PROTEINS OF THE SEEDS OF Ricinus communis.

IV. AMINO ACID SEQUENCE OF THE ALANINE SUBUNIT OF RICIN T FROM THE SEEDS OF CENTRAL ASIAN CASTER OIL PLANT. PEPTIDES FROM LIMITED TRYPTIC HYDROLYSIS

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To establish the primary structure of the CM-alanine subunit of ricin T, its limited tryptic hydrolysis has been performed. With the aid of Aminex Q-150S, followed by high-voltage paper electrophoresis, 14 peptides, amounting in total to 256 amino acid residues, have been isolated. The complete amino acid sequences of nine peptides and the partial sequences of five have been determined.

At the present time, several isoforms of ricin have been isolated from castor beans and characterized: ricin D [1], ricin E [2], ricin variant [3], and ricin T [4]. In all the isoforms, the isoleucine subunits are identical, but differences are found in the amino acid sequences of some sections of the polypeptide chains of the alanine subunits [5]. Consequently, for a definitive answer to the question of the polymorphism of the ricins and also for the functional distinction between them a comparative investigation of the primary structures of these subunits was necessary.

In the present paper we consider the isolation and determination of the structures of tryptic peptides of the carboxymethylated alanine subunit of ricin T.

According to the results of amino acid analysis, the alanine subunit that we investigated contained 256 amino acid residues, including seven lysine and 14 arginine residues [6]. Consequently, on exhaustive cleavage by trypsin one could expect to obtain not less than 22 peptides.

To select the conditions and time of hydrolysis we used the method of peptide maps, which enabled the number of peptides to be judged (Fig. 1). On hydrolysis with trypsin for two hours, 15 peptides were formed, and on hydrolysis for 18 hours more than 30. Therefore, to obtain large peptides convenient for subsequent structural investigations and also to prevent nonspecific cleavage we performed trypsin hydrolysis for two hours.

The hydrolysate was separated with the aid of ion-exchange chromatography on Aminex Q-150 S in a concentration and pH gradient of pyridine-acetate buffers (Fig. 2).

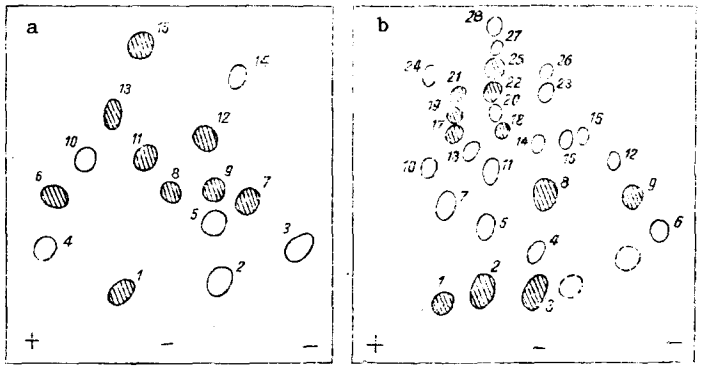

Fig. 1. Peptide map of the CM-alanine subunit of ricin T (the arginine-containing peptides are hatched): a) two-hour hydrolysis by trypsin; b) 18-hour hydrolysis.

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TABLE 1. Peptides Isolated from Fractions of a Limited Tryptic Hydrolysate of the Alanine Subunit of Ricin T

Peptide	Total residues
T-1	T-1-1, T-1-2
T-2	T-2-1 T-2-2,
T-3	T-3-1, T-3-2, T-3-3
T-4	T-4-1, T-4-2
T-5	T-5-1, T-5a-1, T-5b-1, T-6-2
T-6	T-8-3, T-6-1
T-7	T-5-2, T-5a-2, T-5b-2, T-7-2, T-8-4, T-9-2
T-8	T-7-1, T-12-2
T-9	T-8-1, T-12-1
T-10	T-8-2, T-9-1, T-10
T-11	T-9-3.
T-12	T-11, T-12-3, T-12-3
T-13	T-13-1
T-14	T-14

