INVESTIGATION OF THE GLOBULINS OF COTTON SEEDS

VII. PEPTIDES OF A TRIPTIC HYDROLYZATE OF THE 7S-GLOBULIN

É. F. Redina, M. A. Kuchenkova,
and P. Kh. Yuldashev

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In an investigation of the subunits of the 7S-globulin of cotton seeds we showed that their main difference consists in the presence of carbohydrates in subunit II, on the one hand, and in the appearance of acid peptides on its peptide map, on the other hand [1].

In order to examine these differences we have isolated glycopeptides from a tryptic hydrolyzate by precipitation with acetone from a solution acidified to pH 3. The precipitated peptides were dissolved in a 0.1 M solution of ammonium bicarbonate and were separated on a column of Sephadex G-25. The orcinol-positive fraction was studied by the peptide map method. A comparison of the peptide maps of the tryptic hydrolyzates of the protein itself, the subunits, and the glycopeptide fraction showed that the latter is a distinctive fraction of the peptide maps of the subunits.

We previously isolated tryptic peptides of subunits I containing no carbohydrates and obtained their characteristics [2]. Knowing their composition and the acid nature of the individual peptides of subunit II we have carried out a preparative cleavage of the 7S-globulin in order to isolated tryptic peptides in an amount sufficient for determining their amino-acid sequences. To separate the peptides we used ion-exchange chromatography on a column containing Dowex 50WX4.

From the 22 fractions (Fig. 1) by paper chromatography and electrophoresis we isolated 40 peptides homogeneous in relation to their N-terminal amino acids and determined their compositions (Table 1). Peptides T-4-1-1 and T-18-1-2, T-8-3 and T-15-3, and T-13-1-2 and T-16-3 proved to be identical. The Ehrlich reaction showed the presence of one tryptophan-containing peptide.

The fact that a peptide map of the protein showed 20 peptides and of the 37 peptides obtained only 24 included basic amino acids makes it possible to assume that the cleavage of the protein was nonspecific. Definitive conclusions concerning the routes of fragmentation will be made after a study of the amino-acid sequence of tryptic and chymotryptic peptides.

On investigating the fractions from the column by the orcinol reaction, we found carbohydrates only in the first fraction. By paper chromatography we isolated from it two peptides one of which contained mannose and hexosamines in a ratio of 4-5 : 3-4, the total amount of carbohydrates corresponding to their amount in the protein. The composition of the glycopeptide coincided with that of the C-terminal peptide found in subunit (I) containing no carbohydrates, which confirms the hypothesis put forward previously that the difference between the subunits was the fact that one of them contained carbohydrates [1].

EXPERIMENTAL

The reduction and carboxymethylation of the 7S-globulin was performed as described previously [3].

Preparative Separation of the Peptides. We used trypsin ("Spofa," Czechoslovakia) three times recrystallized from ammonium sulfate [4] and treated with 0.01 N HCl to suppress chymotryptic activity.

After carboxymethylation, the protein was desalted on a column of Sephadex G-50 (3.5 × 100 cm) in 50% acetic acid and was concentrated in vacuum at 30°C and dialyzed distilled water.

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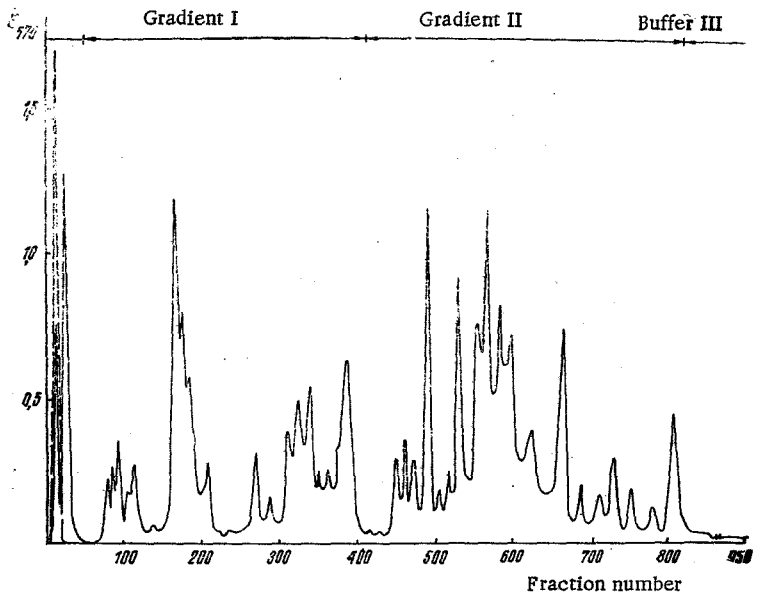

