

A Rapid Procedure for Isolation of the 7S Storage Protein of Cottonseed and Its Characterization¹

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ABSTRACT

Cereal Chem. 61(6): 471-474

A rapid, simple procedure for isolating the 7S component of storage proteins of cottonseed in quantity and uncontaminated by other storage proteins was developed. This procedure, which takes advantage of the solubility characteristics of the storage proteins as a function of ionic strength and temperature, eliminates the lengthy dialysis, concentration, and lyophilization steps of previous methods and thus avoids subjection of the protein to extreme changes in environment, including pH. The storage proteins are first selectively extracted in Tris-NaCl (pH 7.0) solution, following prior removal of the nonstorage proteins by successive

extractions with water. The 12 S and larger storage proteins are then selectively precipitated by 1:3 dilution of the Tris extract with water. The 7S protein is concentrated by cold precipitation (0°C) and purified by gel filtration in the original extracting solution, 0.5 M Tris-0.5 M NaCl (pH 7.0). The entire procedure, including one purification by gel filtration, can be completed within 24 hr. Though essentially pure by most criteria (ultracentrifugation, gel electrophoresis), isoelectric focusing on polyacrylamide gels suggests that the 7S component consists of eight components differing in charge.

In the last decade, the high protein content of cottonseed has prompted many investigations of its flour and protein isolates as food ingredients for human consumption. A more detailed understanding of the structure of the individual proteins is needed to better utilize their functional properties. Cottonseed flour causes a serious color problem in most food systems, whereas the storage proteins of cottonseed, when isolated at a neutral pH, are virtually colorless. The storage proteins also constitute the major fraction of cottonseed proteins (Blouin et al 1981). These proteins consist of two major components and several minor components (Zarins and Cherry 1981). The major proteins were characterized by their sedimentation coefficients 7S and 12S by Martinez (1979).

Rossi-Fanelli et al (1964) were the first to isolate a monodisperse globulin from cottonseed, which they called Acalin A. This is the 7S protein. They reported a molecular weight of 180,000, determined by light scattering. Ovchinnikova et al (1980) published a series of papers on a 7S storage protein, isolated by cold precipitation, from cottonseed variety 108F. Ovchinnikova et al (1975) determined the molecular weight by gel filtration and ultracentrifugation and reported respective values of 125,900 and 130,000. Kuchenkova et al (1977) determined the structure and amino acid sequence of the 7S protein, resulting in a molecular weight of 128,000. Zarins and Cherry (1981) determined the molecular weight of the 7S protein by gel filtration on Sephadex G-200 and reported a molecular weight of 127,000.

The current procedures used to isolate the individual globulins are time-consuming and cumbersome. A new procedure for isolation of the 7S globulin in appreciable quantity followed by purification by gel filtration is reported.

MATERIALS AND METHODS

Preparation of the 7S Protein

Hexane-defatted, glandless cottonseed flour (Southern Regional Research Center) was extracted twice with water to remove the nonstorage proteins. The storage proteins were then obtained by extraction with a solution containing 0.5 M Tris(hydroxymethyl)aminomethane (Tris), 0.5 M NaCl, and 0.2 g/L NaN₃, adjusted to pH 7.0.

The 12S and larger proteins were precipitated by a 1:10 dilution of this extract with distilled water and removed by centrifugation. The diluted extract was then chilled to 0°C, whereupon a precipitate consisting of about 88% of the 7S protein formed. The mixture was centrifuged at 0°C, the supernatant discarded, and the precipitate redissolved in the 0.5 M Tris-0.5 M NaCl solution (pH 7.0).