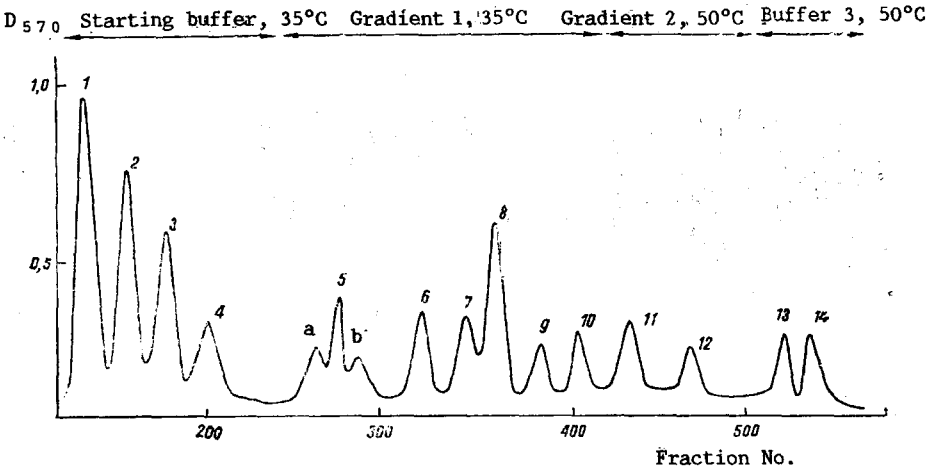

Fig. 2. Chromatographic separation of the peptides of a tryptic hydrolysate on a column of Aminex Q-150 S in pyridine-acetate buffer. Column 1.2 × 100 cm, rate 20 ml/h. Starting buffer: 0.2 M pyridine-acetate, pH 3.1; gradient 1: 0.2 M + 0.5 M pyridine acetate, pH 5.0, 200 ml each, gradient 2: 0.5 M + 2 M pyridine acetate, pH 5.0; volume of the buffers 200 ml each; [final] buffer: 4 M pyridine-acetate, pH 6.4 (100 ml).

The separation of the hydrolysate yielded 16 combined fractions. Analysis of the fractions with the aid of chromatography in a thin layer of cellulose and determination of the N-terminal amino acids showed that three fractions (10, 11, and 14) were homogeneous and did not require further purification. The other fractions consisted of mixtures of peptides. Further purification was performed with the aid of preparative chromatography on Whatman 3 MM paper and by high-voltage paper electrophoresis.

By paper chromatography, fractions T-1, T-2, T-4, and T-6 yielded eight homogeneous peptides, and by electrophoresis in pyridine-acetate buffer at pH 6.4 fractions T-3, T-5, T-5a, T-5b, T-7, T-8, T-9, T-12, T-13 gave 24 peptides, which were designated T-1-1, T-1-2, T-2-1, T-2-2, T-4-1, T-4-2, T-6-1, T-6-2, T-3-1, T-3-2, T-3-3, T-5-1, T-5-2, T-5a-1, T-5a-2, T-5b-1, T-5b-2, T-7-1, T-7-2, T-8-1, T-8-2, T-8-3, T-8-4, T-9-1, T-9-2, T-9-3, T-12-1, T-12-2, T-12-3, T-13-1, T-13-2 and T-13-3.

The homogeneity of the peptides isolated was evaluated both chromatographically and by determinations of their N-terminal amino acid residues. A total of 35 homogeneous peptides were isolated. It must be stated that many of the peptides proved to be identical with one another in amino acid composition and partial amino acid sequence.

Table 1 shows the combined peptides. The results of amino acid analysis and the N-terminal acid residues are given in Table 2.

TABLE 2. Characteristics of the Peptides from the Limited Tryptic Hydrolysate of the CM Alanine Chain of Ricin T

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7
Cys (Cm)	2,45 (2)	2,0 (2)	1,0 (1)		1,0 (1)		
Asp	6,95 (7)	4,55 (5)	1,59 (2)	2,42 (2)	3,56 (4)	3,04 (3)	1,05 (1)
Thr	8,5 (9)	3,77 (4)		1,34 (1)	1,0 (1)	1,0 (1)	
Ser	5,5 (6)	3,92 (4)			2,0 (2)	1,0 (1)	1,5 (2)
Glu	6,93 (7)	0,92 (1)	0,95 (1)	1,98 (2)		4,61 (5)	
Pro	2,05 (2)	0,57 (1)	2,0 (2)				0,98 (1)
Gly	4,75 (5)	1,77 (2)		0,90 (1)		2,23 (2)	
Ala	2,77 (3)	1,77 (2)	1,0 (1)			2,25 (2)	0,95 (1)
Val	5,54 (6)	2,54 (3)	1,0 (1)				
Met		0,98 (1)	0,55 (1)				
Ile	2,77 (3)	0,93 (1)	1,06 (1)	2,42 (2)	1,05 (1)	1,0 (1)	
Leu	7,06 (7)	1,77 (2)		0,90 (1)	1,0 (1)	3,20 (3)	1,12 (1)
Tyr	1,82 (2)	3,82 (4)				1,22 (1)	
Phe	0,65 (1)						
His							
Lys	0,77 (1)	1,06 (1)				1,0 (1)	0,98 (1)
Arg	0,79 (1)	2,06 (2)	1,09 (1)	0,8 (1)	1,0 (1)	1,0 (1)	
Total residues	62	35	11	10	11	21	7
N-terminal amino acid	Thr	Asp	Ala	Glu	Asp	Ala	Ala

