INVESTIGATION OF THE 8.2 S GLOBULIN FROM THE SEEDS OF THE COTTON PLANT

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The main proteins of cotton seed are globulins. In preceding papers [1, 2] we have reported the isolation of an individual 8.2 S protein component. In the present paper we give some results of a study of the physicochemical constants of the 8.2 S globulin.

By the dinitrophenylation method [3] and thin-layer chromatography on silica gel and polyamide [4, 5], a hydrolyzate of dinitrophenylated 8.2 S globulin was found to contain DNP-arginine, ε-DNP-lysine, and traces of DNP-histidine and DNP^{im}-histidine (Fig. 1a). The presence of these DNP amino acids was confirmed by Sakaguti's qualitative reaction and by the reaction with ninhydrin.

For quantitative analysis, the combined DNP-amino acids from the normal butanolic fraction of the hydrolyzate of the DNP derivative of the 8.2 S globulin were decomposed with anhydrous hydrazine to give the free amino acids [7]. These were analyzed on an amino-acid analyzer [ChSAN (Czechoslovak Academy of Sciences) type 6020A]. This showed the presence of 16 moles of lysine and ornithine per 10^5g of the 8.2 S globulin. On hydrazinolysis, DNP-arginine forms ornithine, as has been shown by experimental results with standard DNP-arginine and ornithine.

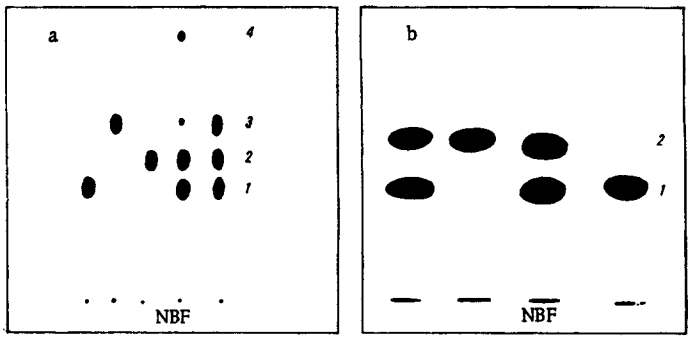

Fig. 1. Chromatogram of the DNP amino acids (plates 10 × 10) in a thin layer of silica gel in the butan-1-ol–25% NH_3 (1 : 1) system (one-dimensional chromatography to a height of 9 cm three times) (a) and in a thin layer of polyamide in the butan-1-ol–concentrated acetic acid (9 : 1) system (b). 1) DNP-arginine (R_f in a thin layer of polyamide 0.30); 2) ε-DNP-lysine (R_f in a thin layer of polyamide, 0.50); 3) DNP-histidine; 4) DNP^{im}-histidine. NBF – butanolic fraction of the hydrolyzate of the 8.2 S globulin (R_f values of the DNP-amino acids from the hydrolyzate of the dinitrophenyl derivative of the 8.2 S globulin in a thin layer of polyamide: 0.30 and 0.50).

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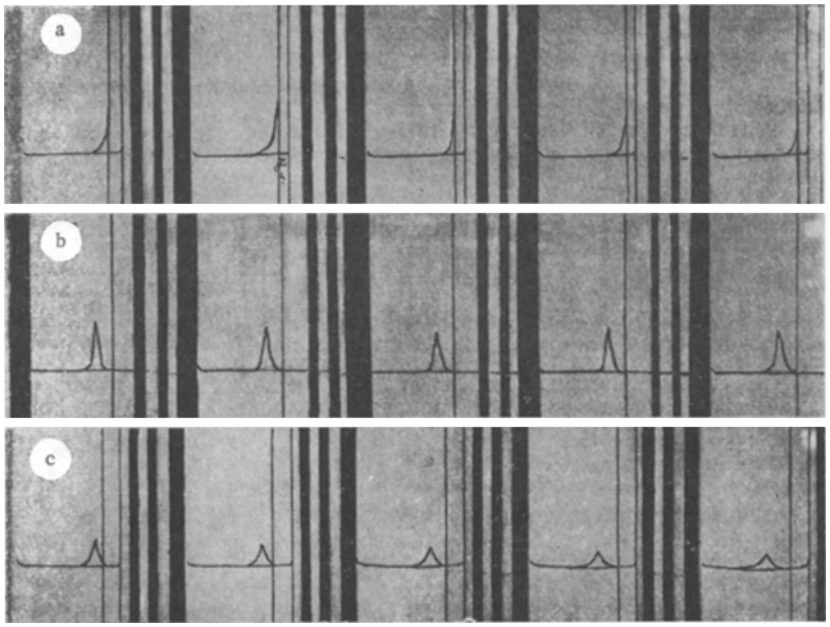

