AN INVESTIGATION OF THE GLOBULINS OF COTTON SEEDS. IV

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The 7S globulin has a quaternary structure and dissociates in 8 M urea into two subunits and two polypeptide chains [1]. In the present work we consider the development of a method for the preparative separation of the subunits and a comparative study of their chemical properties.

We made use of ion-exchange chromatography and gel filtration. Figure 1 shows a graph of the separation of the 7S globulin after incubation in 6 M urea in a column of DEAE-cellulose equilibrated with 0.001 M tris-HCl buffer, pH 8.4, in 6 M urea.

The fractions corresponding to the first peak were eluted with the initial buffer and those corresponding to the second peak with a buffer having a high ionic strength obtained by the addition of 1 M NaCl. After the elimination of the urea by dialysis, the protein of the first fraction precipitated in the form of white flocs, and that of the second remained in solution, the liquid having a creamy color. The further purification of each subunit was performed by rechromatography under the conditions described above and by gel filtration on a column with Sephadex G-200 (0.01 M tris-HCl buffer, pH 8.4, 6 M urea).

The purity of the subunits obtained was checked by electrophoresis in polyacrylamide gel with the addition of 0.1% sodium dodecyl sulfate (SDS). The protein was first incubated in 1% SDS. The presence of a single band on the electrophoretogram showed that each subunit consisted of polypeptide chains of one type.

The results of a comparative study of the chemical compositions of the subunits obtained (Table 1) showed the absence of substantial differences between them. The high content of acidic amino acids, and also of arginine and phenylalanine, must be noted.

By enzymatic cleavage with carboxypeptidase A and hydrazinolysis we found valine as the C-terminal amino acid of the 7S globulin and its subunits. The N-terminal amino acid was previously found to be arginine [3]. Using Edman's method in combination with dansylation, we determined the following N-terminal sequence of both subunits: Arg—Glu—Gly—Phe—Glu—.... These results also showed the similar chemical compositions of the subunits.

The method of peptide maps, which permits the numbers and identities of the subunits to be judged, is widely used for comparing the primary structures of proteins. When the sub-

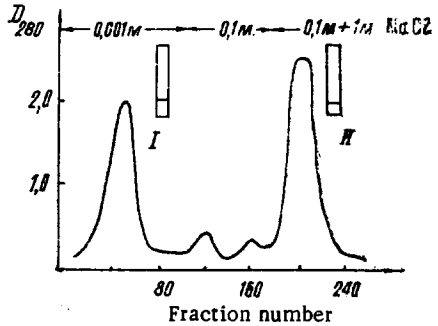

Fig. 1. Separation of the subunits (I and II) of the 7S globulin on DEAE-cellulose.

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TABLE 1. Characteristics of the 7S Globulin Its Subunits

Content, %	7S globulin	Subunits	
		I	II
Amino acids			
lysine	3,3	3,6	2,7
arginine	13,6	14,1	13,9
histidine	3,6	3,8	3,6
1/2 cystine*	0,8	—	—
aspartic acid	9,6	7,9	9,0
threonine	1,9	3,7	3,2
serine	5,1	4,6	5,1
glutamic acid	19,8	21,9	19,8
proline	3,3	2,8	2,6
glycine	3,6	4,1	4,2
alanine	3,1	2,8	2,7
valine	4,2	4,6	4,1
methionine	1,0	0,5	0,7
isoleucine	3,0	3,9	3,6
leucine	7,0	6,6	7,3
tyrosine	5,3	3,7	3,4
phenylalanine	10,3	8,3	10,0
tryptophan*	0,5	—	—
Carbohydrates			
glucose	0,4	0,4	0,2
mannose	1,6	0,6	1,4
hexosamines	1,6	0,4	1,6

*Determined after hydrolysis by p-toluenesulfonic acid [2] only in the globulin.

