AMINO ACID SEQUENCE OF CARDIOTOXIN-ANALOGUE I FROM THE VENOM OF NAJA NAJA ATRA*

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SUMMARY

Cardiotoxin(CTX)-analogue I was obtained in a yield of about 5.3% from the venom of naja naja atra by gel filtration on Sephadex G-50, followed by CM-cellulose column chromatography. When injected intraperitoneally in mice, its LD_{50} was 2.8 (2.45-3.19) μg/g body weight. This toxin was cytotoxic to Yoshida sarcoma cells and produced contracture of the skeletal muscle as did CTX. The amino acid sequence of CTX-analogue I exhibits a high degree of homology with that of CTX from the same venom but differs in 13 positions.

Recently, a number of publications have appeared on the amino acid sequences of snake venom cardiotoxins (1-8). Cardiotoxins(cytotoxins) as a group consist of 60-61 amino acid residues, cross-linked by four disulfide bridges. They are distinguished from neurotoxins by substantial differences in amino acid composition, cytotoxicity to Yoshida sarcoma cells (9-12) and other pharmacological properties (13).

In the course of study of biologically active principles in the venom of Naja naja atra, several basic proteins were isolated by gel filtration on Sephadex G-50 followed by chromatography on CM-cellulose. Some of these proteins possessed cytotoxicity to Yoshida sarcoma cells and produced contracture as well as neuromuscular block as did CTX (9-

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12, 14). In the present communication, the complete amino acid sequence of CTX-analogue I from the venom of Naja naja atra is presented with some of its pharmacological properties.

Materials and Methods

The crude venom was purchased from Sigma Chemical Co., U. S. A. The venom dissolved in 1 % acetic acid was applied on a column of Sephadex G-50 equilibrated in the same elution medium. The protein fraction with molecular weight of about 6000 - 7500 was freeze-dried and applied to a CM-cellulose column, equilibrated with a 0.005 M sodium acetate buffer, pH 5.8. A linear gradient (from 0.005 M sodium acetate buffer, pH 5.8, to 0.5 M sodium aceate buffer, pH 6.5) was then applied at the top of the column. Each fraction was gel-filtered to remove sodium acetate. Homogeneity was ascertained by disc gel electrophoresis.

Table I

Amino Acid Composition of CTX-Analogue I and Peptides Generated by CNBr Cleavage

Amino Acid	CTX-Analogue I	CNBr fragments			
		CB-I	CB-II	CB-III	CB-IV
CM-Cysteine	-	2.6(3)			3.6(5)
Aspartic acid	8.7(8)	1.9(2)			5.3(6)
Threonine	3.4(3)	0.9(1)			1.9(2)
Serine	3.1(3)	0.9(1)			1.8(2)
Glutamic acid	0	0			0
Proline	4.4(4)	1.9(2)			1.9(2)
Glycine	2.0(2)	1.0(1)			1.0(1)
Alanine	2.2(2)	1.9(2)			0
Valine	4.2(4)	0			3.4(4)
Methionine	3.0(3)	0			0
Isoleucine	4.2(4)	1.9(2)			1.8(2)
Leucine	6.6(6)	2.8(3)			3.1(3)
Tyrosine	2.1(2)	0.6(1)			0.9(1)
Phenylalanine	1.3(1)	trace	1.0(1)		0
Tryptophan	0	0			0
Half-Cystine	7.8(8)	0			0
Lysine	7.7(8)	4.3(5)			3.6(3)
Histidine	0	0			0
Homoserine	0	1.0(1)	0.7(1)	1.0	0
Arginine	2.5(2)	0			1.8(2)

Numbers in parentheses represent nearest integers. In CTX-analogue I, CB-I and CB-IV, the value of glycine was taken as 2.0 and 1.0, respectively; and in fragment CB-II, the value of phenylalanine was taken as 1.0.

The first fraction having cytotoxicity to Yoshida sarcoma cells was designated as CTX-analogue I. Its LD_{50} in mice (NIH strain) by intraperitoneal injection was determined according to the method of Litchfield and Wilcoxon (15). The chick's biventer cervicis muscle preparation (16) was used for testing its action on the skeletal muscle.

