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PRIMARY STRUCTURE OF THE ALANINE SUBUNIT OF RICIN T FROM THE SEEDS OF THE CENTRAL ASIAN CASTOR OIL PLANT VI. TRYPTIC PEPTIDES OF THE CYANOGEN BROMIDE FRAGMENTS B-1, B-2, AND B-3

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Tryptic hydrolysates of cyanogen bromide fragments B-1, B-2, and B-3 have been studied. As a result, 23 tryptic peptides have been isolated and characterized and the primary structures of 22 of them have been established.

The isolation of four cyanogen bromide fragments from the carboxymethylated chain of ricin T has been reported previously. Their amino acid compositions and, partially, their N-terminal sequences were investigated and the order of their arrangement in the polypeptide chain of the protein was established [1].

The task of the present work was the isolation and characterization of tryptic peptides from three large fragments (B-1, B-2, and B-3) and the determination of their amino acid sequences.

Peptides B-1, B-2, and B-3 were treated with the proteolytic enzyme trypsin, which possesses the highest action specificity and cleaves peptide bonds formed by the carboxy groups of lysine and arginine. The tryptic hydrolysis of fragments including the above-mentioned amino acids is, therefore, of particular interest for establishing their structures.

Fragments B-1, B-2, and B-3 were hydrolyzed by a method we have described previously [2]. The hydrolysis products were dissolved in 0.2 M pyridine acetate buffer, pH 3.1 (part of the hydrolysate of the B-1 fragment was insoluble) and fractionated on the cation-exchange resin Aminex Q-150S as described in [3]. Elution was conducted with concentration and pH gradients of pyridine acetate buffers. Aliquots of each even-numbered fraction were analyzed by reaction with ninhydrin after alkaline hydrolysis. A chromatographic diagram of the results is given in Fig. 1. Separation yielded 8, 9, and 5 combined fractions from the hydrolysates of B-1 (*a*), B-2 (*b*), and B-3 (*c*), respectively. The peptide compositions of the combined fractions were investigated by chromatography in a thin layer of cellulose (TLC). Their compositions are shown in Fig. 2.

As can be seen from Fig. 2, according to TLC ten fractions — 1, 4, and 8 (*a*), 1, 3, 6, and 9 (*b*), and 1, 4, and 5 (*c*) — were homogeneous and did not require purification. The other fractions, consisting of mixtures of peptides, were additionally purified by PC on Whatman 3 MM paper. As a result, 26 peptides were isolated in the individual form, after which their purity was evaluated by TLC and their N-terminal amino acids were determined by the dansyl method [4].

From the soluble part of the tryptic hydrolysates of the cyanogen bromide fragments we isolated and purified a total of 36 peptides, which considerably exceeded the number theoretically possible.

Determinations of the amino acid compositions and partial N-terminal sequences of the peptides showed that many of them were identical both in composition and in the amino acid sequence of the polypeptide chain. Identical peptide fragments were therefore combined into 22 peptide fractions, which we have denoted as B-1-T-1——B-1-T-8, B-2-T-1——B-2-T-9, and B-3-T-1——B-3-T-5.

From the insoluble part of a hydrolysate of B-1 we isolated the peptide B-1-T-9 by dissolving the residue in a 4 M solution of pyridine in 30% acetic acid.

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TABLE 1. Amino Acid Compositions of the Tryptic Hydrolysates of Fragment B-1

Amino acid	B-1-T-1*	B-1-T-2	B-1-T-3	B-1-T-4	B-1-T-5	B-1-T-6	B-1-T-7	B-1-T-8	B-1-T-9
Cm-Cys		1.9(1)		0.9(1)	0.8(1)			1.1(1)	1.5(2)
Asp	1.8(2)		1.1(1)	2.1(2)	3.7(4)				6.5(7)
Thr	0.9(1)	1.1(1)		1.6(2)	0.9(1)	0.8(1)			8.7(9)
Ser		1.8(2)	1.1(1)	0.9(1)	1.7(2)				6.7(7)
Glu	1.6(2)	1.1(1)	2.9(3)	0.7(1)				1.7(2)	6.8(7)
Pro		0.9(1)							1.7(2)
Gly	1.2(1)	1.7(2)	1.6(2)			1.2(1)			5.6(6)
Ala		0.9(1)	2.1(2)	1.6(2)		1.5(2)			2.6(3)
Val									5.7(6)
Ile	1.6(2)	0.8(1)	1.1(1)	0.9(1)	0.9(1)				2.7(3)
Leu	1.0(1)	0.8(1)	0.9(1)		0.6(1)				6.8(7)
Tyr			0.7(1)	0.6(1)					1.6(2)
Phe									0.7(1)
His									
Lys			0.8(1)			0.8(1)			0.8(1)
Arg	1.0(1)	0.9(1)		0.7(1)	0.9(1)			0.9(1)	
Hse							+		+
Trp**	+		+				+	+	
N-Terminal amino acids	Glu	Ile	Ala	Ile	Asp	Gly	Trp	Thr	Thr
Number of residues	11	12	14	12	11	5	2	5	63

