Isolation and Characterization of the Major Fraction (12 S) of Linseed Proteins

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The major protein of linseed, accounting for 66% of the total proteins, was isolated to homogeneity by gel filtration on Sepharose 6B. The protein has an $s_{20,w}$ value of 12 and contained less than 0.5% carbohydrate and no phosphorus. It has an absorption maximum at 280 nm and fluorescence emission maximum at 320 nm. Circular dichroism studies revealed the protein contained 3% α-helix, 17% β-structure, and the rest aperiodic structure. The 12 S protein showed five nonidentical subunits on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and six subunits each on urea–PAGE in acid and alkaline systems. The intrinsic viscosity value was 3.1 mL/g. Molecular weight estimated by the Archibald method and sedimentation–diffusion measurements was around 294 000. The protein was found to dissociate at acid pH and in low ionic strength buffers.

Linseed (*Linum usitatissimum*) is one of the five major oilseed crops of India. Presently in this country, linseed is crushed by expellers and the deoiled meal contains 25–35% protein. The feed use of linseed meal is limited to ruminants and nonruminants are unable to utilize it due to various antinutritional factors (Madhusudhan, 1984). Our investigations aim at extending it to feed and food uses. We reported in an earlier communication (Madhusudhan and Singh, 1983), a process of demucilaging and dehulling of linseed to produce a demucilaged, dehulled, and defatted linseed meal. The physicochemical characteristics of the total proteins were also studied. The sedimentation velocity pattern of linseed proteins showed that the major protein fraction accounted for nearly 66% of the total proteins. Therefore, it is of interest to isolate this protein and study it in greater detail. This paper describes a method for the isolation of the major fraction of linseed proteins in a homogeneous form and its physicochemical characteristics.

MATERIALS AND METHODS

Materials. Linseed, Khategaon variety, was purchased from M/s Flour and Foods Ltd., Indore, India.

Sepharose 6B and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals, Sweden. Comassie Brilliant Blue R 250, tris(hydroxymethyl)aminomethane, amido black, bovine serum albumin, ovalbumin, pepsin, trypsin, soybean trypsin inhibitor, ribonuclease, *N,N'*-methylenebis(acrylamide), *N*-bromosuccinamide, and 5,5'-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co. Acrylamide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Koch-Light Laboratories, U.K. β-Mercaptoethanol was from Fluka, Switzerland. Sodium dodecyl sulfate (SDS) was recrystallized twice from 50% ethanol.

Methods. Defatted linseed meal was prepared as described earlier (Madhusudhan and Singh, 1983).

Isolation of Total Proteins. Five grams of defatted linseed meal was extracted with 25 mL of 1 M NaCl for 1 h and centrifuged at 6000 rpm for 30 min and the clear supernatant was extensively dialyzed against 0.05 M phosphate buffer, pH 7.6.

Isolation of the Major Protein. Twenty grams of defatted linseed meal was stirred with 300 mL of water for 1 h at room temperature (~28 °C) and centrifuged at 1160*g* for 10 min. The supernatant was set aside and the residue was again extracted twice with 200 mL of water for 1 h each and the water solubles discarded. The residue was finally extracted with 50 mL of 0.05 M phosphate buffer, pH 7.6, containing 1 M NaCl for 1 h, and centrifuged at 1160*g* for 20 min. Ammonium sulfate (20%, w/v) was added to the supernatant and the slurry centrifuged at 2500*g* for 20 min. The pellet was dissolved in 5 mL of 0.05 M phosphate buffer, pH 7.6, containing 0.02% NaN_3, and dialyzed against the same buffer. The protein solution was loaded on a preparative column of Sepharose 6B (2.5 × 93 cm) and fractions corresponding to the major peak were pooled and concentrated. All measurements were done in 0.05 M phosphate buffer, pH 7.6.

Protein Concentration. Protein analysis was done by measuring the absorbance of the protein solution at 280 nm; $E_{1\,cm}^{1\%}$ of the protein was established by plotting the absorbance at 280 nm vs. protein concentration by micro-Kjeldahl method. A factor of 6.25 was used for converting the nitrogen content to protein.

Carbohydrate and Phosphorus Estimation. The carbohydrate and phosphorus contents of the protein were estimated by the methods of Montgomery (1961) and Taussky and Shorr (1953), respectively.

