filtration did not show the presence of higher molecular weight component in the isolated protein fraction and was homogenous by the

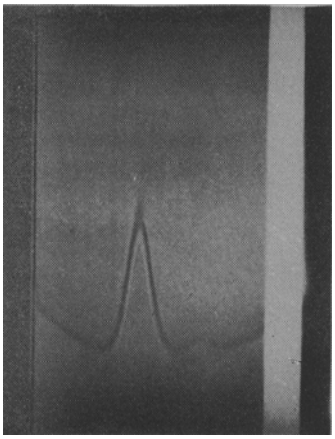

Figure 4. Sedimentation velocity pattern of 7S protein; 1% protein solution in 1 M NaCl. Photograph taken after 60 min at 59,780 rpm; bar angle 65°. Sedimentation proceeds from left to right.

above criteria. Thus by DEAE-cellulose chromatography in buffer A it was possible to isolate the major protein of cottonseed in a homogenous form.

The protein did not contain any phosphorus and was also free from bound and free gossypol. The carbohydrate content of the protein was 1%. Pyrophosphate buffers have been used successfully for minimizing the contamination of nucleic acid and carbohydrate impurities in protein preparations (Eipper, 1972).

The ultraviolet absorption spectrum of the 7S protein is typical of a protein with an absorption maximum at 278 nm and minimum at 250 nm. The ratio of absorbance at 280 to 260 nm was 1.5. This suggested the absence of nucleic acid impurities in the preparation (Layne, 1957) which was also supported by the fact that the protein had no phosphorus. The excitation coefficient of protein at 280 nm, $E^{1\%}_{1\ cm}$, was 6.0.

The protein had fluorescence excitation maximum at 280 nm and emission maximum at 325 nm (figure 5). In proteins containing both tryptophan and tyrosine residues, the fluorescence spectrum of tryptophan alone is observed, irrespective of the wavelength of the exciting light, even in those cases where tyrosine predominates in absorption. The emission maxima of proteins vary between 328 and 342 nm (Teale, 1960). The 7S protein contains 1.28% tryptophan and 2.75% tyrosine (Ibragimov *et al*., 1974). The results suggest that the fluorescence emission maximum at 325 nm is due to the contribution of tryptophan groups which are in the interior of the protein (Shifrin *et al*., 1971; Mills and Creamer, 1975).

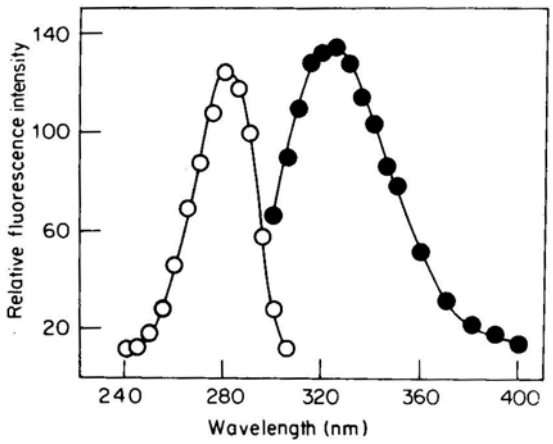

Figure 5. Fluorescence spectrum of 7S protein in buffer A. —O—excitation spectrum; —●—emission spectrum.

ORD and CD spectra

The CD spectrum of the 7S protein in the region of 300-250 nm (figure 6a) has a positive shoulder at 290 nm and peaks at 285, 280, 265 and 258 nm. The peaks at 258 and 265 nm could be assigned to phenylalanine residues; the peaks at 285 nm