* Carbohydrate determined by reaction with orcinol.

** Tryptophan determined with the aid of the Ehrlich reagent.

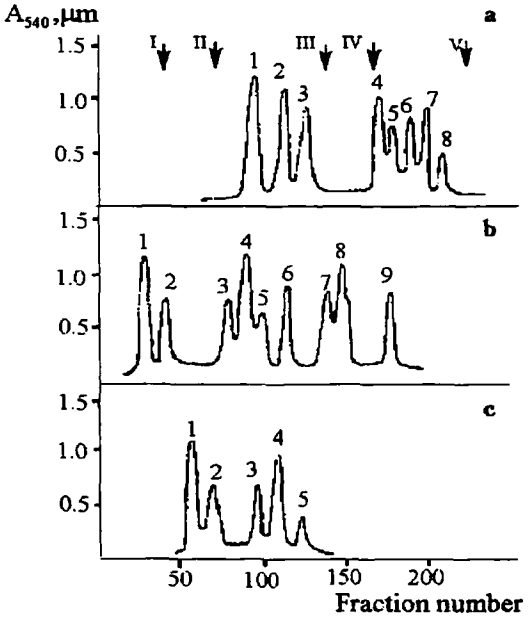

Fig. 1. Chromatography of tryptic peptides from the cyanogen bromide fragments B-1 (*a*), B-2 (*b*), and B-3 (*c*) on a column (0.9 × 60 cm) of Aminex Q-150S in a concentration and pH gradient of pyridine acetate buffers: I) 0.2 M pyridine acetate buffer, pH 3.1; II) I + 0.5 M, pH 5.0; III) II + 2 M, pH 5.0; IV) III + 2 M pyridine; V) 4 M pyridine.

TABLE 2. Amino Acid Compositions of the Tryptic Hydrolysates of Fragment B-2

Amino acid	B-2-T-1	B-2-T-2	B-2-T-3	B-2-T-4	B-2-T-5	B-2-T-6	B-2-T-7	B-2-T-8	B-2-T-9
Cm-Cys						0.9(1)	0.8(1)		0.9(1)
Asp		0.9(1)	1.6(2)	0.8(1)	2.6(3)	1.7(2)	1.6(2)	0.9(1)	
Thr					1.7(2)				1.6(2)
Ser		0.6(1)	0.7(1)		0.7(1)				0.9(1)
Glu					0.6(1)	0.8(1)		0.9(1)	
Pro						0.7(1)		1.6(2)	0.8(1)
Gly	0.8(1)	0.7(1)		0.9(1)		0.8(1)	0.9(1)		0.9(1)
Ala					0.5(1)	0.7(1)			
Val							0.7(1)		2.6(3)
Ile	0.6(1)				0.6(1)	0.8(1)		1.0(1)	
Leu	0.9(1)		0.9(1)		0.8(1)	0.7(1)	0.9(1)		1.2(1)
Tyr									2.7(3)
Phe						0.7(1)			
His						0.6(1)			
Lys		0.9(1)				0.8(1)			
Arg	0.7(1)		0.7(1)	0.6(1)	0.6(1)		0.9(1)	1.2(1)	
Hse									+
Trp*						+			
N-Terminal amino acids	Ile	Ser	Asp	Asp	Ser	Ala	Asp	Asn	Cm-Cys
Number of residues	4	4	5	3	11	12	7	6	14

* Tryptophan determined qualitatively with the aid of the Ehrlich reagent.