Amino Acid Analysis. Protein was hydrolyzed in ampules sealed under vacuum with 6 N HCl containing 0.1% phenol and 0.01% β-mercaptoethanol according to Moore and Stein (1963) at 110 °C for 24, 48, and 72 h. The acid was evaporated under reduced pressure and the amino acid analysis was done with a Yanagimoto LC-5S automatic amino acid analyzer. Tryptophan content was estimated by the method of Spande and Witkop (1967) by using *N*-bromosuccinamide. Tyrosine was also estimated by following the phenolic ionization with increasing pH (Donovan, 1973).

Gel Filtration. This was carried out with a Sepharose 6B column (1.8 × 90 cm) and equilibrated with phosphate buffer. Protein (70 mg) was loaded on the column and eluted with the buffer. Fractions of 3 mL were collected in an automatic fraction collector and the absorbance of the fractions was monitored at 280 nm.

Ion Exchange Chromatography. This was carried out with DEAE-Sephadex A-50 packed into a column (1.8 × 26 cm) equilibrated with phosphate buffer. Protein (100 mg) was loaded on the column and eluted with a linear gradient of 0–0.6 M NaCl in buffer. Fractions of 4 mL were collected in an automatic fraction collector and the absorbance of the fractions was monitored at 280 nm. NaCl concentration was estimated by Mohr's method (Kolthoff and Stenger, 1947).

Polyacrylamide Gel Electrophoresis (PAGE). Electrophoresis was performed in 7.5% gels for testing

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0021-8561/85/1433-0673$01.50/0

homogeneity or dissociation studies in phosphate or tris-glycine buffers. SDS–PAGE was done in 10% gels by the method of Laemmli and Favre (1973) for studying the subunit composition. The molecular weight markers used were bovine serum albumin, ovalbumin, pepsin, trypsin, soybean trypsin inhibitor, and ribonuclease. Urea–PAGE was performed at 4 °C in 10% separating gels at pH 4.5 and 8.7 by a modification of the procedure of Davis (Eipper, 1974). The gels contained 8 M urea and the protein was dissolved in the respective buffers containing 8 M urea prior to electrophoresis. The gels were fixed in 7.5% acetic acid whenever necessary and stained with Coomassie Brilliant Blue R 250 and destained in a mixture of isopropyl alcohol–acetic acid–water (50:75:875). The gels were scanned in a Joyce Loebel Chromoscan 200.

Sedimentation Velocity. These experiments were performed at 56 100 rpm in a Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics and rotor temperature indicator and control (RTIC) system at room temperature (~28 °C). For all the experiments, a 4°, 12-mm duraluminium cell center piece and a 1% protein solution were used. $s_{20,w}$ values were calculated by standard procedure (Schachman, 1959).

Absorption and Fluorescence Spectra. The absorption and fluorescence spectra of the protein were recorded in a Perkin Elmer double beam recording spectrophotometer Model 124 and Perkin Elmer fluorescence spectrofluorimeter Model 203, respectively, at 28 °C.

Circular Dichroism (CD). These measurements were made at room temperature (~28 °C) in a JASCO-J20C automatic recording spectropolarimeter and the instrument was calibrated with d-10 comphorsulfonic acid and the slits were programmed to yield 1-nm band width at each wave length.

Near UV–CD measurements were made with cells of 1-cm path length and protein (1.2–2.0 mg/mL) in phosphate buffer in the range 250–330 nm. Far UV–CD measurements were made with cells of 0.5- or 1.0-mm path length and protein (0.5–0.8 mg/mL) in phosphate buffer in the wavelength range, 200–260 nm. Mean residue ellipticity $[\theta]_{mrw}$ (deg cm^2/dmol) was calculated by the method of Adler et al. (1973), assuming a value of 115 for mean residue weight.

Viscosity Measurement. Reduced viscosity (η_{red}) measurement was done at 30 ± 0.1 °C in a Ostwald Viscometer having a flow time of 182 s with distilled water. Protein solution in buffer was filtered through a 0.45-μm filter before introduction into the viscometer and the flow time was recorded to ±0.1 s with a stop watch. Intrinsic viscosity, $[\eta]$, was determined by plotting $[\eta - \eta_0]/\eta_0 C$ vs. C where η_0 is the viscosity of the solvent, η is the viscosity of the solution, and C is the concentration of protein in g/100 mL (Bradbury, 1970).

