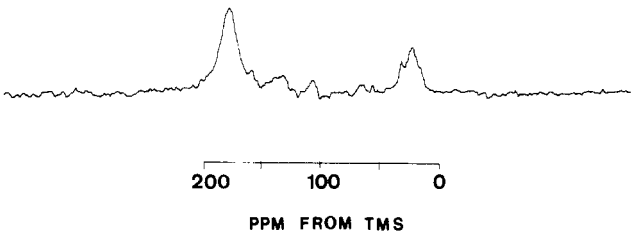

Figure 5. CP/MAS ^{13}C NMR spectrum of red clover seeds by using a 50-μs interruption of the ^{1}H decoupling pulse prior to data acquisition of the ^{13}C signal.

only exception to this rule is the case of methyl groups, which, because of rapid rotational motion, do show strong resonances even under the interrupted decoupling conditions. As can be seen in Figure 5, signals derived from the starch present in red clover seeds were effectively eliminated by interrupted decoupling, while the signal from the carbonyl carbons in the peptide bonds (C-1 in II) is still visible. Methyl signals, presumably from methyl groups in the side-chain R groups of amino acids, also appear in the high-shielding region of Figure 5. It should be noted that the signal-to-noise ratio in Figure 5 was attained only after using 4 times the number of acquisitions used for the normal ^{13}C CP/MAS NMR experiment (Figure 1B). The apparent reduction of overall intensity results largely from the fact that the carbonyl and methyl signals are lower in intensity than the starch signal in the normal spectrum. Also, some small amount of carbon magnetization (roughly one-fourth) is lost from the carbonyl carbons during the interruption of the decoupling, due to neighboring protons, and the methyl carbons are certainly effected by the interruption, even though rapid rotation about the carbon–carbon bond attenuates the effect. Some small signals in the center of the spectrum (110–160 ppm) are also observed in Figure 5 and can be tentatively assigned to substitued aromatic carbons in aromatic amino acids (Opella, et al., 1979).

Although the qualitative importance of the ^{13}C CP/MAS technique is obvious, the quantitative aspects remain to be investigated. Among the factors that require additional study is the extent to which lipid components of varying degrees of mobility can contribute to the ^{13}C spectrum and the analysis. This question has been addressed by Rutar and Blinc (1979). The spin-temperature inversion technique should minimize any contribution due to mobile lipid components. Another factor for additional study is the relative cross polarization efficiencies of the pertinent carbon types in seed spectra. These efficiencies will determine the optimum experimental conditions and the nature of any corrections or calibrations that may be needed for quantitation.

CONCLUSIONS

The use of CP/MAS ^{13}C NMR spectroscopy in the analysis of whole seeds certainly shows great promise. The main limitation would seem to be the length of analysis time. At present, a minimum of 30 min is required per analysis. However, this disadvantage may be offset by the safe, nondestructive nature of the analysis and should be reduced substantially as the equipment and techniques improve. One can expect significant developments in this area during the next few years.

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Isolation and Characterization of the Major Protein Fraction of Sunflower Seeds

E. H. Rahma and M. S. Narasinga Rao*

A method is described for isolating the major protein fraction of sunflower seeds in a homogeneous form. It has an $s_{20,w}$ value of 11.6 S (at 1% protein concentration) and a molecular weight of 300 000. It contains 0.31% carbohydrate and no phosphorus. It consists of at least 10 polypeptide chains. Its structure is predominantly random coil and β structure. Some of the SH groups are buried and are exposed after denaturation of the protein with urea.

The proteins of sunflower seed (*Helianthus annuus*) consist of three protein fractions having sedimentation coefficients of 2, 7, and 11 S (Joubert, 1955; Sabir et al., 1973; Schwenke et al., 1974; Rahma and Narasinga Rao, 1979). The major protein is a high molecular weight protein with a molecular weight of 300 000–350 000 (Sabir et al., 1973; Joubert, 1955). Although there are reports on

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the gel filtration, gel electrophoretic, and ultracentrifugal characteristics of the total proteins of sunflower, such data on individual protein fractions are meager. In this report we describe a method for isolating the major protein fraction in a homogeneous form and also some of its chemical and physicochemical characteristics.