Fig. 2. Results of a molecular-weight determination in the ultracentrifuge; a, b) by Archibald's method; 19,980 rpm, 30°C, initial photograph 3 min, interval 3 min; 8560 rpm, 30°C, initial photograph 5 min, interval 6 min, respectively; c) by the rate-of-sedimentation method; 50,100 rpm, 22°C, initial photograph 7 min, interval 6 min, concentration of protein 10 mg/ml. Solvent – phosphate buffer with μ = 0.5, 20°C, two-sector cell.

The DNP-arginine and ε-DNP-lysine from the hydrolyzate were determined quantitatively by a spectrophotometric method on an SF-4A instrument. The butanolic fraction of the hydrolyzate of the DNP derivative of the 8.2 S globulin was chromatographed in a thin layer of polyamide; the spots of the DNP-arginine and ε-DNP-lysine were scraped off the chromatographic plate with the layer of polyamide and transferred to a 1% solution of $NaHCO_3$. Then the extinctions of the solutions of the DNP amino acids were measured at 360 nm (Fig. 1b).

In 2.5 mg of the 8.2 S globulin we found 0.0090 mg of DNP-arginine and 0.102 mg of ε-DNP-lysine; consequently, 10^5 g of the protein contains 1.05 mole of DNP-arginine (1 mole of arginine is present in 95,000 g of the 8.2 S globulin) and 12.75 mole of ε-DNP-lysine. The molecular weight of the 8.2 S globulin determined by Archibald's method and by the rate of sedimentation [8-9] on an MOM G-120 ultracentrifuge (Hungary) is 100,000 (±10%) (Fig. 2a, b, c). The amino-acid composition of the 8.2 S globulin (in moles) per 10^5 g of protein (per mole of protein) or per mole of N-terminal arginine is given below.

Amino acid	Moles per 10^5 g of protein	Amino acid	Moles per 10^5 g of protein
Lysine	21	Alanine	41
Histidine	25	1/2 cystine	13
Arginine	62	Valine	45
Aspartic acid	54	Methionine	9
Threonine	33	Isoleucine	25
		Leucine	47
Serine	50	Tyrosine	18
Glutamic acid	85	Phenylalanine	49
Proline	36	Tryptophan	11
Glycine	48		

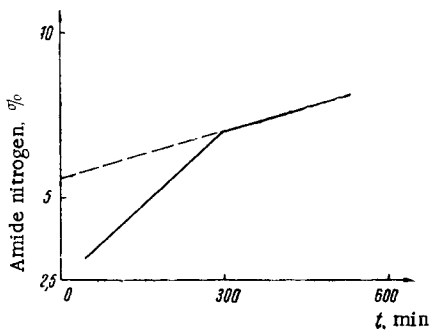

Fig. 3. Content of amide nitrogen (percentage of total nitrogen) in the 8.2 S globulin.

The tryptophan in the 8.2 S globulin was determined spectrophotometrically by the method of Holiday and Ogston [10] and by a modification of the method of Graham et al. [11] using as standards casein and egg albumin. The globulin contains considerable amounts of glutamic and aspartic acids and arginine, serine, and glycine.

In the 8.2 S globulin, the amide groups of the protein were detected by hydrolysis with 2 N hydrochloric acid at 100°C [12], with subsequent determination of the amount of ammonia by Conway's microdiffusion method [13] (Fig. 3). Extrapolation of the curve obtained to zero time [12] showed that the amount of amide nitrogen was 5.6%, or 64 moles of NH_2 groups per mole of protein. Consequently, 46% (or a total of 139 moles) of the dicarboxylic acids (aspartic and glutamic acids) in the 8.2 S globulin are present in the form of amides.