Molecular Weight and Related Parameters. The molecular weight of the protein was estimated by the Archibald method, sedimentation–diffusion and viscosity measurements. The Archibald run was performed by the method described by Narasinga Rao (1961) and the synthetic boundary run was performed by the method described by Chervenka (1973); the speeds used for these runs were 5600 and 6800 rpm, respectively. A 1% protein solution was used for these measurements.

RESULTS AND DISCUSSION

The homogeneity of the isolated protein was determined by the techniques of gel filtration, ion exchange chromatography, PAGE, and ultracentrifugation. In gel filtration, the protein gave a single symmetrical peak eluting at V_e/V_o of 1.89 (Figure 1 part A). This corresponds to the major

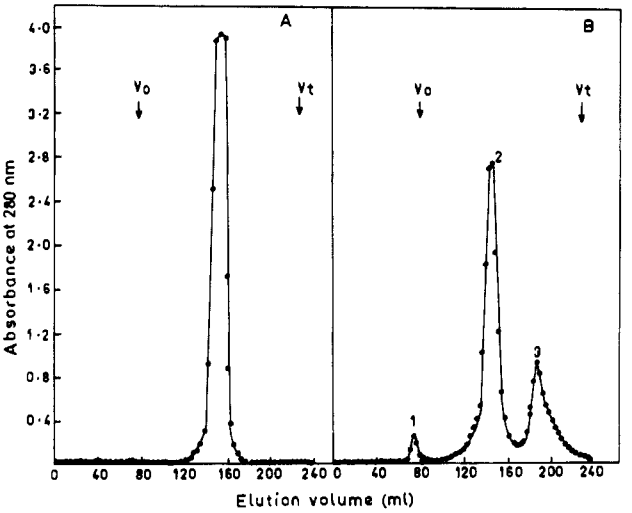

Figure 1. Gel filtration pattern of linseed 12 S protein on Sepharose 6B (0.05 M phosphate buffer, pH 7.6; column, 1.8 × 90 cm). (A) 12 S protein. (B) Total proteins (similar to Madhusudhan and Singh, 1983).

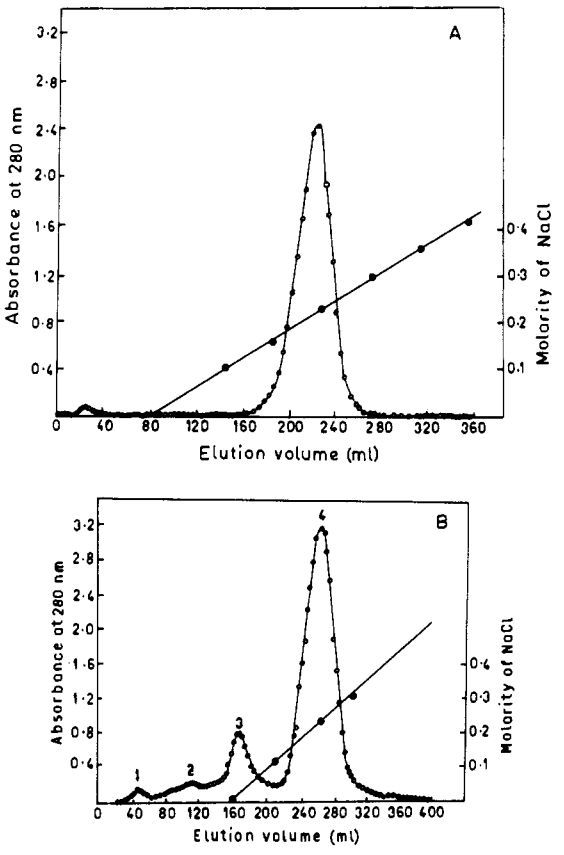

Figure 2. DEAE-Sephadex chromatographic pattern of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6; column, 1.8 × 26 cm). (A) 12 S protein. (B) Total proteins (similar to Madhusudhan and Singh, 1983).

peak (peak 2) in total proteins (Figure 1 part B).

