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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

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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.

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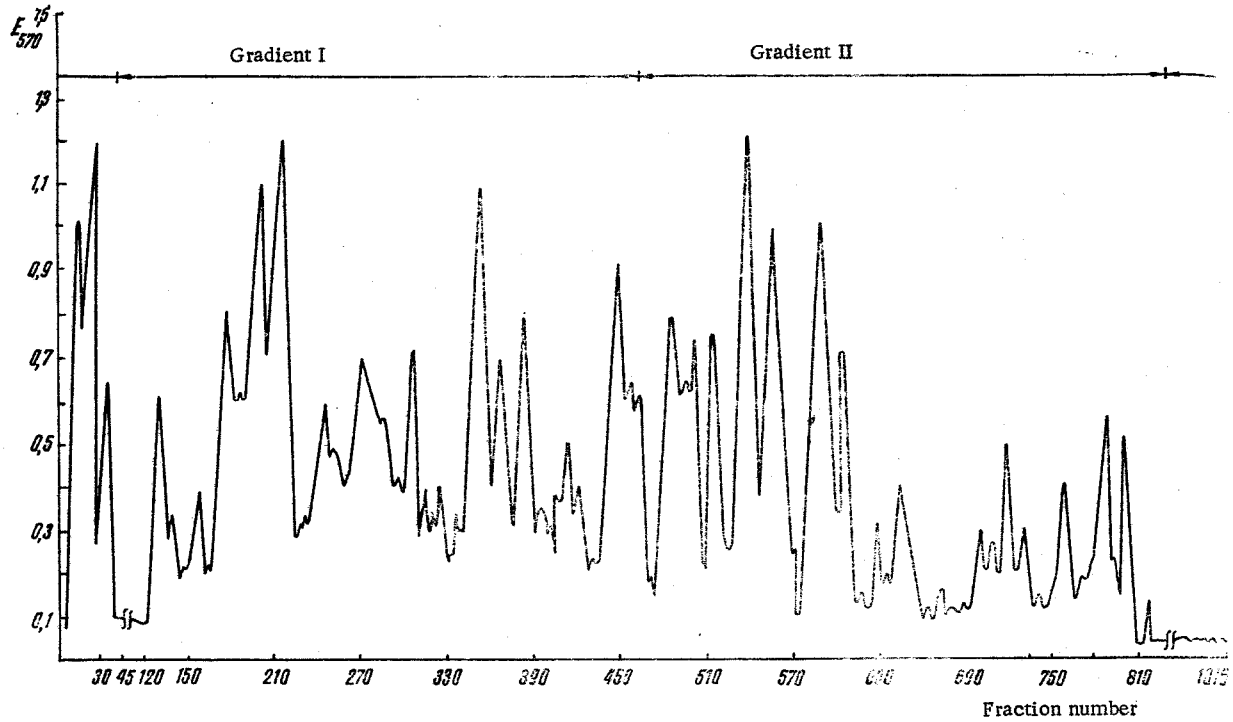

Fig. 1. Separation of a chymotrypic hydrolyzate of CM-7S-globulin on a column of Dowex 50WX4 cation-exchange resin.

EXPERIMENTAL

The isolation and carboxylation of the 7S-globulin has been described previously [2].

Hydrolysis of the CM-7S-globulin by Chmotrypsin. For chymotryptic digestion, 85 μmole of CM-7S-globulin was dissolved in 0.2 M of ammonium bicarbonate, and then chymotrypsin was added to give ratio of enzyme to substrate of 1:50. The enzyme was added in two portions, the second 6 h after the beginning of hydrolysis, which was carried out at 37°C for 18 h. Hydrolysis was stopped by the addition of glacial acetic acid to bring the pH to 3. The hydrolyzate was evaporated and the residue was dissolved in 25 ml of 30% acetic acid and this solution was used for further separation.

Separation of the Products of Chymotryptic Hydrolysis. The products of chymotryptic hydrolysis were fractionated on a column (150 × 2 cm) filled with Dowex 50WX4 cation-exchange resin, the treatment of the resin and the filling of the comumn being as described previously [1]. The rate of elution was 45 ml/h, 15-ml fractions being collected. Before the deposition of the hydrolyzate, the column was washed for two days with 0.2 M pyridine–acetate buffer, pH 3.1, at 35°C. Elution with the starting buffer (0.2 M pyridine–acetate buffer) was continued for 17 h at 35°C. Then the next fractions were eluted with gradient I by passing 0.5 M pyridine-acetat buffer, pH 5.0, (3 liters) through the mixer filled with the starting buffer (3 liters). After the end of gradient I, the temperature in the column jacket was raised to 50°C and after 1 h elution was continued with gradient II by passing 2M pyridine–acetate buffer, pH 5.0, (3 liters) through the mixer containing 0.5 M pyridine–acetate buffer. After the end of gradient II, the column was washed with 2 liters of 2 M pyridine.