Purification of the 7S Protein

The 7S protein was further purified by gel-filtration chromatography at ambient temperature in a 10×90-cm column of Sephadex G-200 (Fig. 1A); 0.5 M Tris-0.5 M NaCl solution (pH 7.0) was used as an eluent. A 7S protein (400–600 mg) sample was injected and eluted upward at 200 ml/hr. The column eluent was monitored at 280 nm and collected in 70-ml fractions. Fractions from the 7S peak were pooled, concentrated by ultrafiltration, and rechromatographed two to three times on Sephadex G-200 until a single symmetrical peak was obtained. The 7S protein used for molecular weight determination, isoelectric focusing, and dissociation studies was purified in a 2.6×95-cm column.

For comparison, the storage proteins were extracted with 0.2 M NaHCO₃-Na₂CO₃ (pH 10.5) after preextraction of the cottonseed flour three times with water. The 7S protein, called 7S' to distinguish it from the 7S protein isolated under neutral conditions, was isolated from the alkaline extract on the same column, except that 0.2 M NaHCO₃-Na₂CO₃ (pH 10.5) was used as eluent (Fig. 1B). The third peak eluted had the same K_{av} 0.32 (partition coefficient) as the 7S component isolated in neutral solution. It was necessary to rechromatograph the 7S' component three to five times to obtain a single symmetrical peak.

Ultracentrifugation

Ultracentrifugation patterns were obtained with a Spinco ultracentrifuge model E, equipped with Schlieren optics, using the analytical rotor at a speed of 59,780 rpm, at 24.6°C in a standardized cell. The solvent was 0.5 M Tris-0.5 M NaCl 0.2 g/L NaN₃ (pH 7.0). The sedimentation coefficient was calculated from the Schlieren pattern and corrected to $S_{20,w}$ by the method of Svedberg and Pederson (1940).

Viscosity, Density, and Partial Specific Volume Determinations

Viscosity was determined at 24.6°C in a modified Ostwald Cannon Fenske ASTM viscometer. Density and partial specific volume were both determined by use of an Ostwald-Sprengel pycnometer.

Stokes Radius Determination

Ribonuclease, myoglobin, ovalbumin, bovine serum albumin, human transferrin, aldolase, and type I bovine thyroglobulin were used as Stokes radius markers. Stokes radius for the 7S protein was

¹This work supported in part with funds from Cotton Incorporated and the National Cottonseed Products Association.

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calculated from the gel-filtration data of Table I by the methods of Porath (1963), and Laurent and Killander (1964).

Calculation of Molecular Weight

The molecular weight of the 7S protein was calculated from the formula of Siegel and Monty (1966):

$$M = 6\pi\eta N a s / (1 - \bar{v}\rho)$$

The parameters of the 7S protein obtained at 24.6°C in 0.5M Tris-0.5M NaCl 0.2 g/L NaN₃ (pH 7.0) were as follows: η = viscosity of medium 1.1086×10^{-2} poises; N = Avogadro's number; a = Stokes radius 4.52×10^{-9} cm (from correlation of Porath); 4.48×10^{-9} cm (from correlation of Laurent and Killander); s = sedimentation coefficient (Tris 24.6°C) 5.88×10^{-13} sec; \bar{v} = partial specific volume 0.738 g/ml; ρ = density of solvent 1.0417 g/ml; and M = molecular weight.

Amino Acid Analysis

Amino acid analysis was accomplished as described by Zarins and Cherry (1981).

TABLE I
Molecular Parameters of Standard Proteins and 7S Protein

Protein	(-log K _{AV}) ^{1/2} ^a	K _d ^{1/3} ^b	Stokes Radius Å
Myoglobin	0.3998	0.8938	18.6
Ribonuclease	0.4051	0.8909	19.2
Ovalbumin	0.5274	0.8163	27.3
Bovine serum albumin	0.6012	0.7657	36.0
Transferrin	0.6369	0.7399	40.2
Aldolase	0.7258	0.6744	46.1
7S Protein	0.7044	...	44.8 ^c
7S Protein	...	0.6904	45.2 ^b

^aFrom Laurent and Killander (1964); Stokes radius = $16.82 + 87.5(-\log K_{AV})^{1/2}$ r = 0.995.