MATERIALS AND METHODS

Sunflower. Russian variety EC 68414, grown in the State of Karnataka, India, was obtained from the local market.

Sunflower Meal. The method of preparation was the same as that described earlier (Rahma and Narasinga Rao, 1979).

Isolation of the Major Protein Fraction. The proteins from the meal were extracted in 10% NaCl solution by using a meal to solvent ratio of 1:10 (w/v). The slurry was stirred for 1 h at room temperature and centrifuged at 4000 rpm for 20 min. To the clear solution we added solid $(NH_4)_2SO_4$ to 6.5% saturation, stirred the solution well, and kept it in the cold (~5 °C) for 30 min. The precipitate was removed by centrifugation and discarded. Ammonium sulfate was added to the supernatant to raise the concentration to 13% saturation, and the suspension was kept in the cold for 30 min. The precipitate was separated by centrifugation and dissolved in 1 M NaCl solution. The protein fraction was reprecipitated with 13% $(NH_4)_2SO_4$ saturation and recovered by centrifugation. It was dissolved in 1 M NaCl solution and dialyzed against several changes of 0.025 M Tris–glycine buffer of pH 8.3.

Gel Filtration. About 50 mg of the protein in the buffer was applied on the column 2.5 × 85 cm) of Sepharose 6B-100 which had been equilibrated with 0.025 M Tris–glycine buffer of pH 8.3 containing 2.5% NaCl. Elution was done with the same buffer. Fractions of 3 mL were collected in an automatic fraction collector, and the absorbance of the fractions was measured at 280 and 328 nm.

Polyacrylamide Gel Electrophoresis. Gels (7.5%) were prepared by the standard procedure (Davis, 1964). Electrophoresis was done in 0.025 M Tris–glycine buffer of pH 8.3 at a constant current of 3 mA/tube. The gels were stained for 2 h with a staining solution containing 1.25 g of Coomassie brilliant blue and destained in 7.5% acetic acid solution.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The method of Weber and Osborn (1969) was used with BSA (69 000), egg albumin (43 000), pepsin, (35 000), α-chymotrypsin (23 800), and lysozyme (14 400) as molecular weight markers.

Ultracentrifugation. The sedimentation velocity experiment was done by using 1% protein in 1 M NaCl solution at room temperature (~28 °C) at 59 780 rpm in a Spinco Model E analytical ultracentrifuge equipped with a Rotor Temperature Indicator and Control (RTIC) unit and phase-plate Schlieren optics. Photographs were taken at 15-min intervals of centrifugation. From the photographs $s_{20,w}$ was calculated by the standard procedure (Schachman, 1959).

Ultraviolet Spectrum. The spectrum of the protein was recorded in the range 240–300 nm with Perkin-Elmer double-beam recording spectrophotometer, Model 124.

Fluorescence Spectrum. For fluorescence measurements protein solution having an absorbance of 0.04 at 280 nm (0.0048%) was used. The emission spectrum was taken in the range of 300–400 nm after excitation at 280 nm. A Perkin-Elmer fluorescence spectrophotometer, Model 203, was used.

Circular Dichroism (CD) Spectrum. CD measurements were made at room temperature (~28 °C) in a JASCO J-20 automatic spectropolarimeter equipped with a xenon arc lamp using a 0.5-mm quartz cell. The spectrum was recorded in the range of 200–300 nm. Measurements were made at two protein concentrations, 0.23 and 0.46 mg/mL, in 1 M NaCl solution because the Tris buffer had absorption in the UV range. Molar residue ellipticity was calculated by the standard procedure (Adler et al., 1973). A value of 115 was used for mean residue weight.