Fig. 1. Separation of a tryptic hydrolyzate of the 7S-globulin on a column of Dowex 50WX4 (the fraction containing carbohydrates is shown by the broken line).

To the resulting solution (175 ml) containing 1.6 g of protein (from its absorption) was added 1.4 g of ammonium bicarbonate to give a 0.1 M solution (pH 8.6) and a solution of trypsin (16 mg, 8 mg after 1 h and 8 mg after 8 h). Hydrolysis was carried out for 16 h, the pH being maintained with NH_4OH solution and the enzyme : substrate ratio being 1 : 50. Then the mixture was evaporated in vacuum at 30°C, and 1/5 of it was taken for the isolation of the glycopeptides.

The treatment of the Dowex 50WX4 (400 mesh) resin, the filling of the column, and the analysis of the eluate were performed as described by Ovchinnikov et al. [5]. The conditions of separation were: column 2 × 150 cm, rate of elution 60 ml/h, 15-ml fractions. Before the beginning of the experiment the column was washed for 48 h with the starting buffer (0.2 M pyridine-acetate buffer, (pH 3.1) at 35°C. The hydrolyzate was dissolved in 20 ml of a solution of acetic acid (pH 2.2) and was deposited on the column under a pressure of nitrogen. Separation was carried out under conditions of gradient elution: 750 ml of the starting buffer, then gradient I obtained by feeding to the mixer containing the starting buffer 0.5 M buffer with pH 4.9 (3 liters each); for gradient II we used 3 liters each of 0.5 and 2M pH 5.0 buffers at 50°C, and for buffer III 2 M pyridine (2 liters). The fractions were analyzed after reaction of an alkaline hydrolyzate with minhydrin and, in addition, 0.5 ml of each even-numbered test-tube was treated with orcinol [6] to detect glycopeptides.

Paper chromatography, and the analysis and elution of the fractions were performed as described previously [2].

Electrophoresis on paper (FN-17, Filtrak, GDR) was performed in pyridine-acetate buffer, pH 6.5, at a voltage of 3000 V (potential gradient 60 V/cm) for 4 h. The detection, elution, purity checking, and determination of the compositions of the fractions and their N-terminal amino acids have been described previously [2].

The isolation of the glycopeptides from the tryptic hydrolyzate of the 7S-globulin was performed by precipitating with a fourfold excess of acetone the hydrolyzate acidified to pH 3 [7], and they were purified on a column of Sephadex G-25 under the conditions described previously for the peptides of subunit I [2].

The orcinol-positive fraction was freeze-dried and studied by the peptide map method, and the amounts of amino sugars and neutral sugars was determined as described previously [1].

SUMMARY

1. The peptides of a tryptic hydrolyzate of the 7S-globulin have been isolated and characterized.

2. The glycopeptide of subunit II has been isolated and its amino-acid and carbohydrate compositions have been determined.

3. It has been shown that the main difference in the subunits of the 7S-globulin consists in the presence of an oligosaccharide in the C-terminal peptide of subunit II.