^bFrom Porath (1963); Stokes radius = $135.69 - 131.09 K_d^{1/3}$ r = 0.995.

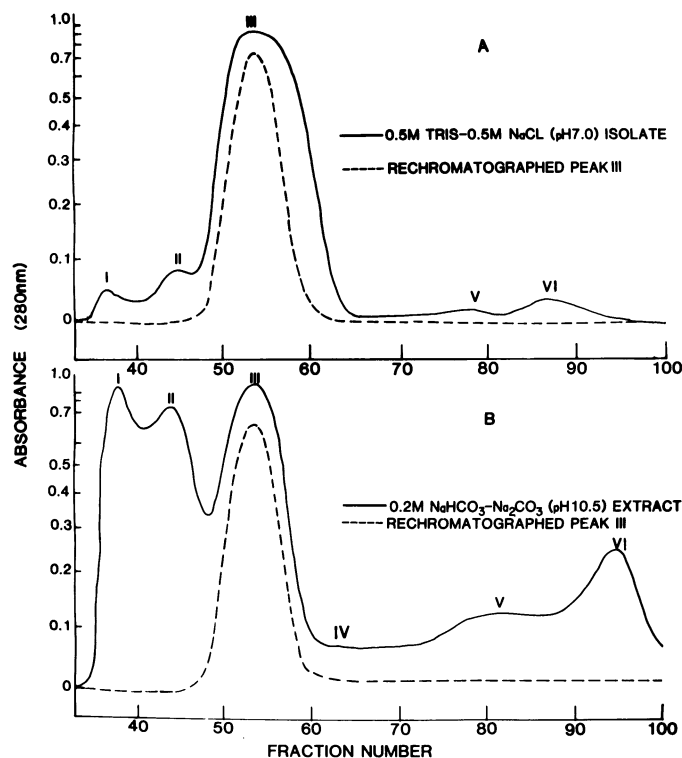


Fig. 1. Sephadex G-200 gel-filtration chromatography of cottonseed protein isolates. A, cryoprecipitated 0.5M Tris-0.5M NaCl (pH 7.0) isolate; B, 0.2M NaHCO₃-Na₂CO₃ (pH 10.5) isolate. --- = rechromatographed 7S protein.

Thin-Layer Gel Filtration (TLG)

Thin-layer gel filtration was conducted on 20 × 40 cm, 0.6-mm thick plates coated with Sephadex G-200. The samples (10 μl of approximately 1% protein solutions) were eluted with the dissociating solutions of the 7S proteins. The procedure used for detecting the spots and plotting migration distances was described by Zarins and Cherry (1981). The same proteins used for Stokes radius markers were used as molecular-weight markers for TLG. In addition, chymotrypsinogen A was used as a marker. Glycoproteins were detected by treating the dried filter paper replicas with periodic acid and Schiff's stain by Kohn's method (Kohn 1960).

Gel Electrophoresis

Gel electrophoresis was performed in cylindrical 7.5% polyacrylamide gels. Samples (0.05 ml) contained 20% sucrose and approximately 6 mg/ml of protein. The electrode compartments were filled with 0.2M citric acid. Power was applied by means of an Ortec 4100 pulsed constant-power supply. Electrophoresis was started (1 hr) at 300 V and 50 pulses per second (pps). The pulse rate was then increased to 100 for 2 hr. The gels were stained with 0.025% Coomassie Blue R-250 in acetic acid-methanol-water (7:20:100; v/v/v) solution containing 6% trichloroacetic acid.

Dissociation

The 7S and 7S' proteins were subjected to dissociating conditions by dialyzing the 7S protein in the original solution against 0.2M citric acid containing 1 ml/L of octanoic acid.