Analysis of the Eluate. The peptides were revealed in the fractions by means of the ninhydrin reaction after alkaline hydrolysis [3]. The numbers of peptides in the fractions were determined roughly by paper chromatography.

Purification of the Peptides. Homogeneous peptides were isolated by descending chromatography and rechromatography on FN-17 paper ("Filtrak," GDR) in the butan-1-ol–pyridine–acetic acid–water (15:10:3:12) system and by high-voltage electrophoresis (pH 6.5; 3000 V; 2 h). The peptides were detected by spraying narrow bands of the chromatogram or the phoretogram with a 0.5% solution of ninhydrin in acetone. The peptides found were eluted from the paper with 10% acetic acid.

Characterization of the Peptides. The amino-acid compositions of the peptides were determined after hydrolysis with 6 N HCl at 110°C for 24 h on a LKB 4101 amino-acids analyzer (Sweden). The N-terminal amino-acids were determined by Gray and Hartley's method in the form of the DNS derivatives [4].

TABLE 1. Characteristics of the Chymotryptic Peptides of the CM-7S-globulin

Peptide	Amino-acid composition															Number of residues	N-terminal amino acid
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe		
XT-1-2-1																	
XT-31-2-1				0,6(1)		0,3	0,9(1)		0,6(1)	0,5	0,36					3	Asp
XT-1-3	0,7(1)			3 4(3)	0,7(1)	0,8(1)	3,5(3)		1 1(1)	1,3(1)	1,0(1)		0,9(1)		0,8(1)	14	Val
XT-2-1-1*	0,8(1)	0,6(1)		0.6(1)		0,6(1)	1,7(2)	(1)	0,5(1)	0,6	0,5(1)		0,8(1)			10	Glu
XT-2-1-2																	
XT-2-3-2	0,75 (1)			0.7(1)		0,4	1,7(2)			0,8(1)			0,5(1)			6	Leu
XT-21-2																	
XT-2-2-2				1,0(1)		0,4	1,0(1)	0,4	0,2	0,4						2	Glu
XT-2-3-1											0,8(1)		1,0(1)			2	Leu
XT-2-4-1-2*				1,1(1)			1,3(1)	(1)		0.9(1)	0,5(1)		0,6(1)		1,0(1)	7	Phe
XT-2-4-1-3				1,3(1)	0,5	0,4	1,5(1)	1,1(1)		0,5(1)	0,48		0,46		1,0(1)	5	Phe
XT-4-2-1				0,8(1)		1,0(1)	1,3(1)		0,9(1)							4	Ser
XT-34-2																	
XT-4-3	0,7(1)	0,6(1)		0,7(1)	0,9(1)	1,0(1)	1,6(2)				0,7(1)						
XT-31-1																8	Thr
XT-4-4-2				1,3(1)			1,0(1)		0,9(1)		0,5		0,4				
XT-5-2						0,3	1,0(1)			1,0(1)	0,7(1)	0,3	0,3		0,8(1)	4	Asp
XT-54-1-2																	
XT-5-3-1				0,5	0,5	0,5		1,0(1)	1,4(1)	1,4(1)	2,4(2)		1,4(1)		1,0(1)	4	Glu
XT-5-3-2			0,9(1)	0,8(1)		0,5			1,3(1)			1,2(1)			0,7(1)	7	Leu
XT-7-7-1					1,0(1)				1,0(1)						0,9(1)	5	Arg
XT-8-3						1,0(1)	1,0(1)		1,0(1)					1,0(1)	1,0(1)	3	Gly