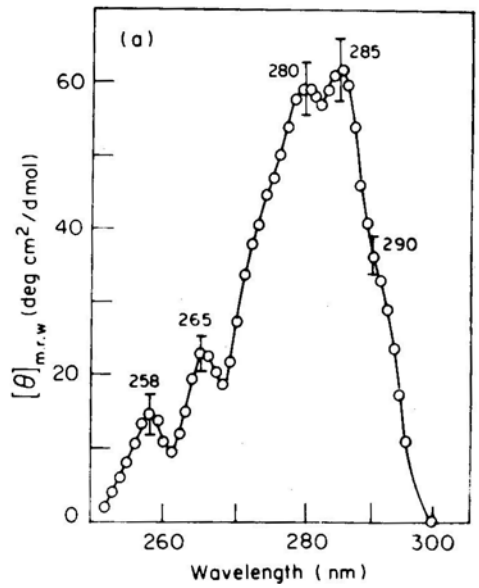

Figure 6a. Near ultraviolet CD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

and 280 nm were possibly due to tyrosine and the shoulder at 290 nm due to tryptophan (Strickland, 1974). Cystine is also known to contribute to the near UV CD spectra of proteins (Strickland, 1974). The 7S protein contains 1.28% tryptophan, 2.75% tyrosine, 6.93% phenylalanine and 1.81% cystine (Ibragimov *et al.*, 1974; Youle and Huang, 1979).

The secondary structure of the 7S protein was determined by measuring its CD spectrum in the region of 260 to 200 nm. The protein exhibited a trough at 208 nm and a shoulder at 225 nm (figure 6b). The helical content of the protein was estimated by the method of Greenfield and Fasman (1969), using the equation

$$\% \text{ α-Helix} = \frac{-[\theta]_{208nm} - 4{,}000}{33{,}000 - 4{,}000} \times 100 \quad (1)$$

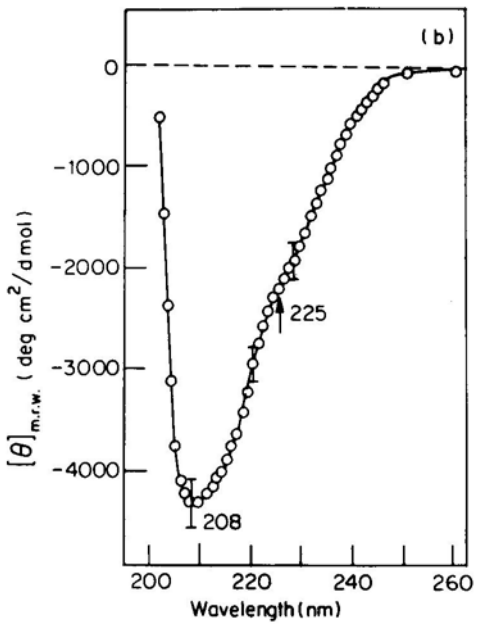

Figure 6b. Far ultraviolet CD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

and by the method of Chen and Yang (1971) using the mean residue ellipticity value at 222 nm, by using the equation

$$[\theta]_{222nm} = -30{,}300\, f_H - 2340 \quad (2)$$

Where f_H = fractional helix content.

Estimation of α-helical content by the two procedures indicated the absence of α-helix in the protein. The proportion of β-structure was estimated by the method of Sarkar and Doty (1966). Taking a mean residue ellipticity value of –23 000 deg

cm^2/d-mol at 218 nm for 100% β-structure, the protein was found to contain 16% β-structure. The exact nature of β-structure is not known and the estimate is approximate. Thus, the secondary structure of the 7S protein consists predominantly of β-structure and random coil. These results are comparable to those on the other oilseed proteins (Jacks *et al*., 1973; Koshiyama and Fukushima, 1973; Jayarama Shetty and Narasinga Rao, 1976; Appu Rao and Narasinga Rao, 1977; Prakash *et al*., 1980; Gururaj Rao and Narasinga Rao, 1981; Rahma and Narasinga Rao, 1981), which are characterized by low α-helical content and predominant β-structure and random coil. Further studies are in progress to characterise the nature of the β-structure of the protein.