Dissociation of the 7S protein in sodium dodecyl sulfate (SDS) solution was accomplished by diluting the protein (15 mg/ml) which was in 0.5M Tris-0.5M NaCl (pH 7.0) with 2.7% SDS in distilled water (v/v, 1:4). This reduction of salt concentration was necessary to retain the protein-SDS complex in solution. This solution was divided into two parts. One part was dialyzed against 0.125M Tris-0.125M NaCl solution containing 2% SDS and 0.2 g/L NaN₃. To the other part, a quantity of β-mercaptoethanol was added so the resultant solution, after dialysis against the same dialysate as above, contained 0.37M β-mercaptoethanol.

In all cases, dialysis was performed for 32 hr at ambient temperature; the dialysate solution was changed six times.

Isoelectric Focusing

Isoelectric focusing was done in cylindrical gels (7 × 80 mm). The gel formula consisted of: 5% w/v acrylamide, 2% crosslinking

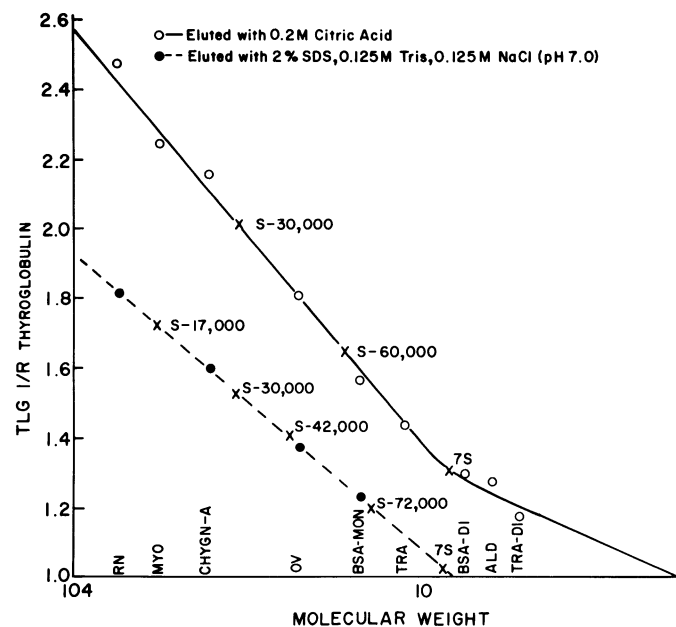


Fig. 2. Plots of marker proteins and 7S subunits based on thin-layer gel filtration for determination of molecular weights of subunits of the 7S protein formed on (—) acid dissociation and (---) SDS dissociation.

(bis); 20% w/v sucrose; 2.5% w/v pH 5–8 Ampholites; 0.05% w/v ammonium persulfate; and 0.06% w/v DMAPN (β -dimethylaminopropionitrile).

The samples were dialyzed against dilute NaOH (pH 10.5) to remove the salts, and 20% w/v sucrose and 10% w/v pH 7–10 carrier ampholyte were then added to the samples.

The power supply used was an Ortec 4100 pulsed constant-power supply. The gels were prefocused for 2 hr at 50 V and 100 pps. The samples (0.25 mg protein per gel) were then layered on top of the gel at the cathode end. The focusing was continued at 50 V for 4 hr, then at 100 V for 16 hr, and finally (to sharpen the bands) at 200 V for 1 hr. The 7S protein precipitated at its isoelectric point, revealing eight white bands in the gel. The bands were excised and soaked in 0.01 *N* KCl solution for 30 min, then the pH was measured. Gels were also scanned in a Schoeffel spectrodensitometer, after the ampholites were removed by soaking the gels overnight in 15% trichloroacetic acid. The proteins were then stained with Coomassie Blue R-250 and scanned again in the spectrodensitometer.

Extinction Coefficient

The extinction coefficient was determined by resuspending accurately weighed, dialyzed, lyophilized 7S protein (moisture-free basis) in the 0.5 *M* Tris–0.5 *M* NaCl solution at five different concentrations and measuring the absorbance at 280 nm by use of a Beckman model 25 spectrophotometer. A value of $E_{1\%}^{1\text{cm}} = 5.95$ was obtained. This value was used to determine the

concentration of the 7S component applied to polyacrylamide gels and TLG plates.