XT-8-4			0,6(1)	0,2	0,38		1,3(1)	0,4	0,8(1)							4	Ser(Thr)
XT-8-5																	
XT-19-5				1,0(1)							1,0(1)		1,3(1)		1,3(1)	4	Gly
XT-12-1					0,32					0,9(1)						3	Asp
XT-14-4-2			1,1(1)	0,48	0,64(1)	0,7(1)	1,1(1)		1,0(1)		0,7(1)				1,0(1)	2	Ala
XT-15-1	0,8(1)		0,6(1)	2,1(2)		1,3(1)	3,2(3)		1,0(1)	1,3(1)	1,0(1)		0,7(1)		0,9(1)	7	Val
XT-16-2			0,9(1)	0,9(1)	0,8(1)	1,4(1)	0,42	1,1(1)	0,4	0,8(1)	0,8(1)	0,5	0,5			12	Ala
XT-17-1	0,8(1)			0,9(1)		1,1(1)	1,8(2)		0.7(1)		0,35				0,8(1)	8	Val
XT-17-3			0,4(1)	1,0(1)		0,6(1)	1,9(2)		0,5(1)	0,8(1)						6	Glu
XT-17-4-3		0,8(1)	0,2	0,3	1,1(1)		0,4	1,3(1)	1,2(1)	0,8(1)	1,9(2)				0,5(1)	8	Glu
XT-17-5											0,6(1)		1.0(1)		1,3(1)	8	Val
XT-18-1									1,1(1)				1,0(1)	1,0(1)	1,0(1)	3	Phe
XT-19-2					1,1(1)	1,0(1)				1,2(1)						3	Leu
XT-19-6													1,0(1)		0,8(1)	4	Ser
XT-20-2			1,0(1)	0,5			1,5(1)						0,8(1)			1	Leu
XT-23-1	0,8(1)			0,8(1)		0,7(1)	1,5(1)		1,0(1)	0,7(1)	0,7(1)		0,8(1)		1,0(1)	4	Phe
XT-25-1-1	1,2(1)		0,9(1)			1,3(1)	3,4(3)	0,5(1)		0,9(1)					0,8(1)	9	Leu
XT-25-2-2	0,6(1)		0,3	0,8(1)		0,8(1)	1,5(1)		0,6(1)			1,1(1)				8	Arg
XT-26-1	0,3		0,4			0,6(1)	1,0(1)		1,0(1)						0,7(1)	7	Glu
XT-26-2	0,4		1,1(1)	0,9(1)		0,8(1)	2,4(2)	0,8(1)	1,1(1)	0,9(1)	0,2	0,3	0,3			3	Gly(Ser)
XT-26-3			0,9(1)	1,2(1)			1,0(1)		1,0(1)		0,2	0,9(1)			0,7(1)	9	Ala
XT-27-1															1,1(1)	6	Glu
XT-27-2							1,0(1)									1	Glu
XT-27-3				1,2(1)			1,8(2)									3	Glu
XT-28-1	0,8(1)		0,6(1)	1,7(2)		0,4	1,6(2)		0,5	0,9(1)	0,3		1,1(1)			8	Glu
XT-27-5†				0,4		0,5	0,5		0,4		1,4(1)	0,4	0,5		0,2	2	Val
XT-28-2-1	0,9(1)							0,4									
XT-28-2-2				0,9(1)	0,9(1)	0,8(1)			0,3	1,2(1)					1,1(1)	6	Asp
XT-29-1							1,0(1)									1	Glu
XT-29-2-1	0,6(1)		0,7(1)	0,3			1,6(2)			0,7(1)			0,5(1)			6	Arg
XT-30-1			0,9(1)	0,6(1)			0,9(1)		0,6(1)							4	Asp
XT-30-2			0 9(1)	0,5(1)		0,9(1)	1,4(2)		0,9(1)		1,2(1)					7	Ser
XT-30-3-1	0,3	0,5	0,7(1)	1,3(1)		0,5(1)	1,2(1)		1,1(1)	0,3				0,8(1)	0,9(1)	7	Phe
XT-30-3-2**	0,7(1)	1,3(1)	0,7(1)	1,4(1)			1,6(2)		1,1(1)	0,9(1)	0,7(1)	0,9(1)	0,7(1)	0,8(1)	0,7(1)	13	Val
XT-31-2-1				0,9(1)			1,1(1)		1,1(1)		1,1(1)					3	Gly
XT-31-2-2												0,5					
XT-37-2		0,3	0,7(1)	1,2(1)		0,3	1,4(1)		1,1(1)						0,8(1)	5	Glu
XT-32-1-1	1,1(1)	0,9(1)															
XT-32-2-1				0,9(1)	0,8(1)	0,8(1)	2,0(2)		0,3						0,5	7	Ser(Thr)

TABLE 1 (continued)