Figure 7 shows the far UV-ORD spectrum of the 7S protein in the region 280-210 nm. The protein exhibited a trough at 233 nm, a shoulder at 221 nm and a cross over point at 215 nm. A similar trough (230-235 nm) has been reported with other oilseed proteins (Fukushima, 1968; Catsimpoolas *et al.,* 1970; Appu Rao and Narasinga Rao, 1976; Appu Rao and Narasinga Rao, 1977; Jayarama Shetty and

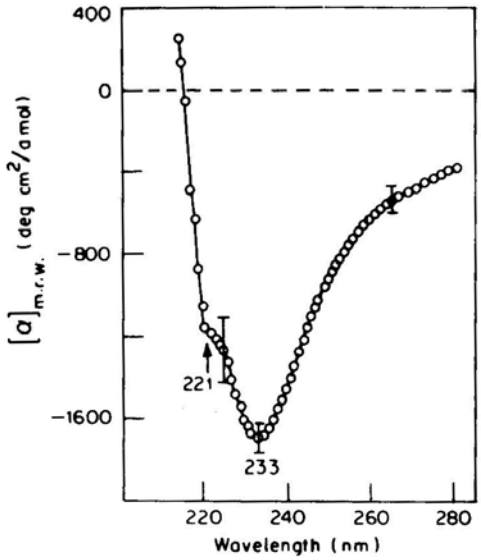

Figure 7. Far ultraviolet ORD spectrum of 7S protein in buffer A. The data points are average values of atleast three determinations, the error bars indicating ±mean deviation.

Narasinga Rao, 1978; Gururaja Rao and Narasinga Rao, 1978; Gururaja Rao and Narasinga Rao, 1981). The trough at 233 nm could be due to either *α*-helix or anti-parallel β-structure (Fasman and Potter, 1967). The CD measurements suggest that the protein contains very little of α-helix. So the observed trough at 233 nm could possibly be due to antiparallel β-structure. Using a value of 6400 deg cm^2/d mol at 230 nm for 100% β-structure (Sarkar and Doty, 1966), the proportion of β-structure in the 7 S protein was estimated as 25%. This could be an overestimate due to contribution of both the antiparallel β-structure and random coil to the rotation at this wavelength.

The optical rotation was also measured in the region 650-330 nm and the data analyzed by the Moffitt-Yang equation (Moffitt and Yang, 1956) to determine the secondary structure. The equation is

$$[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \quad (3)$$

where [α] is the specific rotation at wavelength λ and λ_0, a_0 and b_0 are constants. Using λ_0=212 nm, [α] $[\lambda^2 - \lambda_0^2]/\lambda_0^2$ was plotted against $\lambda_0^2/]\lambda^2-\lambda_0^2]$. The slope, b_0, was found to be zero, which indicated the absence of α-helical structure in the protein.

Analytical SDS-polyacrylamide disc gel electrophoresis of 7 S protein in 0.025 M tris-glycine buffer of pH 8.3 containing 0.1% SDS (figure 8) showed that it contained atleast seven non-identical subunits and the molecular weights were 66,000; 57,000; 37,000; 34,000; 21,300; 19,800 and 18,000.

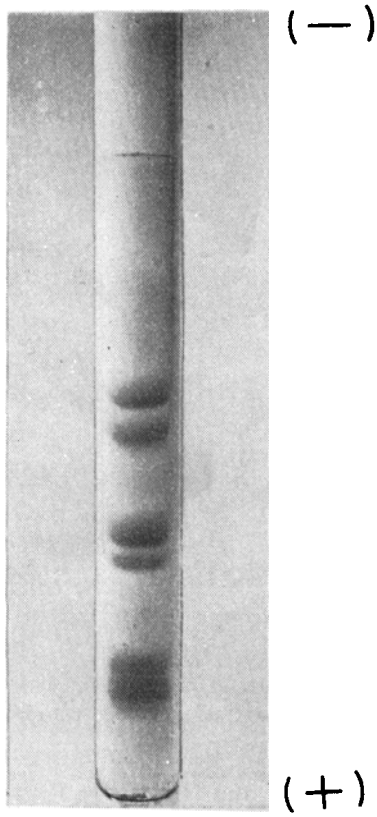

Figure 8. SDS-polyacrylamide disc gel electrophoresis pattern of 7S protein (0.025 M tris-glycine buffer of pH 8.3, containing 0.1% SDS).