RESULTS AND DISCUSSION

The isolate obtained by cryoprecipitation of the 7S protein in the 0.5 *M* Tris–0.5 *M* NaCl (pH 7.0) solution (Fig. 1A) was separated by gel filtration in a Sephadex G-200 column into four minor peaks and one major peak, the 7S protein. The isolate obtained by extraction of cottonseed flour with 0.2 *M* NaHCO_3 – Na_2CO_3 (pH 10.5) buffer (Fig. 1B) was separated into three major and three minor peaks (peak IV obscured by background absorption). The dashed line in both figures represents the rechromatographed, isolated 7S and 7S' protein, respectively. Molecular weight of the 7S protein was calculated from the formula of Siegel and Monty (1966), using values of the Stokes radius obtained by means of the Porath (1963) correlation (144,710 daltons), and the Laurent and Killander (1964) correlation (143,421 daltons). Sedimentation coefficient, corrected to the standard state of water ($S_{20,w}$), was 7.39. The 7S protein isolated by the two methods had identical amino-acid composition and electrophoretic mobility and showed identical patterns on isoelectric focusing. However, the 7S protein did not dissociate as readily as did the 7S' in acid solutions. Chromatography of the two proteins, dialyzed against 0.2 *M* citric acid on Sephadex G-200 TLG plates developed with 0.2 *M* citric acid, showed that the 7S protein dissociated only slightly. The major portion remained in the undissociated state; there were two weak, diffuse spots due to subunits. The dissociation pattern of the 7S' protein showed two fairly well-defined spots of molecular weights 30,000 and 60,000 (Fig. 2). Figure 3 shows representative results of the 7S proteins dialyzed against 0.2 *M* citric acid, analyzed by polyacrylamide gel electrophoresis. Sample A (Fig. 3) shows that the major portion of the 7S protein is still in the undissociated state. The two slower moving bands seem to be due to aggregates (possibly formed in the gel). The six bands moving faster than the 7S protein are due to subunits. The 7S' protein (Fig. 3B) appears to