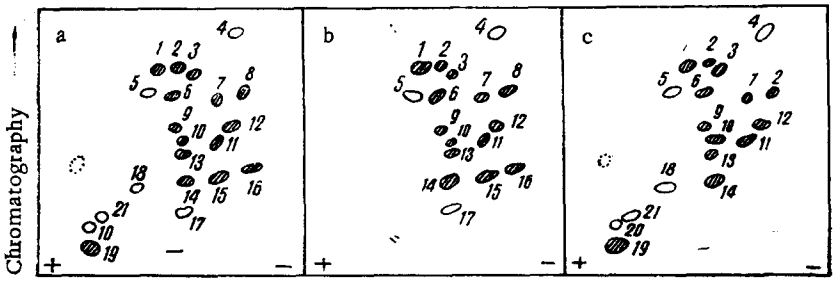

Fig. 2. Peptide maps of the CM-7S-globulin (a), and the CM-subunits I (b) and II (c). The arginine-containing peptides are hatched.

units are identical, the number of peptides detected on the map will be smaller by a factor that is a multiple of the number of subunits.

The 7S globulin contains about 140 lysine and arginine residues. On the map of a tryptic hydrolyzate of the globulin 20-21 peptides were detected, and in the case of the subunits 17-18 peptides each (Fig. 2).

The map of subunit (II) lacked the neutral peptide (17) and two basic peptides (15 and 16), and that of the subunit (I) lacked the acid peptides (18-21).

According to the amino-acid composition, for the 7S globulin a high content of aspartic and glutamic acids is characteristic. The passage of the acid peptides (18-21) into the region of neutral and basic peptides is possible through the presence of the amides of the amino acids mentioned. The study of the primary structure will lead to a definitive answer to this question.

The 17-20 peptides detected in tryptic hydrolyzates of globulin and its subunits permit the assumption that the globulin consists of eight polypeptide chains of similar amino-acid compositions. The results obtained agree well with information published previously on the routes of the dissociation of the 7S-globulin molecule in 8 M urea and 1% SDS [1]. It was then shown that the subunits differ in molecular weight. On the basis of the results of a comparison of the properties of the subunits, the presence of a nonprotein component in the 7S globulin could be assumed. From the spectra of the products of its reaction with L-

cysteine in sulfuric acid [4], we established the presence in the protein of carbohydrates the composition and amounts of which were found by gas–liquid chromatography (see Table 1). The globulin molecule contains about 2% of neutral sugars — glucose and mannose in a ratio of 1:4 — and also about 2% of hexosamines.

The difference in the molecular weights of the polypeptide chains composing the globulin and its subunits is probably due to different amounts of carbohydrates.

At the present time a study of the primary structure of the peptides of the tryptic hydrolyzates has begun.

EXPERIMENTAL METHOD

Separation of the Subunits of the 7S Globulin on a Column of DEAE-Cellulose. About 4 g of the 7S globulin obtained as described previously [1] was dissolved in 70 ml of 0.001 M tris-HCl buffer, pH 8.4, in 6 M urea. The protein was incubated at 50°C for 2 h and was then deposited on a column (4 × 50 cm) equilibrated with the same buffer. Elution was performed stepwise with the initial buffer, 0.1 M tris-HCl buffer, and 0.1 M tris-HCl with the addition of 1 M NaCl. The rate of elution was 27-30 ml/h. The fractions obtained were subjected to spectrophotometry on an SF-16 instrument at 280 nm, and an elution graph was plotted. The fractions belonging to a single peak were combined, dialyzed for 2-3 days, and freeze-dried. The yield (from the absorption) of peak I was 940 mg and of peak II 2630 mg.

Rechromatography of Subunit I. Subunit I (1 g), eluted as the first peak in the chromatography of the 7S globulin dissociated in urea on a column of DEAE-cellulose, was dissolved in 8 ml of 0.001 M tris-HCl buffer, pH 8.4, in 6 M urea, and the solution was incubated at 50°C for 2 h and deposited on a column (3 × 32 cm) of DEAE-cellulose equilibrated with the initial buffer. Elution was performed with the same buffer solutions as in the chromatography of the 7S globulin. The rate of elution was 22 ml/h and the fraction volume 11 ml.

Rechromatography of Subunit (II). Subunit II (1 g), issuing as the second peak in the chromatography of the urea-dissociated 7S globulin on a column of DEAE-cellulose, was dissolved in 5 ml of 0.001 M tris-HCl buffer, pH 8.4, in 6 M urea, incubated at 57-60°C for 2 h, and deposited on a column of DEAE-cellulose (3 × 32 cm). The conditions for rechromatography were similar to those for subunit I.