In DEAE-Sephadex chromatography, a single symmetrical peak eluting at a salt concentration of 0.24 M NaCl was obtained. Total proteins gave four peaks eluting at 0 M (peak 1 and 2), 0.005 M (peak 3), and 0.24 M (peak 4) NaCl concentration; the isolated protein and the major peak of total proteins (peak 4) eluted at 0.24 M NaCl concentration (Figure 2 parts A and B).

Electrophoresis of the protein gave a band as against three bands obtained with total proteins in phosphate buffer (Figure 3 parts A and B).

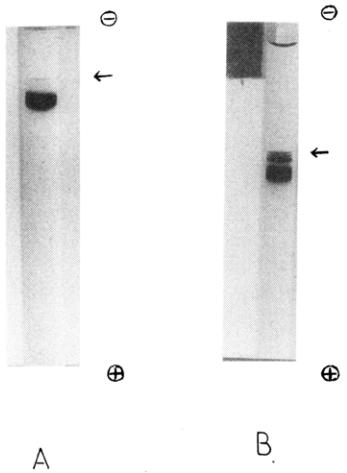

Figure 3. Polyacrylamide gel electrophoresis pattern of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6; 7.5% gels). (A) 12 S protein. (B) Total proteins.

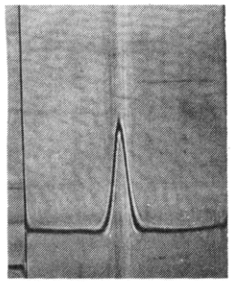

Figure 4. Sedimentation velocity pattern of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6). Photograph taken 32 min after attainment of a speed of 56 100 rpm. Bar angle 70°. Sedimentation proceeds from left to right.

A sedimentation velocity experiment showed a single peak (Figure 4) with $s_{20,w}$ value of 12. Thus, the protein appears to be homogeneous by the above physicochemical techniques. The protein contained less than 0.5% carbohydrate and no phosphorus.

The amino acid composition of the 12 S protein is shown in Table I. The protein contained a high amount of aspartic acid (11.3 g/16 g of N), glutamic acid (19.8 g/16 g of N), and arginine (12.5 g/16 g of N) like the globulins of other oilseed proteins (Prakash and Nandi, 1978; Gururaj Rao, 1980; Schwenke et al., 1979). It appears that the lysine content of this protein was higher than α-globulin, arachin, and helianthinin, and arginine was higher than glycinin, helianthinin, and rapeseed 12 S protein (Badley et al., 1975; Shetty, 1975; Prakash and Nandi, 1978; Schwenke et al., 1979, 1981). From the spectrophotometric titration of protein in the pH range 9–12.5, the number of tyrosine residues ionized at various pH values were calculated. This showed a gradual increase in absorbance below pH 10.5 and above this, there was a steep increase. At pH 12.5, the curve attained a plateau region and nearly 42 phenolic groups were titrated at this pH. This value agrees with the data from the amino acid composition.

The $E_{1\,cm}^{1\%}$ of the 12 S protein was 7.6 in phosphate buffer. The absorption spectrum of the protein (Figure 5) is typical of a protein with the absorption maximum at 280 nm and minimum at 250 nm. The 280 to 260 ratio was 1.64 suggesting the abscence of nucleic acid impurities (Layne, 1957) in the preparation. This was supported by the fact that the protein contained no phosphorus. The fluorescence emission spectrum showed a maximum at 320 nm when excited at 280 nm and this could be due to the

Table I. Amino Acid Composition of Major Protein (12 S) of Linseed

amino acid	g/16 g of N	no. of residues/ mol protein[a]
Asp[b]	11.3	289
Thr[c]	3.9	113
Ser[c]	5.1	172
Glu[d]	19.8	451
Pro	4.5	136
Gly	4.8	247
Ala	4.8	199
Val	5.6	166
$^1/_2$ Cys[c]	1.4	18
Met[c]	1.7	38
Ile	4.6	120
Leu	5.8	151
Tyr	2.3	41
Phe	5.9	118
Lys	3.1	71
His	2.5	54
Arg	12.5	235
Try[e]	1.3	20

[a] A molecular weight of 294 000 determined by the Archibald method was used in calculation. [b] Includes aspargine. [c] Values are at 0 h of hydrolysis by extrapolating 24, 48, and 72 h of hydrolysis values. [d] Includes glutamine. [e] Estimated by the method of Spande and Witkop (1967).