TABLE 1. Characteristics of Tryptic Peptides of the CM-7S-Globulin

Peptide	Amino-acid composition															Number of residue	N-terminal amino acid
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe		
T-1-1				1,60(2)	0,77(1)	0,74(1)				0,90(1)	1,44(2)	0,68(1)	0,66(1)			9	Asp
T-2-1				0,20		0,20	1,00(1)				0,10			0,10		1	Glu
T-3-3-1							0,88(1)			0,93(1)			0,96(1)		1,17(1)	4	Leu
T-4-1-1			1,00(1)		0,70(1)	0,30										2	Thr
T-18-1-2							0,77(1)			0,65(1)			0,63(1)			6	Leu
T-4-1-2	0,74(1)			1,84(2)			0,86(1)					1,16(1)				2	Glu
T-4-1-4							0,93(1)			0,63(1)			0,64(1)			7	Leu
T-5-1-1*	0,84(1)			1,85(2)						1,27(1)			0,56(1)			5	Leu
T-5-2			0,64(1)			1,11(1)	1,53(1)			1,12(1)	0,86(1)	0,10				7	Val
T-6-1	0,71(1)		0,30	1,43(2)		0,83(1)	1,25(1)									2	Lys
T-7-1-3*	(1)								0,94(1)							4	Gly
T-8-1-1-1			0,91(1)			0,63(1)	1,14(1)		0,20		0,40				0,83(1)	3	Glu
T-8-2-2			1,00(1)	0,40	0,1	0,1	1,20(1)										
T-8-3	0,30	0,20	0,72(1)	1,40(1)	0,3	0,76(1)	1,30(1)	(1)	0,90(1)	0,70(1)	1,75(2)		0,87(1)		0,64(1)	11	Ser
T-15-3		0,2	1,00(1)	0,93(1)	0,2	0,2		(1)		1,00(1)	2,14(2)		0,2		0,3	6	Asp
T-8-4		0,95(1)			0,85(1)			0,90(1)	0,95(1)	1,05(1)	1,50(2)				0,92(1)	8	Val
T-8-5-1			1,08(1)	1,43(1)		0,97(1)	0,90(1)		0,90(1)					0,70(1)		6	Tyr
T-9-1-1		0,60(1)	0,93(1)	0,93(1)		1,20(1)	1,72(2)									7	His
T-9-4†									1,09(1)				1,46(1)	1,00(1)		3	Leu
T-9-6-1																5	Ser
T-10-1-2	0,90(1)	1,00(1)		1,11(1)		0,83(1)	1,15(1)		1,00(1)	1,00(1)	0,61(1)					4	Gly
T-10-3-2-1	1,00(1)								1,11(1)		0,82(1)		1,00(1)		0,96(1)	8	Val
T-10-4-1		0,77(1)	0,66(1)	0,50		0,77(1)	0,72(1)					0,65(1)				6	Asp
T-12-1		0,65(1)	0,72(1)	1,75(2)			0,76(1)										
T-13-1-2			0,92(1)	1,22(1)			1,30(1)		0,94(1)			0,68(1)			0,90(1)	6	Gly
T-16-3			1,10(1)	0,95(1)		0,3	1,28(1)		0,95(1)		0,4				0,69(1)	5	Gly
T-13-2					0,93(1)	0,78(1)	1,10(1)									3	Thr
T-14-2-1			(1)													1	Arg
T-14-2-3			1,22(1)			1,41(1)			0,78(1)		0,80(1)					4	Val
T-14-3-3			1,00(1)								1,24(1)					2	Val
T-16-1-2				0,40			0,30							0,88(1)	1,12(1)	2	Phe
T-16-4	0,83(1)	0,93(1)		1,00(1)	0,94(1)		1,00(1)		1,22(1)						1,29(1)	5	Thr
T-17-1-2	0,4	0,93(1)	0,60(1)	0,74(1)			2,50(2)		0,48	0,48		0,40		0,20		7	Phe
T-17-2-1	1,00(1)	0,30	0,50	0,40	0,30		2,12(2)									3	Glu
T-17-2-3				0,86(1)		1,50(1)	1,00(1)								0,86(1)	3	Ser
T-17-3-1			1,00(1)	0,40			1,20(1)			1,21(1)						3	Phe
T-17-4-1			0,78(1)							1,00(1)	1,50(1)		1,19(1)			2	Ala
T-19-1		0,83(1)	1,20(1)			1,41(1)	1,00(1)	(1)					0,60(1)			9	Val
T-20-1-2*		0,20	1,00(1)			0,20	0,20									2	Leu
T-20-2-2																	

*The peptides each contain one CmCys residue.