Gel Filtration of Subunits I and II of the 7S Globulin through a Column of Sephadex G-200. In each case, 100 mg of the subunit obtained after rechromatography was dissolved in 2 ml of 0.01 M tris-HCl buffer, pH 8.4, in 6 M urea, and the solution was incubated at 56-60°C for 2 h and was deposited on a column (2.5 × 100 cm) of Sephadex G-200 equilibrated with the same buffer. The rate of elution was 6 ml/h, and the fraction volume 3 ml.

Determination of the Amino-Acid Compositions of the Subunits. Samples (4 mg) of the subunits were each dissolved in 5 ml of 6 N HCl. Hydrolysis was performed in vacuum-sealed tubes at 110°C for 24 h. The hydrolyzate obtained was dissolved in 1 ml of citrate–phosphate buffer pH 2.2. The amino acids were analyzed on an AAA-881 amino-acid analyzer.

Electrophoresis in Polyacrylamide Gel. This was performed as described previously [1].

Determination of the C-Terminal Amino Acid. A solution of 30 mg of the protein in 6 ml of 0.01 M phosphate buffer (pH 8.0; $\mu = 0.5$) was treated with 1 ml of a solution of carboxypeptidase A ($D_{280} = 0.7$). The mixture was incubated at 27°C and 1-ml samples were taken after 1, 2, 4, 8, 18, and 24 h and were acidified to pH 1 to stop the reaction; after evaporation, the residue was dissolved in 80% ethanol and the amino acids that it contained were determined on an amino-acid analyzer. A graph of the dependence of the amount of amino acids on the time of incubation was plotted from the results obtained.

Reduction and S-Carboxymethylation of the 7S Globulin (RCM-7S-Globulin) and Its Subunits. These were performed as described previously [1].

Tryptic Digestion of the RCM-7S-Globulin and Its Subunits; Preparation of Peptide Maps. The RCM-7S-globulin (50 mg) (or an RCM-subunit, 20 mg) was suspended in 2 ml of acetate–ammonia buffer, pH 8.8, 0.5 ml of a solution of trypsin (1 mg of "Serva" TPCK-trypsin in 1 ml of acetate–ammonia buffer) was added, and the protein was digested at 37°C. After an hour, another 0.5 ml of the trypsin solution was added. The enzyme–protein ratio was 1:50. Digestion was performed for 6 h, the pH being maintained with ammonia. Then the mixture was centrifuged and freeze-dried, and the residue was dissolved in 0.5 ml of the initial buffer.

The hydrolyzate (5-10 μliters) was deposited on a plate 20 × 20 cm with a thin layer of FND cellulose (GDR) and was chromatographed twice in the butanol—acetic acid—pyridine—water (15:3:10:12) system. After drying, the plates were subjected to electrophoresis in pyridine acetate buffer, pH 6.5: voltage 900 V, current strength 20-25 mA, time 40 min. The spots on the chromatogram were revealed with a 0.1% solution of ninhydrin in ethanol with the addition of 4% of collidine and 20% acetic acid.

Determination of the Neutral Sugars in the 7S Globulin and Its Subunits. The protein (50 mg) was hydrolyzed in 3 N HCl in vacuum-sealed tubes at 100°C for 3 h. The hydrolyzate was freed from amino acids and amino sugars by treatment with dry Dowex 50 × 4 resin and was then investigated for neutral sugars by gas—liquid chromatography.

Determination of the Hexosamines in the 7S Globulin and Its Subunits. Use was made of the Elson—Morgan reaction in Cessi's modification. The 7S globulin (20 mg) or one of the subunits (10 mg) was hydrolyzed with 2 N HCl in vacuum-sealed tubes for 16 h. Then the reaction mixture was evaporated in a rotary evaporator and the residue was dissolved in 2 ml of 0.5 N Na_2CO_3.

The N-terminal sequence was determined by Edman's method in combination with dansylation [5]. The size of the samples of subunits used was 5 mg.

SUMMARY

1. A method has been developed for the preparative separation of the subunits of the 7S globulin of cotton seeds.

2. The similarity of the chemical structures of the subunits has been shown by the peptide map method and by a comparison of amino-acid compositions and of N- and C-terminal amino acids. It is suggested that their main difference consists in their carbohydrate contents.

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