Amino acid	T-8	T-9	T-10	T-11	T-12	T-13	T-14
Cys (Cm)	1,0 (1)		1,0 (1)			1,0 (1)	
Asp	3,86 (4)	1,61 (2)	1,65 (2)		2,04 (3)	2,34 (2)	1,0 (1)
Thr	1,98 (2)			1,05 (1)	2,0 (2)		
Ser	2,54 (3)	0,85 (1)			1,04 (1)		
Glu	0,98 (1)	1,65 (2)			1,0 (1)	1,0 (1)	
Pro	0,98 (1)	2,54 (3)				0,65 (1)	
Gly	3,04 (3)	1,65 (2)	1,95 (2)	1,0 (1)		1,05 (1)	0,85 (1)
Ala	1,0 (1)				1,05 (1)	1,0 (1)	
Val	2,04 (2)	0,60 (1)	3,04 (3)	1,95 (2)			
Met	0,65 (1)						
Ile	1,65 (2)	1,95 (2)	0,60 (1)		0,84 (1)	0,88 (1)	
Leu	4,05 (4)	3,65 (4)	0,65 (1)		0,94 (1)	0,87 (1)	
Tyr	1,0 (1)	0,65 (1)					
Phe	1,05 (1)	0,98 (1)				0,64 (1)	
His	0,98 (1)	0,94 (1)				0,75 (1)	
Lys	1,0 (1)			0,95 (1)		0,85 (1)	
Arg			1,54 (2)		1,0 (1)		0,95 (1)
Total residues	29	20	12	5	11	12	3
N-terminal amino acid	Ile	Glu	Ile	Gly	Ser	Ala	Asp

TABLE 3. Amino Acid Sequences of the Peptides of the Limited Tryptic Hydrolysate

Peptide	Amino acid sequence	Number of residues
T-1*	Thr-Ser-Leu-Val-Asp-Thr-Leu-(CmCys2, Asp6, Thr7, Ser5, Glu7, Pro2, Gly5, Ala3, Val5, Ile3, Leu5, Tyr2, Phe1, Lys1, Arg1)	62
T-2	Asp-Asn-Ser-Leu-Arg-Ser-Asp-(CmCys2, Asp2, Thr4, Ser2, Glu1, Pro1, Gly2, Ala2, Val3, Met1, Ile1, Leu1, Tyr4, Lys1, Arg1)	35
T-3	Ala-Asp-Val-CmCys-Met-Asn-Pro-Ile-Pro-Glu-Arg	11
T-4*	Glu-Ile-Trp-Asx-Asx-Gly-Thr-Ile-Leu-Glx-Arg	11
T-5	Asp-Asn-CmCys-Leu-Thr-Ser-Asp-Ser-Asn-Ile-Arg	11
T-6	Ala-Glu-Gln-Trp-Asp-Glu-Gln-Gly-(Asp2, Thr1, Ser1, Glu1, Gly1, Ala1, Ile1, Leu3, Tyr1, Lys1, Arg1)	22
T-7	Ala-Ser-Asn-Pro-Ser-Leu-Lys	7
T-8	Ile-Leu-Ser-CmCys-Gly-Pro-Ala-(Asp4, Thr2, Ser2, Glu1, Gly2, Ile1, Met1, Leu3, Val2, Tyr1, Phe1, Lys1, Arg1)	29
T-9	Glu-Ile-Leu-Tyr-Pro-Val-Trp-Gly-(Asp2, Ser1, Glu1, Pro2, Gly1, Ile1, Leu3, Phe1, His1)	21
T-10	Ile-Val-Gly-Arg-Asn-Gly-Leu-CmCys-Val-Asn-Val-Arg	12
T-11	Gly-Thr-Val-Val-Lys	5
T-12	Ser-Gln-Thr-Asp-Ala-Asp-Asn-Ile-Thr-Leu-Arg	11
T-13	Ala-Asp-Pro-Gly-Asn-Phe-Leu-Glu-Ile-Trp-His-CmCys-Lys	13
T-14	Asp-Gly-Arg	3

*Carbohydrate-containing peptides.

To determine the N-terminal acid sequences of the peptides we used Edman's method with identification of the amino acids in the form of their 1-dimethylaminonaphthalene-5-sulfonyl (DNS) derivatives [7, 8], or of the phenylthiohydantoin (PTH) derivatives for determining dicarboxylic amino acids and their amines [8]. Tryptophan- and carbohydrate containing peptides were determined qualitatively with the aid of the Ehrlich reagent [10] and by the reaction with orcinol [11]. Tryptophan was detected in peptides T-1, T-8, T-9, and T-13, and carbohydrate in peptides T-1 and T-4.

The structures of the carbohydrate-containing peptides were determined by the method of Konisberg and Hill [12]. The results of the determination of the amino acid sequences of the peptides isolated are given in Table 3. In the course of the determination of the amino acid sequences, an uncleaved Arg-Ser bond was detected in peptide T-2 and an uncleaved Arg-Asn bond in peptide T-10, while lysine and arginine residues were detected in peptides T-1, T-2, T-6, and T-8. The stability of the peptide bonds of Lys and Arg with dicarboxylic amino acids has been reported previously [13-16].