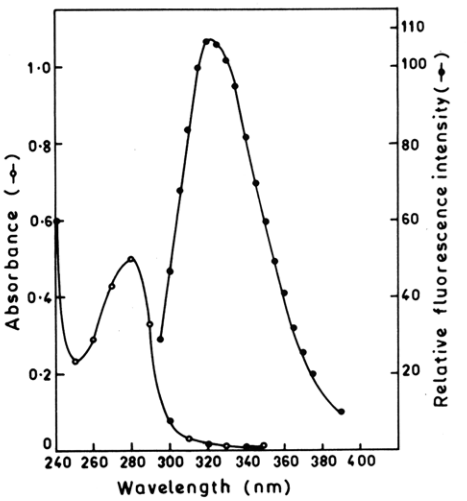

Figure 5. Ultraviolet absorption and fluorescence emission spectrum of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6).

buried tryptophan groups in the apolar environment of the protein (Shifrin et al., 1971; Mills and Creamer, 1975).

The near UV–CD spectrum in the wavelength range 250–330 nm showed positive peaks at 284 and 290 nm, a shoulder at 275 nm, and negative peaks at 259 and 265 nm (Figure 6). The major peak at 284 nm and the minor peak at 290 nm probably originate from tyrosine and tryptophan residues, respectively. The band at 302 nm was assigned to tryptophan and might arise from an assymmetric environment around tryptophan residues (Strickland, 1974). The amino acid composition of the 12 S protein showed 2.3 g of tyrosine and 1.3 g of tryptophan per 16 g of N. The far UV–CD spectrum exhibited a trough at 207 nm and a shoulder at 215 nm in the scan range 200–260 nm (Figure 7). The spectrum is characterized by a major negative band. As is evident, no fine structure is present in the spectrum, indicating probably the dominance of aperiodic and β-pleated structure. The helical content computed from the methods of Greenfield and Fasman (1969) and Chen and Yang (1971) were 4% and 3%, respectively. The proportion of β-structure was estimated to be 17% by the

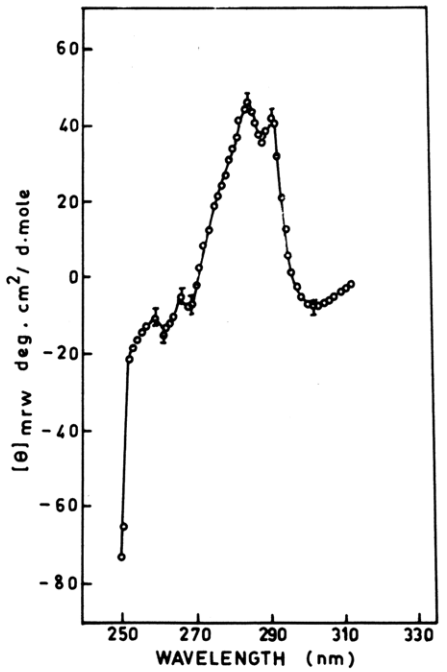

Figure 6. Near UV–CD spectrum of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6).

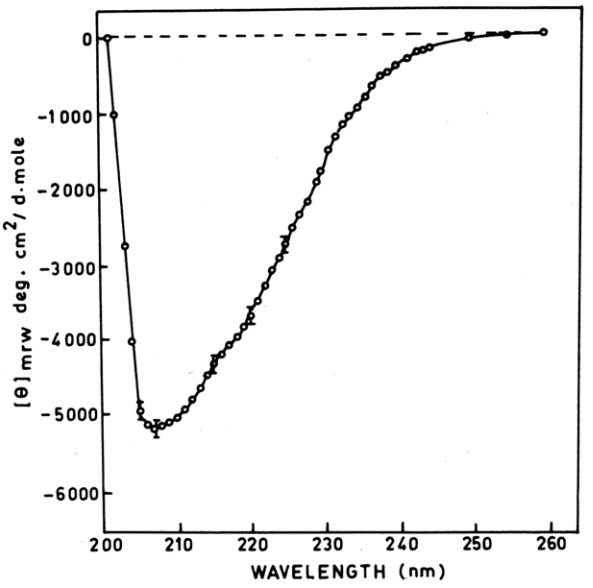

Figure 7. Far UV–CD spectrum of linseed 12 S protein (0.05 M phosphate buffer, pH 7.6).

method of Sarkar and Doty (1966). The low percentage of α-helical regions and the CD bands arising principally from β-pleated sheet and aperiodic structure in our present investigation are in general agreement with CD spectra of other major storage proteins of oilseeds (Prakash and Narasinga Rao, 1984).