EXPERIMENTAL

Preparation of the Dinitrophenyl Derivative of the 8.2 S Globulin. A solution of 50 mg (0.5 μmole) of the protein in 15 ml of phosphate buffer with an ionic strength of 0.5 was brought to a pH of 9 with 0.4 N NaOH solution, and 0.2 ml of DNPB was added; the flask with the reaction mixture was sealed and was left in a thermostat at 40°C with magnetic stirring overnight. On the following day, the excess of DNPB was eliminated from the reaction mixture by three extractions with peroxide-free ethyl ether, and the residue was acidified with 4 N HCl to pH 1.

The acid solution of the DNP-protein was transferred to a tube, to which an equal volume of concentrated HCl was added. The air was evacuated from the tube (with the contents frozen) by means of a water pump, the tube was sealed, and hydrolysis was performed at 105-110°C for 24 h. The hydrolyzate was diluted with water, and the DNP-amino acids were extracted with ether and butanol. The ethereal and butanolic fractions of the hydrolyzate were studied in thin layers of silica gel and polyamide.

DNP-amino acids were found only in the butanolic fraction. (A set of DNP-amino acids of natural origin was used as markers.)

Cleavage of the DNP-Amino Acids. An ethanolic solution of the n-butanolic fraction of the hydrolyzate of the DNP derivative of the 8.2 S globulin (1 ml, containing the equivalent of 10 mg of protein) was evaporated in a rotary evaporator at 60-70°C. Then the dry residue was treated with 3-4 drops of anhydrous pyridine. The flask with the reaction mixture was tightly sealed and was left in the thermostat at 70-80°C for 2 h. The excess of hydrazine was eliminated under vacuum in a desiccator over concentrated sulfuric acid. The amounts of free amino acids in the dry residue were determined in an amino-acid analyzer.

Determination of Tryptophan. A. Spectrophotometric Method. The absorptions of a solution of 9.4 mg of the protein in 10 ml of a 0.1 N solution of caustic soda were measured at 280 and 305 nm on an SF-4A instrument. The absorptions of the solution at the wavelengths mentioned were 0.920 and 0.310, respectively.

The concentration of tryptophan in the protein solution was calculated by means of the formula $M_{tryp} = (0.207\ \Sigma_{280} - 0.280\ \Sigma_{305}) \cdot 10^{-3}$.

B. By Hydrolysis in 0.1 N Caustic Soda Solution. A solution of 50 mg (0.5 μmole) of the protein in 50 ml of a 0.1 N solution of caustic soda was evaporated to dryness in a porcelain dish on the boiling water bath. The dry residue was dissolved in 5 ml of water. To 2 ml of the aqueous solution and to a standard solution of tryptophan, each in a 25-ml measuring flask, were added 0.25 ml of a 5% solution of p-dimethylaminobenzaldehyde (in 10% sulfuric acid), 0.3 ml of a 0.3% aqueous solution of ferric chloride, 0.3 ml of a 0.01% aqueous solution of potassium dichromate, and 12.5 ml of concentrated hydrochloric acid. The flasks were left in the dark for 4 h and their contents were made up to the mark with water.

The standard solution had a concentration of 0.2 mg/ml (in 0.1 N caustic soda solution). The optical density of the solution with respect to the standard solution was measured in an FÉK-56 photocolorimeter.

Determination of Amide Nitrogen. Samples of the 8.2 S globulin (102.0 and 100.7 mg) were transferred to 20-ml round-bottomed flasks and each was covered with 5 ml of 2 N hydrochloric acid. The flasks were attached to reflux condensers and heated in the boiling water bath for 3 h. Then the heating was stopped and the flasks containing the hydrolyzates were cooled in ice, and 2-ml samples were transferred to 25-ml measuring flasks. Heating was resumed, and samples were taken after every 2 h, the reaction mixture being cooled previously in each case. After the sample had been diluted with water (25 ml in each case), the ammonia content was determined.

CONCLUSIONS

1. It has been established that in the 8.2 S globulin the N-terminal amino acid is arginine; its molecular weight found from the N-terminus is 95,000, and by ultracentrifuging 100,000.

2. On heating with hydrazine, DNP-arginine is converted into ornithine.

3. The 8.2 S globulin consists of 672 amino acid residues.

4. The amount of amide nitrogen in the 8.2 S globulin is 5.6% of the total protein nitrogen.

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