Ibragimov *et al.* (1969) and Martinez (1979) have reported the molecular weight of the 7 S protein as 140,000. Since in SDS-PAGE experiments, the bands were not of equal intensity and the stoichiometry of the subunits was not established, no attempt was made to determine the molecular weight from these experiments.

Discussion

The methods reported in the literature for the isolation of the 7 S protein of cottonseed use (1) the effect of salt and temperature on the solubility of the protein fractions; (2) repeated chromatography on ion-exchange resins or (3) sucrose-density gradient centrifugation. Some of the procedures exposed the protein to extreme changes in pH and temperature. The method described in this report avoids the use of extremes of pH or temperature. Further the various authors do not state if the isolated 7S protein was free from gossypol and nucleic acid impurities. Apart from other effects, bound gossypol can alter the conformation of the protein (Maliwal, Appu Rao and Narasinga Rao,, unpublished data). In the present investigation we obtain a 7S protein preparation which is free from bound gossypol (figure 2). This observation is supported by CD spectral evidence also. When gossypol binds to bovine serum albumin it induces extrinsic CD bands at 385-390 nm and 300 nm. (Maliwal, Appu Rao and Narasinga Rao, unpublished data). The CD spectrum of the 7S protein in the region 400-300 nm (not shown in Figure 6a) did not indicate peaks at 390 or 300 nm. Thus the method appears to be superior to the earlier methods, especially when the protein is used for conformational studies.

The colouring matter contributed mainly by gossypol and gossypol-like pigments was removed on the DEAE-cellulose column. As mentioned earlier, sodium pyrophosphate buffer seems to have minimised the contamination of nucleic acids and carbohydrate impurities during isolation.

There is some variation in the sedimentation coefficient values reported earlier for the 7 S protein. Species variation, differences in the method of isolation of the protein and the conditions used for ultracentrifugation could have contributed to these variations. The observed $S_{20,W}$ value of the protein in 1 M NaCl at 1% protein concentration was 6 S. Rossi-Fanelli *et al*., (1964) have reported an $S^{o}_{20,\ w}$ value of 9.2S in 0.3M NaCl at pH 7.0; Naismith (1956) has reported an $S_{20,\ w}$ value of 8S in phosphate-NaCl buffer of pH 7.8 and I=0.5; Ibragimov *et al.* (1969) have reported a sedimentation constant of 7 ±0.25 S in 1.25 M NaCl at pH 7.3; and Youle and Huang (1979) have reported, by the density gradient technique, a value of 5 S in 1 M NaCl.

SDS-PAGE experiments showed that the protein consisted of atleast 7 non-identical subunits with molecular weights ranging from 66,000 to 18,000. Further work is in progress to quantitate their proportion and to characterize them.

References

Adler, A. J., Greenfield, N. J. and Fasman, G. D. (1973) *Methods Enzymol.,* **27,** 675.
Appu Rao, A. G. and Narasinga Rao, M. S. (1976) *J. Agric. Food Chem.,* **24,** 490.
Appu Rao, A. G. and Narasinga Rao, M. S. (1977) *Indian J. Biochem. & Biophys.,* **14,** 26.
Catsimpoolas, N., Wang, J. and Berg. T. (1970) *Int. J, Protein Res.*. **2.** 277.
Chen, Y. H. and Yang, J. T. (1971) *Biochem. Biophys. Res. Commu.,* **44,** 1285.
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.,* **121,** 404.
Eipper, B. A. (1972) *Proc. Nat. Acad. Sci. U.S.,* **69,** 2283.