†The peptide contains tryptophan (determined qualitatively by the color reaction with the Ehrlich reagent).

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INVESTIGATION OF THE GLOBULINS OF COTTON SEEDS

VIII. CHYMOTRYPTIC HYDROLYSIS OF THE 7S-GLOBULIN. ISOLATION AND PURIFICATION OF A CHYMOTRYPTIC PEPTIDE

N. L. Ovchinnikova, M. A. Kuchenkova, and P. Kh. Yuldashev

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We have previously described the tryptic hydrolysis of the carboxymethylated 7S-globulin (CM-7S-globulin) isolated from cotton seeds and the separation, purification, and characterization of the tryptic peptides [1]. To show the complete primary structure of the CM-7S-globulin it is necessary to obtain overlapping peptides. With this aim we have studied the products of the cleavage of the CM-7S-globulin by chymotrypsin. According to an amino-acid analysis, a chymotryptic hydrolyzate should be expected to contain 23-25 peptides, but a peptide map showed the presence of 30-35 peptides. The appearance of additional peptides can be explained by the presence in the 7S-globulin of a large number of dicarboxylic acids present mainly in the form of amides and by the capacity of chymotrypsin for cleaving bonds formed by the carboxy groups of glutamine and asparagine.

To fractionate the chymotryptic hydrolyzate of the CM-7S-globulin we used ion-exchange chromatography on Dowex 50WX4 cation-exchange resin in a gradient of pyridine-acetate buffers. As a result of the separation we obtained 56 fractions (Fig. 1), each of which consisted of a complex mixture of peptides. Further purification was carried out by preparative paper chromatography in the butan-1-ol–pyridine–acetic acid–water (15 : 10 : 3 : 12) system. All the fractions were separated in this way, and from fractions Nos. 1, 4, 8, 12, 17, 21, 25, 27, 30, 35, 37, 38, 39, 46, 50, and 54 we isolated the following peptides in the pure form: XT-1-3, XT-4-1, XT-8-3, XT-8-4, XT-8-5, XT-12-1, XT-17-3, XT-19-2, XT-19-5, XT-19-6, XT-21-2, XT-25-1, XT-25-2, XT-XT-27-1, XT-27-2, XT-27-5, XT-30-1, XT-35-1, XT-37-2, XT-38-1, XT-39-1, XT-46-1, XT-50-1, XT-54-2, XT-56-2.

To purify the peptides XT-1-2, XT-2-1, XT-2-2, XT-2-3, XT-2-4, XT-4-4, XT-7-7, XT-10-1, XT-21-3, XT-23-1, XT-23-3, XT-28-2, XT-27-1, XT-31-2, XT-32-2, XT-34-1, XT-36-1, XT-36-2, XT-38-2, XT-39-3, XT-40-1, XT-41-1, XT-41-2, XT-51-1, XT-53-1, XT-54-1, XT-54-3, XT-55-3 we used high-voltage paper electrophoresis in pyridine acetate buffer. To obtain the peptides XT-2-4-1, XT-4-2, XT-4-3, XT-4-4-2, XT-4-4-1, XT-4-5, XT-13-1, XT-14-1, XT-14-2, XT-14-4, XT-16-1, XT-17-1, XT-23-1-1, XT-24-1, XT-29-2, XT-30-2, XT-30-3, XT-26-4, XT-31-2, XT-31-1-1, XT-32-2-1, XT-33-3, XT-33-4, XT-35-1, XT-36-2, XT-36-1-2, XT-38-2-1 in the pure form we used rechromatography in the same system as for the preparative chromatography. The homogeneity of the peptides isolated was evaluated chromatographically from the appearance of one spot on paper chromatography and high-voltage electrophoresis, and also by a determination of the number of N-terminal amino-acid residues. The results of the amino-acid analyses and of the determinations of the N-terminal amino acids are given in Table 1.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent.

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