# AN INVESTIGATION OF THE GLOBULINS OF COTTON SEEDS. I

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Methods for isolating a number of individual globulin components from cotton seeds have been reported previously [1-4]. By thin-layer gel chromatography we have found in cotton seeds five globulin fractions differing in molecular weight.

The defatted flour, which had been washed with water to eliminate albumins, was extracted with a 10% solution of sodium chloride, and the extract was deposited on a glass plate coated with a thin layer of Sephadex G-150 Super Fine equilibrated with a 10% solution of sodium chloride. Chromatography was performed for 42 h, and then a paper replica was taken (Fig. 1). It can be seen from the figure that the cotton seeds contain two main globulin components (3 and 4) amounting to 55 and 38% of the total amount of protein. The molecular-weight distribution of all five components according to the results of thin-layer gel chromatography is given below:

| Protein | $R_f$, cm | Mol. wt. |
|---|---|---|
| 1. Bovine serum albumin | 13,2 | 68 000 |
| 2. Ox heart lactate dehydrogenase | 16,4 | 140 000 |
| 3. Catalase | 17,5 | 240 000 |
| Globulin fractions | | |
| 1 | 9 | — |
| 2 IV | 12,8 | — |
| 3 III | 16,1 | 128 800 |
| 4 IV | 19,5 | >300 000 |
| 5 V | 21,3 | >500 000 |

Fraction III was investigated further. Three methods were used to isolate it in which the protein from the salt extract was precipitated by changing the ionic strength of the solution or the temperature or by the addition of various amounts of ammonium sulfate. The protein fractions obtained were analyzed by gel chromatography in a thin layer of Sephadex G-150 Super Fine as described above.

The protein obtained by reprecipitation with a change in the temperature of the solution [1] possessed a higher solubility and contained a smaller amount of high-molecular-weight impurities than the proteins isolated by the other two methods mentioned above. The yield of protein was 2% of the weight of the defatted flour. To determine the degree of purity of the globulin obtained we used gel filtration in a column of Sephadex G-200 and chromatography on DEAE-cellulose. The amount of impurity found did not exceed 10% in either case. To determine the molecular weight of the globulin by gel filtration in a column of Sephadex G-200 we took the same standard proteins as for gel filtration in a thin layer; the molecular weight of the globulin so determined was 125,900, while according to ultracentrifugation it was 130,000, the sedimentation coefficient being 7 S.

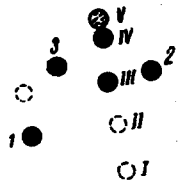


Fig. 1. Separation of cotton-seed globulins in a thin layer of Sephadex G-150 Super Fine.

By spectrophotometric titration according to Boyer [5] we found seven free SH groups in the protein component under study. After the reduction of the globulin with β-mercaptoethanol, the number of SH groups had increased to 21. This makes it possible to assume the presence of seven S–S bonds in it. On subsequent carboxymethylation of the reduced globulin, titratable SH groups had completely disappeared.

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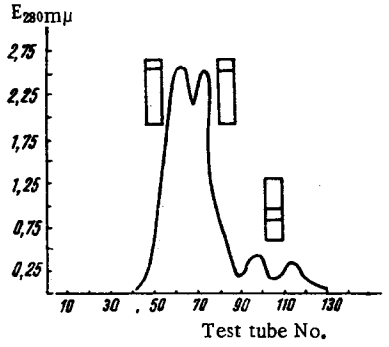


Fig. 2

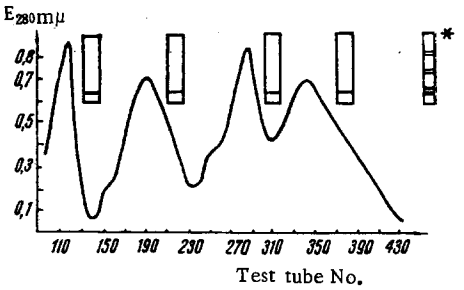


Fig. 3

Fig. 2. Separation on a column of Sephadex G-200 of the globulin dissociated in 8 M urea.

Fig. 3. Separation on a column of Sephadex G-200 of the globulin dissociated in 1% dodecyl sulfate (* – an electrophoretogram of the protein dissociated in 1% dodecyl sulfate).

By dansylation and dinitrophenylation methods we determined the amount of N-terminal amino acid – arginine (6 moles per mole of protein). The results of a comparison using thin-layer gel chromatography, ion-exchange chromatography, and a determination of the N-terminal acids, of the 8.2 S globulin isolated previously [2] and the globulin that we had obtained showed their identity.

In order to determine the quaternary structure of the globulin we used electrophoresis in polyacrylamide gel in the presence of 8 M urea and 0.1% sodium dodecyl sulfate (SDS). The electrophoretograms obtained in the presence of 8 M urea showed two intense bands in the upper part of the gel and two weak bands corresponding to proteins with higher mobilities. In the presence of 0.1% SDS two intense bands were found at the end of the gel and four low-intensity bands above.