Thus, as the result of limited tryptic hydrolysis, 14 individual peptides comprising a total of 256 amino acid residues were isolated. The complete structures of nine and the partial structures of five peptides have been established.

EXPERIMENTAL

Trypsin from the USA, Aminex Q-150S and paper for PC and for EP from the United Kingdom, FN-17 paper from the GDR, KSK silica gel with a particle size L5/40 μ [sic] from Czechoslovakia, dansyl chloride from Serva, and polyamide plates (6 × 6 cm) from the United Kingdom were used. The remaining reagents were used after preliminary purification.

Tryptic Hydrolysis. A solution of 950 mg of the protein in 10 ml of 0.2 M ammonium acetate buffer, pH 8.0, was treated with a trypsin solution to give an enzyme:substrate ratio of 1:50 (by weight). Hydrolysis was carried out at 37°C for 2 h. After its termination, the hydrolysate was acidified to pH 3.1 and was freeze-dried.

Peptide Maps. A solution of 0.4 mg of the freeze-dried tryptic hydrolysate in 100 μl of 50% pyridine deposited on a 20 × 20 cm plate with a thin layer of type FND cellulose (GDR) was chromatographed in the butan-1-ol—water—acetic acid—pyridine (15:12:3:10) system. This was followed by electrophoresis for 45 minutes in puridine-acetate buffer, pH 6.5, at U = 800 V, I = 25 mA, on a cooled plate. The spots were revealed with a 0.1% solution of ninhydrin in acetone. Ehrlich's reagent was used to detect tryptophan-containing peptides [10].

Chromatography on a Column of Aminex Q-150 S. A solution of 950 mg of the products of tryptic hydrolysis in 100 ml of 0.2 M pyridine-acetate buffer, pH 3.1 (starting buffer) was deposited on a 1.2 × 100 cm column (Sweden) equilibrated with the same buffer. The preparation of the ion-exchanger, deposition, and the analysis of the eluates were carried out as described in [18].

The peptides were purified both by chromatography and by electrophoresis on Whatman 3MM and FN-17 papers. Chromatography was conducted in the butan-1-ol—acetic acid—pyridine—water (15:3:10:12) system. Paper electrophoresis was carried out for 20 min in the pyridine-acetic acid—water (100:4:896) system at pH 6.4 and 5.3 with a voltage of 1500 V at −7°C. The chromatogram was dried at 30°C, cut into strips, and stained with 0.2% ninhydrin in acetone. Then the corresponding zones were eluted successively with a 50% solution of pyridine and a 1% solution of ammonia.

Amino Acid Analysis. Samples were hydrolyzed with 5.7 N HCl at 110°C for 24 h. Amino acid analysis was performed on a LKB-4101 amino acid analyzer. Cystine was determined in the form of carboxymethylcysteine in hydrolysate of the reduced and carboxymethylated peptide. To determine the tryptophan content of a peptide, the protein was hydrolyzed with p-toluenesulfonic acid [19].

SUMMARY

1. On limited trypsin hydrolysis, the CM-alanine subunit of ricin T yielded 14 individual peptides comprising a total of 256 amino acid residues.

2. The complete amino acid sequence of nine peptides and the partial sequences of five peptides have been established.

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USE OF THE SILYLATION REACTION IN SYNTHESIS OF FRAGMENT 1-4 OF THE ACTH SEQUENCE

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A new effective method of synthesizing fragment 1-4 of the ACTH sequence ensuring a high overall yield of the desired product is proposed. This result is achieved thanks to the wide use of the silylation reaction in the synthesis, which has permitted a considerable simplification of the process and the avoidance of the formation of by-products. The peptides synthesized have been characterized by their angles of optical rotation, chromatographic mobilities, and melting points. A table of chemical shifts in the ^{13}C NMR spectra of the final and intermediate compounds is given.

In recent years, the silylation reaction has found ever wider employment in organic synthesis [1]. Its use considerably simplifies the synthesis, since it permits the reaction to be performed with free amino acids. Such an approach eliminates the stage of obtaining esters of the amino acids and their hydrolysis in an alkaline medium. Furthermore, the trimethylsilyl group ensures the protection of the free hydroxy groups of amino acids [2] and, which is of no little importance, is easily eliminated during the usual acid-alkali washings.

Known methods of obtaining fragment 1-4 of the sequence of ACTH are, as a rule, either multistage, since they involve the preparation of amino acid derivatives with protected side chains [3, 4] or, where amino acid derivatives with unprotected side chains are used, lead to the desired product with a low overall yield of the order of 20% [5, 6].

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