The preparation of reduced and S-carboxymethylated (RCM)-CTX-analogue I was carried out by the method reported by Crestfield (17). Amino acid analyses of the toxin and the peptides obtained by cyanogen bromide cleavage or tryptic digestion of the RCM-toxin were carried out by standard method. Sequential degradation were conducted by the modified Edman procedure (18-19), and the phenylthiohydantoin amino acids were identified by thin layer chromatography in several solvent systems (20-22). Carboxypeptidase digestion was performed according to standard procedures (23). Cyanogen bromide cleavage (24) of RCM-toxin was carried out in 70 % formic acid, and the resulting peptides were fractionated on Sephadex G-25 in 0.2 M acetic acid. The fragments obtained were further purified on a column of DEAE-cellulose. Tryptic peptides were separated by high vol-

Table II

Amino Acid Composition of Tryptic Peptides of CB-I

Amino Acid	T-1	T-2	T-3	T-4	T-5	T-6	CB-I
CM-Cysteine			0.7(1)	0.5(1)	0.9(1)		3
Aspartic acid			1.0(1)		1.1(1)		2
Threonine				0.5(1)			1
Serine		0.9(1)					1
Glutamic acid							0
Proline		0.7(1)		0.8(1)			2
Glycine				1.2(1)			1
Alanine		0.9(1)		1.1(1)			2
Valine							0
Methionine							0
Isoleucine		1.3(2)					2
Leucine	0.8(1)	0.7(1)	1.0(1)				3
Tyrosine			0.5(1)				1
Phenylalanine							trace
Tryptophan							0
Lysine	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)		5
Homoserine						1.0	1
Arginine							0

The values of the amino acids underlined were taken as 1.0. The numbers in parenses represent the nearest integers.

tage paper electrophoresis at pH 3.6, and paper chromatography in a solvent system of 1-butanol-acetic acid-water = 4:1:5 (v/v), water saturated phenol, or n-butanol-acetic acid-pyridine-water = 15:3:10:12.

RESULTS AND DISCUSSION

The LD_{50} of CTX-analogue I in mice by intraperitoneal injection was estimated to be 2.8 (2.45-3.19) μg/g body weight. CTX-analogue I produced a marked contracture followed by neuromuscular block in the chick biventer cervicis muscle preparation, as did CTX, at a concentration of 10 ug/ml. The molecular weight of CTX-analogue I was estimated by gel filtration to be about 7000. Based on this value and on the amino acid analysis, one molecule of CTX-analogue I contains about 60 amino acid residues: Asp 8.7, Thr 3.4, Ser 3.1, Glu 0, Pro 4.4, Gly 2.0, Ala 2.2, Half-Cys 7.7, Val 4.2, Met 3.0, Ile 4.2, Leu 6.6, Tyr 2.1, Phe 1.3, Lys 7.8, Arg 2.5.

Twentynine stepwise Edman degradations of RCM-CTX-analogue I revealed the amino-terminal sequence to be : H-Leu.Lys.Cys.Asn.Lys.Leu.Ile.Pro.Ile.Ala.Ser.Lys.Thr.Cys.Pro.Ala.Gly.Lys.Asn.Leu.Cys.Tyr.Lys.Met.Phe.Met.Met.Ser.Asp.. The carboxy-terminal sequence was examined by the use of

Table III

Amino Acid Composition of Tryptic Peptides of CB-IV

Amino Acid	T'-1	T'-2	T'-3	T'-4	T'-5	T'-6	CB-IV
CM-Cysteine				1.8(2)	1.8(2)	0.6(1)	5
Aspartic acid		1.0(1)	1.2(1)	1.5(1)	2.3(2)	1.0(1)	6
Threonine			0.9(1)		1.1(1)		2
Serine		1.1(1)	1.0(1)				2
Glutamic acid							0
Proline			1.2(1)	0.9(1)			2
Glycine				1.0(1)			1
Alanine							0
Valine		1.0(1)	1.0(1)	1.0(1)	1.4(1)		4
Methionine							0
Isoleucine			1.0(1)	0.8(1)			2
Leucine		2.4(2)	1.2(1)				3
Tyrosine					0.9(1)		1
Phenylalanine							0
Lysine		1.0(1)	1.0(1)	1.0(1)			3
Homoserine							0
Arginine	1.0				1.0(1)		2