Peptide	Amino-acid composition															Number of residues	N-terminal amino acid
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe		
XT-33-2-2			1,2(1)	0,9(1)		0,3	0,6(1)		0,6(1)			0,9(1)			1,1(1)	6	Arg
XT-33-3-2			1,2(1)				1,2(1)			0,4		1,0(1)	1,2(1)			4	Glu
XT-35-1-1	0,9(1)		1,4(1)			2,2(2)	4,0(4)		1,0(1)	1,0(1)					0,9(1)	11	Arg
XT-36-1-1			0,9(1)	0,3	0,8(1)	0,6(1)	1,1(1)	0,4	0,7(1)					0,6(1)		6	Glu
XT-36-1-2-2			1,0(1)				1,0(1)					0,5(1)	0,5(1)			4	Glu
XT-38-1		0,9(1)	0,9(1)	0,9(1)			2,2(2)		1,0(1)						1,2(1)	7	His
XT-39-1																	
XT-38-2-3	0,5(1)		0,7(1)	1,4(1)	1,1(1)	0,8(1)	0,9(1)		1,0(1)	0,6(1)	0,4(1)	0,3	0,5(1)		0,9(1)	10	Arg
XT-38-2-4	0,8(1)		0,3	0,5	0,4		1,1(1)		1,0(1)				0,5			3	Gly
XT-39-3-1	0,7(1)			1,2(1)	0,6(1)	0,8(1)	0,8(1)		0,8(1)	0,9(1)	0,3		0,5		0,9(1)	8	Gly
XT-44-2			1,0(1)							1,0(1)		0,8(1)	0,8(1)			4	Ile
XT-51-2																	
XT-46-1		0,9(1)	1,0(1)			1,1(1)	1,4(1)		2,0(2)				1,0(1)	0,7(1)		8	Leu
XT-50-1			0,8(1)								0,9(1)		1,3(1)			3	Val
XT-55-3-1						0,7(1)	1,2(1)		0,9(1)					0,4(1)	0,5(1)	5	Ser
XT-55-3-2			1,0(1)				0,4			0,9(1)					1,0(1)	3	Ala
XT-56-2			1,0(1)	0,1		·			0,2	1,0(1)			1,0(1)		1,0(1)	4	Leu

*The peptides each contain one CM-cysteine residue.

†The peptide contains one methionine residue.

SUMMARY

From a chymotryptic hydrolyzate of the CM-7S-globulin of cotton seeds we have isolated and characterized in relation to amino-acid composition and N-terminal amino acids 65 homogeneous peptides, 38 of which contain arginine or lysine residues and are overlapping.

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ISOLATION OF AN INTRACELLULAR LIPASE FROM THE HEAT-TOLERANT FUNGUS Rhizopus microsporus UzLT-1 AND ITS PROPERTIES

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The comprehensive study of the intracellular and extracellular enzymes secreted by microorganisms is important for explaining the physiological roles of particular enzymes and the routes of their biosynthesis and secretion by the cells. We have previously reported that the fungus Rhizopus microsporus UzLT-1 discharges into the surrounding medium two extracellular lipases [1] and have described methods for their purification [2]. In the present paper we give a method for the isolation and purification of an intracellular lipase from the mycelium from the fungus Rhizopus microsporus, UzLT-1.

The fungus was grown by a known method [1]. The fungal mycelium was separated from the culture liquid by filtration and was washed with distilled water until the filtrate was clear. The washed mycelium was broken down in 0.005 M phosphate buffer, pH 7.5, with glass beads in a mortar and was then separated by vacuum filtration on a Buchner funnel. The enzyme was concentrated with the aid of dry washed Molselekt G-25 (from the firm "Reanal"). All the subsequent purification was carried out at 2-4°C. The concentrated enzyme solution was dialyzed first against distilled water and then against 0.005 M phosphate buffer, pH 7.5. The precipitate that deposited after dialysis was separated by centrifuging. The supernatant liquid, containing 39.84 units of lipase activity, was deposited on a column of DEAE-Sephadex A-50 equilibrated with the initial buffer. The protein was eluted with phosphate buffer, using a stepwise concentration gradient from 0.005 to 0.2 M followed by the use of the buffer containing 0.3 M NaCl.

Three protein fractions were obtained (Fig. 1). The first fraction, eluted by the initial buffer, and the third, eluted by 0.2 M phosphate buffer containing 0.3 M NaCl, exhibited lipase activity.

The fraction corresponding to the first active peak (Fig. 2) was concentrated and dialyzed against 0.005 M acetate buffer, pH 5.6 overnight. The dialyzed enzyme solution was filtered and deposited on a column of SE-Sephadex C-50 previously equilibrated with 0.005 M acetate buffer, pH 5.6.

Under these conditions, the bulk of the inactive protein was washed out with the free volume. The column was washed with the initial buffer at the rate of 30 ml/h until the absorption of the eluate at 280 nm reached 0.01. The lipase was eluted with acetate buffer with stepwise increasing concentration to 0.05 M, pH 5.6. The results of purification are given in Table 1. After purification an electrophoretically homogeneous fraction of lipase the specific activity of which was 16 times greater than the specific activity of the first extract was obtained.

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