SDS–PAGE showed that the protein contained at least five nonidentical subunits with molecular weights of 11 000, 18 000, 29 000, 42 000, and 61 000 (Figure 8). The proportion of the bands differed as is obvious and the bands corresponding to the molecular weights 18 000 and 42 000 were more intense. Hence, no attempt was made to calculate molecular weight from the data. Urea–PAGE at acidic and alkaline systems showed six subunits each (Figure 9 parts A and B). From the mobility of the bands, the protein is assumed to contain one acidic, two neutral, and three basic subunits. The intrinsic viscosity of the 12 S protein in phosphate buffer was 3.1 mL/g. Globular

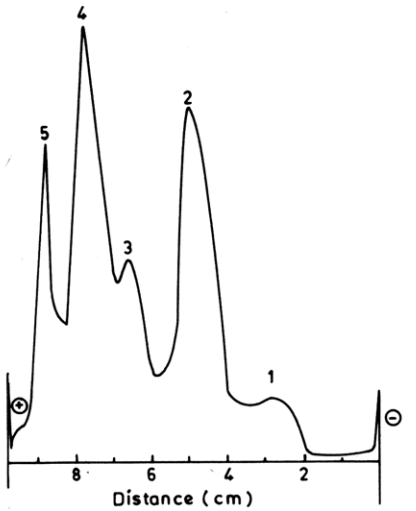

Figure 8. Densitometric tracings of SDS–PAGE of linseed 12 S protein. Molecular weight of subunits: 1, 61 000; 2, 42 000; 3, 29 000; 4, 18 000; 5, 11 000 (10% gels).

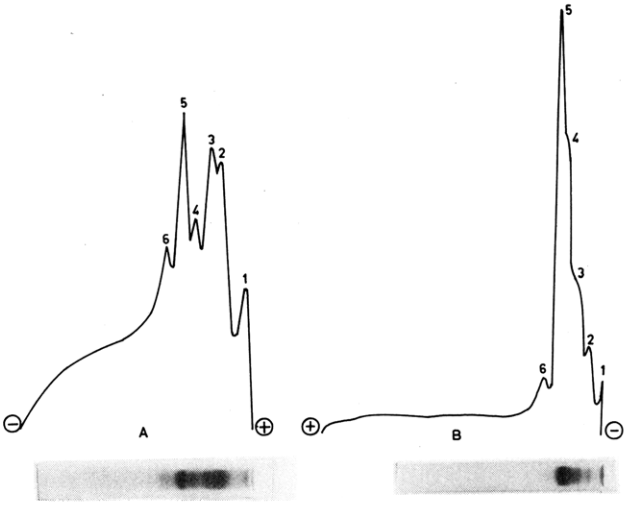

Figure 9. Densitometric tracings of urea–PAGE of linseed 12 S protein in acid and alkaline systems (10% gels). (A) 0.07 M β-alanine–acetate buffer, pH 4.5. (B) 0.01 M Trisglycine buffer, pH 8.7.

Table II. Molecular Weight and Related Data of the Major Protein (12 S) of Linseed[a]

parameter	value
diffusion coefficient, $D_{20,w}$	3.7×10^{-7} cm²/s
molecular weight	
Archibald method	294 000
from viscosity data	252 000
from sedimentation–diffusion	298 000
Stoke's radius (r)	58.31×10^{-8} cm
frinctional ratio (f/f_o)	1.322

[a] Partial specific volume ($\bar{V}$) was assumed as 0.73.

proteins generally have values in the range 3.0–4.0 mL/g (Tanford, 1961). The molecular weight and other parameters are shown in Table II. From the values, the difference in molecular weights obtained by viscosity and other measurements is evident. Calculation of molecular weight from viscosity is subject to various limitations (Tanford, 1961). The value of 1.322 for f/f_o suggests a globular conformation for the protein when compared with the values for standard proteins (Cantor and Schimmel, 1980).