To determine the molecular weights of the subunits corresponding to the bands mentioned we used electrophoresis in polyacrylamide gel by the method of Weber and Osborn [6]. The standard proteins used were catalase cleaved in 1% SDS, pepsin, and cytochrome. The following values of the molecular weights were obtained: 70,790; 56,230; 38,020; 35,480; 18,620; and 15,800. On electrophoresis in the presence of dissociating agents the reduced and carboxylated protein gave no new bands, which shows the absence of disulfide bonds between the polypeptide chains. On studying the globulin dissociated in 8 M urea by gel chromatography in a thin layer in the presence of proteins of known molecular weights, we found two components in the cleaved globulin, with mol. wt. 54,950 and 40,740. On preparative separation in a column of Sephadex G-200 of the protein dissociated in 8 M urea, we obtained four fractions the electrophoretograms of which are shown in Fig. 2: The first two, present in major amount (80%), were found by gel chromatography in a thin layer of Sephadex and the two minor components (18%) of the deposited protein were not found in the thin layer because of their small amount. The molecular weights of the minor components isolated from the column (23,000 and 17,380) were determined by gel chromatography in a thin layer of Sephadex G-75 Super Fine.

In the preparative separation of the protein cleaved in 1% SDS on a column of Sephadex G-200 four fractions were obtained (Fig. 3) the percentage ratios of which were 20, 25, 18, and 22; their molecular weights (37,000, 35,000, 18,750, and 1700) were determined by ultracentrifugation using the method of unestablished equilibrium.

The two fractions of low molecular weight were dialyzed against distilled water to eliminate SDS and were freeze-dried, and their molecular weights were determined by gel filtration through a column of Sephadex G-75 equilibrated with 0.01 M tris buffer, pH 8.2, with the addition of sodium chloride to $\mu$ 0.5. As the standard proteins we used cytochrome in the monomeric and dimeric forms, and also ox serum albumin. The free volume of the column was determined with respect to dextran blue. The molecular weights found were 67,610 and 47,860. On comparing the results obtained, we assume that the globulin consists of two types of polypeptide chains with molecular weights of about 18,500 and 16,000; the molecular weights of 70,790, 56,230, 35,480, and 38,020 found on electrophoresis in the presence of 0.1% SDS and of 67,610 and 47,860 determined by gel filtration relate to tetrameric and dimeric forms of the two polypeptide chains mentioned.

## EXPERIMENTAL METHOD

Thin-Layer Gel Chromatography. The defatted flour (1 g) was washed three times with distilled water and centrifuged, and the solid matter was extracted with 5 ml of 10 % sodium chloride solution for 30 min and was centrifuged. The concentration of protein in the extract obtained was 55-60 mg/ml.

For chromatography we used glass plates (20 × 40 cm) on which a 0.5-mm layer of Sephadex G-150 Super Fine in sodium chloride solution had been deposited. The plates were equilibrated for 12 h. The proteins in a concentration of 20-40 mg/ml were deposited in the form of a single drop. The chromatographs were run for 40-42 h. The angle of slope of the plates was 15°.

A paper replica was treated with a 0.1 % solution of Bromophenol Blue in a mixture of methanol and acetic acid (9 : 1). Then a calibration curve of the dependence of $R_f$ on log M was plotted, and the molecular weight was determined from this. For the globulin components we obtained molecular weights of 68,000, 128,000-130,000, 320,000-340,000, and above 400,000. For calibration we took proteins of known molecular weights: bovine serum albumin (68,000), catalase (240,000), and lactate dehydrogenase (140,000).

Isolation of the Main Globulin of Cotton Seeds. The defatted flour (500 g) was extracted with a 10 % solution of sodium chloride buffered with phosphates to pH 7.4 in a ratio of 1 : 30 for 12 h. The mixture was centrifuged. The supernatant liquid was dialyzed against flowing water for two days. The precipitate that had deposited was separated off and washed, with cooling, with 0.05 M Na phosphate buffer, pH 7.4, and recentrifuged. The precipitate was dissolved at 30° C in a 0.3 M solution of sodium chloride buffered with phosphates to pH 7.4, the solution was centrifuged, and the supernatant liquid was cooled to −2° C and below. The precipitate that deposited was centrifuged and reprecipitated similarly three more times. The protein obtained was freeze-dried. Yield 1.8 % on the defatted flour.

Purification on a Column of Sephadex G-200. A solution of 100 mg of protein in 3 ml of phosphate buffer, $\mu$ 0.5, pH 7.4, was deposited on a column (2 × 200 cm) on Sephadex G-200 equilibrated with the same buffer. The rate of elution was 6 ml/h. The protein was eluted in two fractions. The material corresponding to the individual peaks was dialyzed and freeze-dried. The yields of fractions 1 and 2 amounted to 9 % and 90 %, respectively, of the protein deposited.