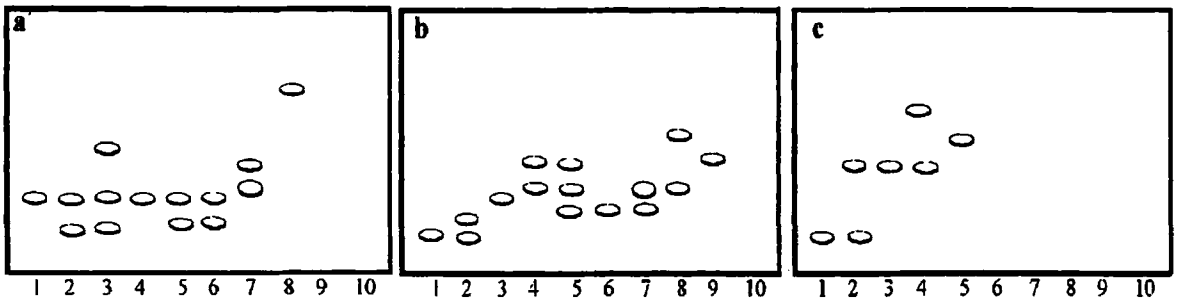

Fig. 2. Thin-layer chromatography of the tryptic hydrolysates of cyanogen bromide fragments B-1 (*a*), B-2 (*b*), and B-3 (*c*) on a cellulose plate in the butan-1-ol—pyridine—acetic acid—water (15:10:3:10) system.

The tryptophan contents of the peptides were determined by the reaction with *p*-dimethylaminobenzaldehyde [5] and the carbohydrate contents by the reaction with orcinol [6]. Tryptophan residues were detected in the peptides B-1-T-4, B-1-T-3, B-1-T-7, B-1-T-8, B-1-T-9, B-2-T-6, and B-3-T-4. Carbohydrates were detected in peptides B-1-T-1 and B-1-T-9.

Thus, as a result of separation on a column of the ion-exchange resin Aminex Q-150S followed by PC of the products of the tryptic hydrolysis of the cyanogen bromide fragments B-1, B-2, and B-3 we have isolated the 23 peptides the compositions of which are given in Tables 1—3. The amino acid sequences of the peptides isolated were determined by the Edman method in the DNS modification, and the PHT derivatives of the amino acids in a thin layer of silica gel [7] and on a polyamide plate by Woods' method [8]. Tryptophan in the peptides was determined in a thin layer of cellulose with the Ehrlich reagent [9] after each step of the degradation of the peptide fragments.

TABLE 3. Amino Acid Compositions of the Tryptic Hydrolysates of Fragment B-3

Amino acid	B-3-T-1	B-3-T-2	B-3-T-3	B-3-T-4	B-3-T-5
Cm-Cys					
Asp		0.9(1)	4.5(5)		1.8(2)
Thr			0.9(1)		
Ser		1.6(2)	0.9(1)	0.7(1)	
Glu					1.7(2)
Pro		0.9(1)			2.7(3)
Gly			0.7(1)	0.8(1)	0.6(1)
Ala		0.9(1)			
Val			1.6(2)		1.2(1)
Ile			0.9(1)		1.9(2)
Leu		0.9(1)	2.6(3)	1.9(2)	1.6(2)
Tyr			0.9(1)		0.8(1)
Phe	0.7(1)			0.6(1)	
His				0.7(1)	
Lys	0.8(1)	0.6(1)			
Arg			0.9(1)		
Hse					
Trp*					+
N-Terminal amino acids	Phe	Ala	Asn	His	Glu
Number of residues	2	7	16	6	15

*Tryptophan determined qualitatively with the aid of the Ehrlich reagent.

TABLE 4. Amino Acid Sequences of Peptides from Tryptic Hydrolysates of the Cyanogen Bromide Fragments B-1, B-2, and B-3