Dissociation Studies. While checking the homogenity of 12 S protein, PAGE was performed in alkaline and

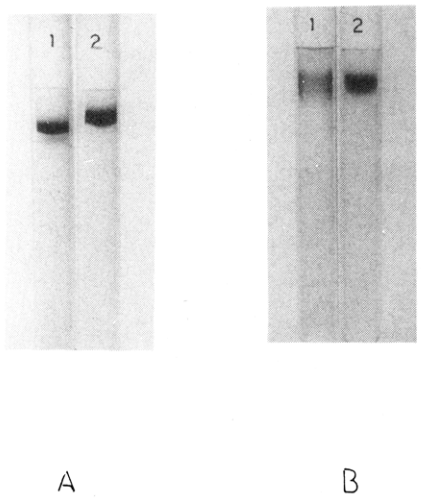

Figure 10. PAGE pattern of linseed 12 S protein in trisglycine and phosphate buffer systems. (A) Trisglycine buffer, pH 8.3: 1, 0.025 M; 2, 0.15 M. (B) Phosphate buffer, pH 7.6: 1, 0.0083 M; 2, 0.05 M.

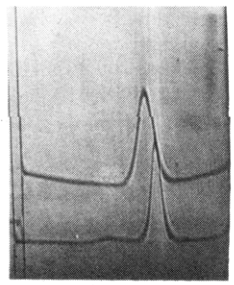

Figure 11. Sedimentation velocity pattern of linseed 12 S protein in trisglycine buffer. Top: 0.025 M trisglycine buffer containing 1 M NaCl, pH 8.3. Bottom: 0.025 M trisglycine buffer, pH 8.3. Photographs taken 52 min after attainment of 56 100 rpm. Bar angle 70°. Sedimentation proceeds from left to right.

acidic buffers at pH 8.3 and 3.6, respectively. Electrophoresis could not be performed at pH 4.5 due to the precipitation of protein at this pH. When PAGE was performed in 0.025 M trisglycine buffer, pH 8.3, two bands were observed (Figure 10 part A), whereas at acidic pH in 0.05 M β-alanine–acetate buffer, pH 3.6, PAGE yielded a number of bands. Both these buffers are of low ionic strength compared to that of 0.05 M phosphate buffer, pH 7.6, with μ 0.15. In 0.05 M phosphate buffer, the protein gave a single band. To determine whether this behavior was due to ionic strength, PAGE of the 12 S protein was performed in trisglycine buffer of pH 8.3 and μ 0.15 and a single band was obtained (Figure 10 part A). Also, in phosphate buffer of low ionic strength, μ 0.025, two bands were obtained (Figure 10 part B). From these results, it may be concluded that low ionic strength has an effect on the integrity of the 12 S protein, yielding more than one band at low ionic strength and a single band at higher ionic strengths.

Confirmatory evidence was obtained from sedimentation velocity experiments in 0.025 M trisglycine buffer, pH 8.3, without and with 1 M NaCl. In the absence of NaCl, the 12 S protein dissociated into 10 S and 7 S components (Figure 11), the proportion of these being 90% and 10%, respectively. From the above experiments, it could be concluded that the oligomeric protein dissociates at low ionic strength and such an effect has been reported in soybean, sunflower, and rapeseed (Wolf and Briggs, 1958; Schwenke, 1975; Schwenke et al., 1983). The major protein on PAGE at pH 3.6 revealed a number of bands, possibly due to dissociation. Dissociation of oligomeric seed proteins at acid pH has been reported in various oilseeds (Prakash and Nandi, 1977; Kishore Kumar Murthy and Narasinga Rao, 1984; Srinivas, 1984; Sripad, 1984).

ACKNOWLEDGMENT

We thank Dr. M. S. Narasinga Rao for his valuable suggestions and Dr. T. Ogawa for the amino acid analysis. One of us (K.T.M.) thanks the Council of Scientific and Industrial Research, New Delhi, for the award of a fellowship.

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Received for review October 4, 1984. Accepted March 4, 1985.