Reduction and Carboxymethylation of the Globulin. The pH of a solution of 3.5 g of the globulin in 25 ml of a freshly prepared 8 M solution of urea which had been recrystalized from 75 % ethanol was brought to 8.5 with a 1 N solution of lithium hydroxide. Then 6 ml of mercaptoethanol was added and the pH was restored to 8.5 with the 1 N solution of lithium hydroxide. Reduction was performed in a beaker protected from the light in a current of nitrogen for 4 h with continuous stirring. Then the protein was precipitated with a 10-fold excess of cold acidified ethanol (39 parts of ethanol + one part of 1 N hydrochloric acid at 4-6°C) and was centrifuged at 3000 rpm for 15 min. After this it was washed with acidified ethanol three times and dissolved in 8 M urea, the pH of the solution was brought to 8.5 with a 1 N solution of lithium hydroxide, and 1.5 g of monoiodoacetic acid (20-fold excess for 1 SH group) in 10 ml of 8 M urea, pH 8.5, was added. The alkylation reaction was continued for 30 min under the same conditions as for the reduction process. The protein was precipitated with acidified ethanol, washed twice, and centrifuged. The precipitate was dissolved in 8 M urea and dialyzed against distilled water for two days. The protein purified by dialysis was freeze-dried. Yield 0.43 g (12 %).

Determination of Free SH Groups. p-Chloromercuribenzoate (purified by two reprecipitations with 1 N hydrochloric acid from a 1 N solution of caustic soda, the precipitate being washed twice with water and with acetone and dried over phosphorus pentoxide) (20 mg) was dissolved in 160 ml of a buffer prepared by mixing 3 M dipotassium phosphate with 2 M monopotassium phosphate in a ratio of 39 + 1.5 ml. Urea was added to the resulting solution to a concentration of 6 M. Samples of 1.25, 2.5, 5, 6.25, 8.7, 10, 12.5, 15, 16.2, 18, 20, 25, 30, 40, 47, and 50 ml were taken from the initial solution and their volumes were made up to 50 ml in each case.

To each of a number of test tubes with ground-in stoppers were added 4 ml of the p-chloromercuribenzoic acid solution and 1 ml of protein solution with a concentration of 1-2 mg/ml. The samples were incubated at room temperature for 30 min in a current of nitrogen and were photometered at 250-280 cm. Then graphs of the dependence of the extinction at 250 nm on the number of moles of added p-chloromercuribenzoate were plotted. The absorption at 280 nm was used to calculate the accurate concentration of protein. The point of inflection on the graph corresponded to the number of sulfhydryl groups.

Study of the Dissociation of the Protein in 8 M Urea and 1 % SDS. The protein was dissolved in 8 M urea or 1 % SDS and incubated at 47-50° C for 45 min. In the electrophoresis of samples dissociated as

described above, the solutions for the preparation of the gels were treated with dodecyl sulfate to a concentration of 0.1 % or with urea to a concentration of 8 M. Electrophoresis was performed for 3 h (5 mA per tube) using tris-glycine buffer. The plates for gel chromatography in a thin layer of Sephadex G-150 Super Fine were equilibrated with 8 M urea. Gel chromatography was performed by the method described.

Separations of the Protein Dissociated in 8 M Urea on a Column of Sephadex G-200. The protein (300 mg) which had been incubated in 8 M urea was deposited on a column of Sephadex G-200 (2 × 200 cm) equilibrated with 8 M urea. The rate of elution was 3 ml/h. According to the elution graph, four fractions were obtained containing 107, 130, 15, and 18 mg of protein. The fractions were dialyzed and freeze-dried.

Separation of the Protein Dissociated in 1 % SDS on a Column of Sephadex G-200. A solution of 75 mg of the protein in 1 % SDS was deposited on a column of Sephadex G-200 (2 × 100 cm) equilibrated with 0.1 % SDS. The rate of elution was 4.65 ml/h. Four fractions were obtained with yields of 20, 25, 16, and 22 % of the protein deposited, respectively.

By the method of unestablished equilibrium at 22,000 and 8000 rpm molecular weights of 37,000, 35,000, 18,750, and 17,000 were found for the polypeptides obtained.

## SUMMARY

1. Cotton seeds contain five globulin components, two of which are the main representatives; the molecular weights of the latter are 130,000 and more than 300,000.

2. The globulin with a molecular weight of 130,000 (N-terminal amino acid arginine) has a quaternary structure: it consists of two types of polypeptide chains.

## LITERATURE CITED

1. A. Rossi-Fanelli, Biochem. Biophys. Res. Commun., 15, 110 (1964).
2. Kh. Mirzarakhmatov and P. Kh. Yuldashev, Khim. Prirodn. Soedin. 397 (1968).
3. V. M. Mikhailov, N. N. Karavaeva, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 395 (1968).
4. A. P. Ibragimov, Sh. Yunuskhanov, and A. V. Tuichiev, Biokhimiya, 34, 1107 (1969).
5. P. D. Boyer, J. Amer. Chem. Soc., 76, 7331 (1954).
6. K. Weber and M. J. Osborn, J. Biol. Chem., 244, 4406 (1969).