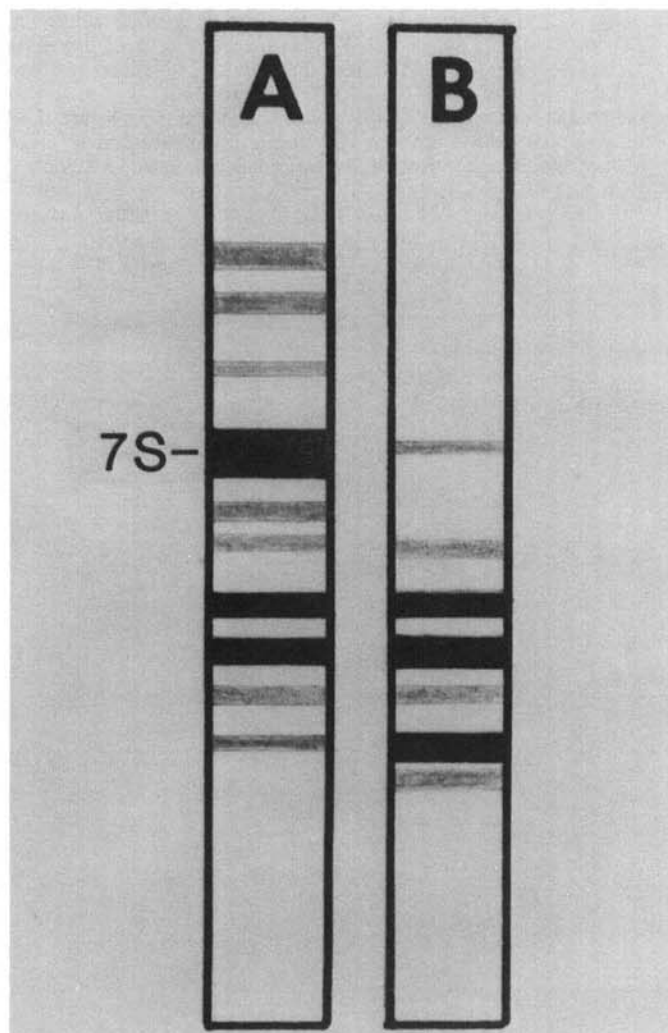


Fig. 3. Gel-electrophoretic patterns of the 7S protein dialyzed against 0.2 *M* citric acid. **A**, isolated from cryoprecipitated isolate in 0.5 *M* Tris–0.5 *M* NaCl (pH 7.0) solution; **B**, isolated from 0.2 *M* NaHCO_3 – Na_2CO_3 (pH 10.5) buffer extract.

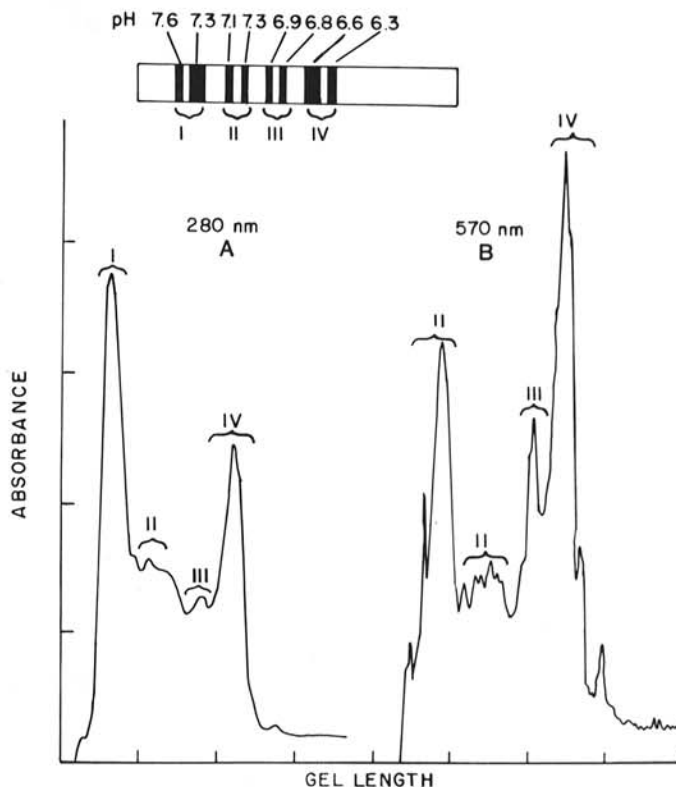


Fig. 4. Disk electrofocusing of the 7S protein in the pH range between 5 and 8. The densitometer tracings were obtained with a Schoeffel model SD 3000 spectrodensitometer. **A**, unstained, scanned at 280 nm; **B**, stained, scanned at 570 nm.

be almost totally dissociated. There is only a trace of a band indicating the undissociated protein; there is no indication of aggregation. The six bands moving faster than the 7S protein are due to subunits. Dialyzing the 7S' protein for 24 hr against the Tris solution, before acid dissociation, did not alter its dissociation pattern; neither did dialyzing the 7S protein against the bicarbonate buffer increase its degree of dissociation in citric acid. During the Tris extraction, the high salt concentration appears to have neutralized the surface charges of the 7S protein molecule, strengthening the intramolecular forces holding the subunits together.

Chromatography of the two 7S proteins on Sephadex G-200 TLG plates eluted with 0.125M Tris-0.5M NaCl containing 2% SDS (pH 7.0) showed identical dissociation patterns. The samples not containing β -mercaptoethanol showed spots corresponding to molecular weights of 17,000, 30,000, 42,000, 72,000, and greater than 100,000 (Fig. 2). This last spot is absent from the samples containing β -mercaptoethanol. Dieckert et al (1981) observed similar subunits of acalin A on SDS polyacrylamide gels, and Ovchinnikova et al (1975) reported similar subunits obtained by dissociating their 7S protein in 8M urea. When the TLG prints were stained with periodic-acid Schiff's reagent, all the spots gave a positive response, indicating that they are glycoproteins. The 7S protein probably consists of basic subunits of molecular weights 15,000-17,000, and the larger subunits consist of two or more of these subunits.