Fasman, G. D. and Potter, J. (1967) *Biochem. Biophys. Res. Commun.,* **27,** 209.
Fukushima, D. (1968) *Cereal Chem.,* **45,** 203.
Greenfield, N. and Fasman, G. D. (1969) *Biochemistry,* **8,** 4108.
Gururaj Rao, A. and Narasinga Rao, M. S. (1978) *Can. Inst. Food Sci. Technol. J.,* **11,**155.
Gururaj Rao, A. and Narasinga Rao, M. S. (1981) *Int. J. Peptide Protein Res.,* **18,**154.
Ibragimov, A. P., Yunuskhanov, Sh. and Tuichiev, A. V. (1969) *Biochem.* (*USSR*)*,* **34,** 897.
Ibragimov, A. P., Turshunbaev, P., Tuichiev, A. V. and Yunuskhanov, Sh. (1974)*Biochem.* (*USSR*)*,* **39,** 186.
Jacks, T. J., Barker, R. H. and Weigang (Jr.), O. E. (1973) *Int. J. Peptide Protein Res.,* **11,** 305.
Jayarama Shetty, K. and Narasinga Rao, M. S. (1976) *Analytical Biochem.,* **73,** 459.
Karon, M. L., Adams, M. E. and Altschul, A. M. (1950) *J. Phys. Colloid Chem.,* **54,** 56.
Koshiyama, I. and Fukushima, D. (1973) *Cereal Chem.,* **50,**114.
Laemmli, U. K. (1970) *Nature,* **227,** 680.
Layne, E. (1957) *Methods Enzymol.,* **3,** 447.
Martinez, W. H. (1979) *J. Am. Oil Chem. Soc.,* **56,** 280.
Martinez, W. H., Berardi, L. C. and Goldblatt, L. A. (1970)*J. Agric. Food Chem.,* **18,** 961.
Mills, O. E. and Creamer, L. K. (1975) *Biochim. Biophys. Acta,* **379.** 618.
Moffitt, W. and Yang, J. T. (1956) *Proc. Nat. Acad. Sci., U.S.,* **42,** 596.
Montgomery, R. (1961) *Biochim. Biophys. Acta,* **48,** 591
Naismith, W. E. F. (1956) *J. Appl. Chem.*, **6,** 283.
Nath, J. P., Subramanian, N. and Narasinga Rao, M. S. (1980) *J. Agric. Food Chem.,* **28,** 844.
Phillips, R. D. and Martinez, W. H. (1973) *Cereal Sci. Today,* (Abs. No. 77), **18,** 293.
Pons (Jr.), W. A. and Guthrie, J. D. (1949) *J. Am. Oil Chem. Soc,* **26,** 671.
Pons (Jr.), W. A., Hoffpauir, C. L. and O'Connor, R. T. (1950) *J. Am. Oil Chem. Soc,* **27,** 390.
Prakash, V. Nandi, P. K. and Jirgensons, B. (1980) *Int. J. Pep. Prot. Res.,* **15,** 305.
Rahma, E. H. and Narasinga Rao, M. S. (1981) *J. Agric. Food Chem.,* **29,** 518.
Rossi-Fanelli, A. and Antonini, E., Brunori, M., Bruzzeri, M. R., Caputo, A. and Satriani, F. (1964) *Biochem. Biophys. Res. Commun.,* **15,** 110.
Sarkar, P. K. and Doty, P. (1966) *Proc Nat. Acad. Sci. U.S.,* **55,** 981.
Schachman, H. K. (1959) in *Ultracentrifugation in Biochemistry* (New York: Academic Press), p. 242.
Shifrin, S., Luborsky, S. W. and and Grochowski, B. J. (1971) *J. Biol. Chem.,* **246,** 7708.
Strickland, E. H. (1974) CRC Critical Reviews in *Biochemistry,* **2,**113.
Taussky, H. H. and Shorr, E. (1953)*J. Biol. Chem.,* **202,** 675.
Teale, F. W. J. (1960) *Biochem. J.,* **76,**381.
Weber, K. and Osborn, M. (1969) *J. Biol. Chem.,* **244,** 4406.
Youle, R. J. and Huang, A. H. C. (1979) *J. Agric. Food Chem.,* **27,** 500.
Zarins, A. and Martinez, W. H. (1974) *Cereal Sci. Today,* (Abs. No. 127), **19,** 402.