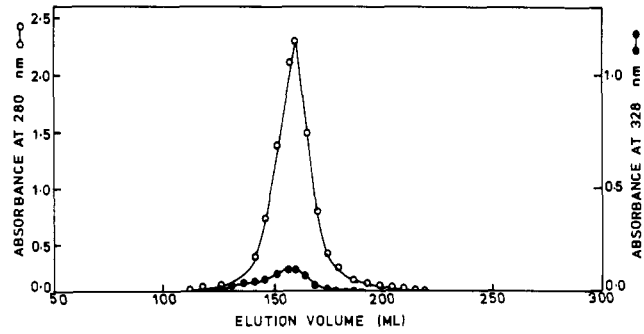

Figure 1. Gel filtration pattern of the protein.

Viscosity. The intrinsic viscosity of the protein was determined in 1 M NaCl solution at 30 ± 0.1 °C with an Ostwald viscometer having a flow time of 185 s with distilled water. Intrinsic viscosity was obtained from the intercept of the plot of $(\eta/\eta_0 - 1)/C$ vs. C, where η is the viscosity of the solution, η_0 is that of the solvent, and C is the protein concentration in g/100 mL.

Carbohydrate Content. Carbohydrate was estimated by the method of Montgomery (1961) using protein solution in 1 M NaCl.

Phosphorus Content. This was estimated by the method of Taussky and Shorr (1953).

Chlorogenic Acid Content. Chlorogenic acid was estimated by the procedure of Pomenta and Burns (1971).

Sulfhydryl-Group (SH) Estimation. The SH content of the protein was estimated by the procedure described by Beveridge et al. (1974) using 1% protein solution. Measurements were also made in the presence of various concentrations of urea.

RESULTS AND DISCUSSION

Two methods have been reported for the isolation of the major protein fraction of sunflower. The method of Joubert (1955) did not give a homogeneous protein as judged by gel electrophoresis and sedimentation velocity techniques. It showed contamination with low molecular weight proteins. On the other hand, the protein isolated by the method of Schwenke et al. (1974) had poor solubility and was not very useful for physicochemical studies. Further, the method was time consuming.

The homogenity of the protein prepared by the method described in this paper was determined by gel filtration, gel electrophoresis, and ultracentrifugation. In gel filtration (Figure 1), it gave a single symmetrical peak with an elution volume corresponding to that of the major fraction of the total proteins (Rahma and Narasinga Rao, 1979). In gel electrophoresis (Figure 2), a single, fairly sharp band in the position of the major protein fraction of the total proteins was observed. A single symmetrical peak was observed in the sedimentation velocity pattern (Figure 3). Its $s_{20,w}$ was found to be 11.6 S. The protein was homogeneous by the above three techniques. Thus, a homogeneous protein preparation could be obtained by the procedure described in this paper. Further, the solubility of the protein in buffer and salt solutions was good, unlike

Figure 2. Gel electrophoresis pattern of the protein.

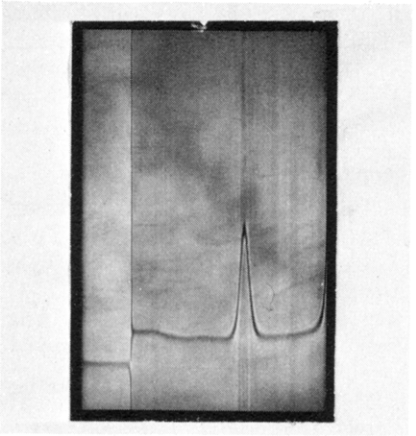

Figure 3. Sedimentation velocity pattern of the protein. The photograph was taken after 30-min centrifugation at 59 780 rpm (sedimentation proceeds from left to right).

Table I. Carbohydrate, Phosphorus, Chlorogenic Acid, and Sulfhydryl Content of Sunflower Total Proteins and 11S Protein

constituent	% 11S protein	% total protein
carbohydrate	0.31	1.30
phosphorus	0	0.026
chlorogenic acid	0.31	0.41
sulfhydryl	2.0[a]	3.5[a]

[a] Number of residues per 300 000 g of protein.

that of the protein obtained by the method of Schwenke et al. (1974).