The isoelectric focusing pattern (IEF) (Fig. 4) shows that the 7S protein consists of eight components that appear as four doublets differing in charge with isoionic points ranging from pH 6.3 to 7.6. The storage proteins store nitrogen in the form of glutamine and asparagine. Kuchenkova et al (1977) found that the 7S protein consisted of equal amounts of paired peptides with identical amino-acid sequence; in some cases, however, the corresponding amides were found instead of glutamic and aspartic acid.

CONCLUSION

The cryoprecipitation method gives 7S protein of higher purity than previously published methods. The immediate transfer of the concentrated 7S protein to a preparative Sephadex G-200 column

avoids extreme environmental changes and allows minimum time for the 7S protein to react with other proteins, possibly enzymes.

LITERATURE CITED

- BLOUIN, F. A., ZARINS, Z. M., and CHERRY, J. P. 1981. Protein functionality in foods. J. P. Cherry, ed. Am. Chem. Soc. Symp. Ser. 147. The Society, Washington, DC.
- DIECKERT, J. W., WALLACE, R. W., and DIECKERT, M. C. 1981. Chemistry and biology of the cottonseed globulins. Proc. Beltwide Cotton Production Res. Conf. Page 351. National Cotton Council, Memphis, TN.
- KOHN, J. 1960. Cellulose acetate electrophoresis and immuno-diffusion techniques. Page 56 in: Chromatographic and Electrophoretic Techniques, Vol. 2. I. Smith, ed. Heineman, London.
- KUCHENKOVA, M. A., REDINA, E. F., OVCHINNIKOVA, N. L., YULDASHEV, P. Kh. 1977. The globulins of cottonseed 12. The structure of the 7S globulin. Chem. Nat. Compd. Engl. Transl. 13:570.
- LAURENT, T. C., and KILLANDER, J. 1964. A theory of gel filtration and its experimental verification. J. Chromatogr. 14:317.
- MARTINEZ, W. H. 1979. Functionality of vegetable proteins other than soy. J. Am. Oil Chem. Soc. 56:280.
- OVCHINNIKOVA, N. L., KUCHENKOVA, M. A., and YULDASHEV, P. Kh. 1975. An investigation of the globulins of cottonseeds. 1. Chem. Nat. Compd. Engl. Transl. 10:413.
- OVCHINNIKOVA, N. L., KUCHENKOVA, M. A., and YULDASHEV, P. Kh. 1980. Study of globulins from cottonseeds 23. Isoelectric focusing of the 7S globulin in a borate-polyol system. Khim. Prir. Soedin. 4:577, and references therein.
- PORATH, J. 1963. Some recently developed fractionation procedures and their applications to peptide and protein hormones. Pure Appl. Chem. 6:233.
- ROSSI-FANELLI, A., ANTONINI, E., BRUNORI, M., BRUZZESI, M., CAPUTO, A., and SATRANI, F. 1964. Isolation of a monodisperse fraction from cottonseeds. Biochem. Biophys. Res. Commun. 15:110.
- SIEGEL, L. M., and MONTY, K. J. 1966. Determination of molecular weights and fractional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Applications to crude preparations of sulfite hydroxylamine reductases. Biochim. Biophys. Acta 112:346.
- SVEDBERG, T., and PEDERSEN, K. O. 1940. The Ultracentrifuge. Oxford University Press, New York.
- ZARINS, Z. M., and CHERRY, J. P. 1981. Storage proteins of glandless cottonseed flour. J. Food Sci. 46:1855.

[Received February 2, 1984. Accepted May 18, 1984]