Peptide	Amino acid sequence
B-1-T-1*	Glu-Ile-Trp-Acx-Acx-Glu-Thr-Ile-Leu-Glu-Arg
B-1-T-2	Ile-Ser-CmCys-Gly-Pro-Ala-Thr-Ser-Gly-Glu-Arg
B-1-T-3	Ala-Glu-Gln-Gln-Trp-Ala-Leu-Tyr-Gly-Asp-Ser-Ile-Lys
B-1-T-4	Ile-Tyr-Asn-CmCys-Asn-Tyr-Ala-Thr-Ser-Ala-Gln-Arg
B-1-T-5	Asp-Asp-CmCys-Leu-Thr-Ser-Asp-Ser-Asn-Ile-Arg
B-1-T-6	Gly-Thr-Val-Val-Lys
B-1-T-7	Thr-Hse
B-1-T-8	Trp-Gln-Gln-Asp-Arg
B-1-T-9*	Thr-Ser-Leu-Val-Leu-Ala-Asp-Thr (CmCys2, Asp6, Thr7, Ser6, Glu7, Pro, Gly6, Ala2, Val5, Leu5, Ile3, Tyr2, Phe1)
B-2-T-1	Ile-Val-Gly-Arg
B-2-T-2	Ser-Asp-Gly-Lys
B-2-T-3	Asp-Asn-Ser-Leu-Arg
B-2-T-4	Asp-Gly-Arg
B-2-T-5	Ser-Gln-Thr-Asp-Ala-Asp-Asn-Ile-Thr-Leu-Arg
B-2-T-6	Ala-Asn-Pro-Gly-Asn-Phe-Leu-Glu-Ile-Trp-His-CmCys-Lys
B-2-T-7	Asp-Gly-Leu-CmCys-Asn-Val-Arg
B-2-T-8	Asn-Pro-Ile-Pro-Glu-Arg
B-2-T-9	CmCys-Val-Thr-Thr-Leu-Tyr-Pro-Ser-Gly-Val-Tyr-Val-Hse
B-3-T-1	Ala-Ser-Asn-Pro-Ser-Leu-Lys
B-3-T-2	Phe-Lys
B-3-T-3	Asn-Ser-Gly-Thr-Ile-Leu-Asp-Tyr-Asn-Asp-Leu-Val-Leu-Asp-Val-Arg
B-3-T-4	Glu-Ile-Leu-Tyr-Pro-Val-Trp-Gly-Asn-Pro-Asn-Glu-Ile-Pro-Leu
B-3-T-5	His-Leu-Phe-Leu-Gly-Ser

* Carbohydrate-containing peptides.

To determine the structures of the carbohydrate-containing peptides we used Edman's method in Konisberg's modification [10].

The results of determination of the amino acid sequences of the tryptic peptides isolated are given in Table 4.

As can be seen from Table 4, the hydrolysis of fragment B-1 formed nine peptides, and the complete structures of eight of them were determined. The N-terminal peptide fragment was B-1-T-4, and the C-terminal fragment B-1-T-7. The sequence of eight amino acid residues was established for peptide B-1-T-9.

Fragment B-2 yielded nine tryptic peptides, and the structures of all of them were established. The N- and C-terminal peptides were B-2-T-8 and B-2-T-9, respectively. From a hydrolysate of fraction B-3 we obtained five tryptic peptides and established the structures of all of them. The N-terminal peptide was B-3-T-2, and the C-terminal one B-3-T-5.

EXPERIMENTAL

Tryptic Hydrolysis. The cyanogen bromide fragments B-1, B-2, and B-3 (300 mg in each case) were dissolved in 10 ml of 0.2 M ammonium acetate buffer, pH 8.0. Trypsin was added to this solution in a ratio of enzyme to substrate of 1:50 by weight. Hydrolysis was conducted at 37°C for 6 h. After its end, the hydrolysate was acidified to pH 3.1 and was lyophilized.

The chromatographic separation of the hydrolysates was achieved as described previously [3] on a column of the ion-exchange resin Aminex Q-150S. The fractions were purified by PC on Whatman 3 MM paper in the butan-1-ol—pyridine—acetic acid—water (15:10:3:12) system and the chromatogram was dried at 30°C. Strips were cut out and the spots were revealed with a 0.2% solution of ninhydrin in acetone.

Amino acid compositions were determined on a 399 T amino acid analyzer (Czech Republic) after the hydrolysis of the samples with 5.7 N HCl at 110°C for 24 h.

Determination of Tryptophan. To detect tryptophan-containing peptides, a chromatogram was sprayed with a freshly prepared 1% solution of *p*-dimethylaminobenzaldehyde in acetone with concentrated acid (9:1) and was left at room temperature for 10—15 min. The tryptophan-containing peptides gave a purple-red coloration.

Carbohydrate-containing peptides were determined with the orcinol reagent [6].

N-Terminal amino acids and amino acid sequences were determined as described in [7].

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