The carbohydrate content of the protein expressed as glucose was 0.31% (Table I) and was thus low. The carbohydrate content of sunflower 11S protein has not been reported.

The 11S protein did not contain any phosphorus. This agreed with the observation that the 280 to 260 nm absorbance ratio was 1.55. Thus, sunflower 11S protein prepared by our method was completely free from nucleic acid or phytate impurities. There does not appear to be any literature reports on the phosphorus and phytate content of sunflower 11S protein.

The chlorogenic acid (CGA) content of the 11S protein was 0.31% (Table I). This represents the bound CGA since free CGA would be removed during dialysis. The CGA content of total proteins was 0.41%. Thus, a major portion

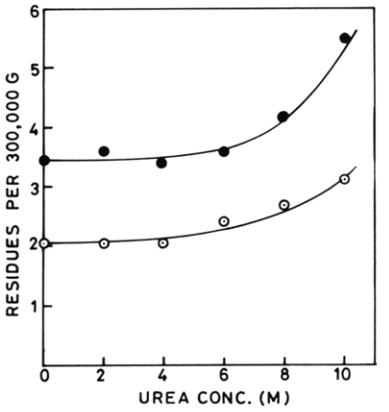

Figure 4. Sulfhydryl content as a function of urea concentration. (⊙) 11S protein; (●) total proteins.

of CGA in the protein was bound to the 11S protein. Sabir et al. (1973) have reported that CGA is bound with the low molecular proteins and that ~70% of it diffuses during dialysis. On the contrary, the gel filtration data of sunflower total proteins (Rahma and Narasinga Rao, 1979) and of the 11S fraction (Figure 1) clearly suggest that most of the CGA of sunflower proteins was associated with the 11S protein.

The sulfhydryl content of 11S protein was 2.0 residues/300 000 g of protein and that of the total proteins 3.5 residues/300 000 g (Table I). All the SH groups were not available for estimation in the native state. When the protein was denatured in urea solution, the number of SH groups increased (Figure 4). This was also true of the total proteins (Figure 4). In 10 M urea solution, 11S protein had an SH content of 3.2 residues compared to 2.0 residues in the native state. Thus, one group was buried inside the protein molecule and became available for interaction only when the protein was denatured.

The absorption spectrum of the protein was typical of a protein with a maximum at 280 nm and a minimum at 255 nm. The ratio of the absorbance at 280 nm to that at 260 nm was 1.55. From this value it could be concluded that the protein was free from nucleic acid impurities (Layne, 1957). This was compatible with the observation that the protein did not contain phosphorus.

The fluorescence emission spectrum of the protein gave a maximum at 325 nm. Free tryptophan gives an emission maximum around 350 nm and tyrosine around 305 nm (Chen et al., 1969). Teale (1960) has reported that the fluorescence emission of proteins having both tryptophan and tyrosine residues is more characteristic of tryptophan, and tyrosyl emission is suppressed. The tryptophan and tyrosine content of the 11S protein has not been determined. The observed maximum at 325 nm indicated a greater contribution of tryptophan residues.

The CD spectrum of the protein in the region 260–200 nm is given in Figure 5. It consisted of two minima, one at 208–210 nm and another at 223–225 nm. The wavelengths of the minima correspond to those of a completely α-helical conformation. However, the ellipticity values corresponding to the two minima in 100% α helix are of equal magnitude (Greenfield and Fasman, 1969). The ellipticity value at 208–210 nm was -4300 deg cm^2 dmol^{-1} and that at 223–225 nm -2700 cm^2 dmol^{-1}. Polypeptides with 100% β structure also show a minumum around 220 nm, and the ellipticity value of the β structure at this wavelength is lower than that of α helix.