The value of the amino acid underlined were taken as 1.0. The numbers in parentheses represent the nearest integers.

carboxypeptidase A. After 3 and 24 hr incubation, the liberation of amino acids were found to be Asn:CM·Cys = 3:1 and 1:1, respectively. From the results, the carboxy-terminal sequence was determined to be -Cys·Asn-OH. The sequence was ascertained by separation of the tryptic peptide derived from the carboxy-terminal sequence. RCM-CTX-analogue I was cleaved by cyanogen bromide in 70% formic acid for 24 hr at 37° and the resulting peptides were fractionated by gel filtration on a column of Sephadex G-25. High molecular weight fragments CB-I and CB-IV were further purified by chromatography on a column of DEAE-cellulose. Low molecular weight fragments CB-II and CB-III, which appeared in the column volume of gel filtration procedure were further purified by paper electrophoresis and by paper chromatography. The amino acid compositions of these fragments are given in Table I.

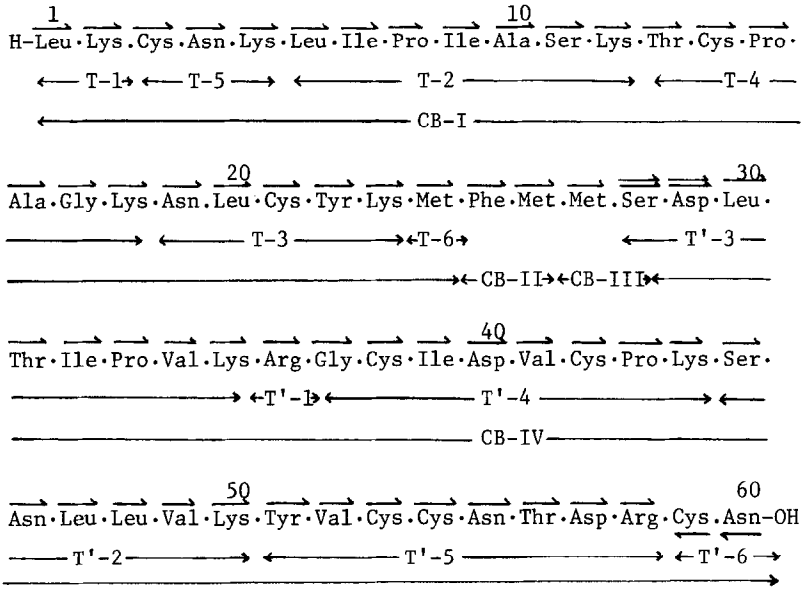

Fig. 1. Amino Acid Sequence of CTX-Analogue I from Formosan Cobra Venom (Naja naja atra)

Horizontal arrows above and below amino acid residues denote the sequences of CNBr fragments derived from RCM-toxin and tryptic peptides. Right- and left-handed arrows show that the sequence was elucidated, respectively, by Edman degradation or by the action of carboxypeptidases A and B, T and T' represent peptides produced by tryptic digestion of fragments CB-I and CB-IV, respectively.

The carboxy-terminal fragment CB-IV, from which homoserine was absent, contained 34 amino acid residues. Stepwise Edman degradation gave the first 32 amino acid residues, viz. -Ser·Asp·Lys·Thr·Ile.Pro·Val·Lys·Arg·Gly.Cys·Ile·Asp.Val·Cys·Pro·Lys·Ser·Asn·Leu.Leu.Val.Lys·Tyr·Val·Cys·Cys·Asn·Thr·Asp·Arg·. The central fragments CB-II and CB-III, located between the amino and carboxy-terminal fragments, were determined to be phenylalanylhomoserine and single homoserine, respectively. On the basis of above results, the amino acid sequence of CTX-analogue I was found to be CB-I-II-III-IV.

To ascertain the sequence, fragments CB-I and CB-IV were then digested by trypsin, and the resulting peptides were separated by high voltage paper electrophoresis and by paper chromatography. The amino acid composition of the peptides was determined by amino acid analyses and is shown in Tables II and III. On the basis of these results the primary structure of CTX-analogue I can now be expressed as shown in Fig. 1.

The amino acid sequence exhibits a high degree of homology with CTX but differs in 13 positions.

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