Calculation of the α-helical content of the 11S protein from its ellipticity value at 208 nm using the equation of Greenfield and Fasman (1969) showed the value to be

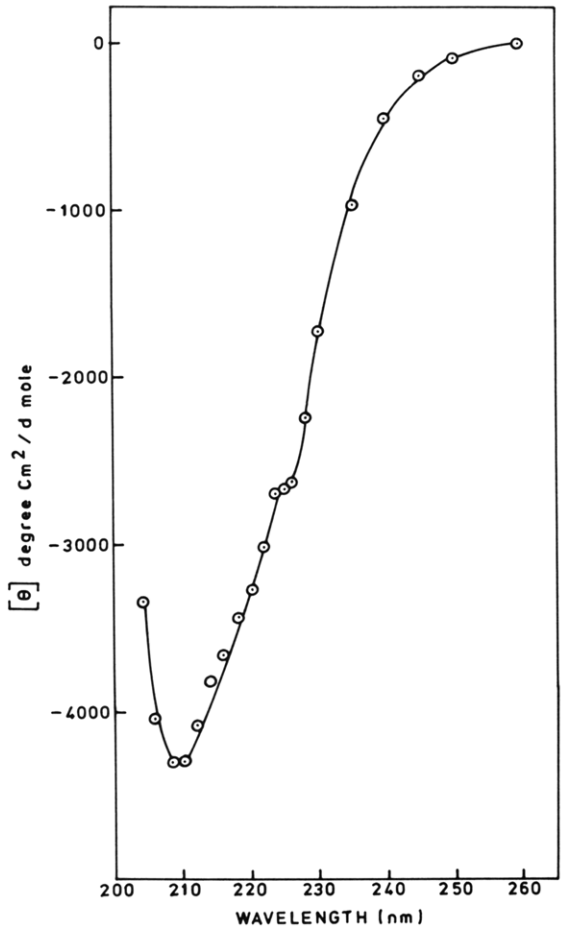

Figure 5. Circular dichroism spectrum of the protein.

~1%. Thus the major structures appeared to be β structure and random coil.

The intrinsic viscosity of the protein was found to be 0.036 dL/g in 1 M NaCl. Tanford (1969) has reported that globular proteins have an intrinsic viscosity in the range of 0.03–0.04 dL/g. Thus the protein appeared to be globular in shape and had a compact structure. Recently, Plietz et al. (1978) have reported from small-angle X-ray scattering measurements that the 11S protein approximates to an oblate ellipsoid of revolution with $a/b = 0.6$.

The 11S protein of sunflower is reported to have a high molecular weight of 300 000–350 000 (Joubert, 1955; Sabir et al., 1973). From the $s_{20,w}$ and intrinsic viscosity, the molecular weight can be calculated by using (Schachman, 1959)

$$M_r = \frac{4690 s_{20,w}^{3/2} [\eta]^{1/2}}{(1 - \bar{\nu}\rho)^{3/2}}$$

where M_r is the molecular weight, $[\eta]$ is the intrinsic viscosity, $\bar{\nu}$ is the partial specific volume, and ρ is the density. If we assume $\bar{\nu} = 0.75$ (Tanford, 1969), the molecular weight value calculated would be 280 000. Strictly, in this equation the value of $s_{20,w}$ at zero protein concentration should be used. This value may be expected to be 12.2 S; then the calculated molecular weight would be 303 000, which falls in the range of reported values (Sabir et al., 1973; Joubert, 1955).

Figure 6. NaDodSO$_4$–polyacrylamide gel electrophoretic pattern of the protein.

Since the protein had a high molecular weight, it was likely to be oligomeric and consist of subunits (Klotz, 1966). Therefore, NaDodSO$_4$–polyacrylamide gel electrophoresis was performed to determine the number and molecular weight of the subunits. It consisted of 10 major bands; 2 minor bands were also observed (Figure 6). The molecular weights of the major bands were 81 000, 70 800, 50 100, 45 700, 37 000, 32 400, 25 700, 19 000, 12 900, and 9100. Since all the bands were not of equal intensity and also some of them could be due to small impurities in the 11S protein, no attempt was made to calculate the molecular weight of the protein from NaDodSO$_4$–polyacrylamide gel electrophoretic data.

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