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

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1. Introduction

The toxic action of snake venoms in humans bitten by snakes and after injection into experimental animals causes disturbances of brain, nerve, and muscle function (neurotoxic action); disturbances of heart function (cardiotoxic action); tissue damage (cytotoxic action); hematological disturbances (hemorrhage, anemia, changes in the coagulability of the blood and hemolysis); and finally shock [1]. However, the specific content of toxin components and enzymes in each venom, as well as the mechanism of action and the disturbances, vary from one species to another. The main pharmacological actions of *Elapidae* venoms are neurotoxic, cardiotoxic and cytotoxic actions [2]. In addition to neurotoxins, their venoms contain cardiotoxin (CTX) (=cytotoxin) as their major protein constituents [3].

Studies on the structure–activity relationships of biologically active components in the venom of *Elapidae* snake are being made in our laboratory. Recently, we fractionated the venom of *Naja naja atra* and isolated at least four CTXs, named CTX-analogues I, II, III and IV in the homogeneous states by polyacrylamide disc-gel electrophoresis [4]. The determination of the complete amino acid sequence aid a great help to demonstrate the functionally important amino acid residues. In previous papers, we reported the primary structures of CTX-analogues I [5], II [6], and III [4], which was identical to CTX reported by Narita and Lee [7]. In this communication, we report the complete amino acid sequence of CTX-analogue IV.

2. Materials and methods

Lyophilized venom of *Naja naja atra* was purchased from Sigma Chemical Co., Ltd., USA. Bovine trypsin (three times recrystallized) was obtained from Worthington Biochemical Corp., USA. Sephadex G-25 and G-50 were the products from Pharmacia Fine Chemicals, Sweden. Carboxymethyl(CM)-cellulose was obtained from Seikagaku Kogyo Co., Ltd., Japan.

The venom dissolved in 1% acetic acid was applied to a column of Sephadex G-50 and eluted with 1% acetic acid as an eluent. Due to the molecular weight of CTXs (cytotoxins) isolated from the venom of *Naja naja* [3], the protein fraction with molecular weight of about 6 000–7 500 was freeze-dried and applied to a column of CM-cellulose equilibrated with 0.005 M sodium acetate buffer, Ph 5.8. The column was eluted with a gradient formed from the equilibration buffer in the mixing vessel and 0.5 M sodium acetate, pH 6.5, in the reservoir. Each fraction was pooled, lyophilized, and then gel-filtered on a column of Sephadex G-25 to remove sodium acetate. The fourth fraction with cytotoxicity to Yoshida sarcoma cells was named CTX-analogue IV. To further purify the CTX-analogue IV fraction obtained, the sample dissolved in 0.2 M sodium

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acetate buffer, pH 6.0, was applied to a column of CM-cellulose equilibrated with the same buffer. A gradient (from 0.2 M sodium acetate buffer, pH 6.0, to 0.8 M sodium acetate buffer, pH 6.8) was then applied at the top of the column. The pooled protein fraction forming the major peak was lyophilized and the dried sample was gel-filtered to remove sodium acetate. Homogeneity was examined by polyacrylamide disc-gel electrophoresis according to the method of Williams et al. [8]. Its LD_{50} value in mice (NIH strain) by subcutaneous injection was determined by the method of Litchfield and Wilcoxon [9]. Cytotoxicity to Yoshida sarcoma cells was determined by the method of Braganca et al. [10] with some modifications.

Preparation of the reduced and S-carboxymethylated(RCM)-CTX-analogue IV was performed as usual method described by Crestfield et al. [11]. Amino acid analyses of CTX-analogue IV, the fragments generated by cyanogen bromide cleavage and the tryptic peptides were carried out by standard method. Cyanogen bromide cleavage of the RCM-toxin was performed in 70% formic acid by the method of Gross and Witkop [12]. The resulting fragments were applied to a column of Sephadex G-50 equilibrated with 0.2% acetic acid and were eluted with the same elution medium. The degradative Edman procedure as modified by Iwanaga et al. [13] was adopted to determine the sequence of the RCM-toxin and the fragments CB-III generated by cyanogen bromide cleavage of the RCM-toxin. The phenylthiohydantoin amino acids were identified by thin layer chromatography in several solvent systems [14,15]. The trypsin digestion of the fragments CB-I and CB-III was carried out at 37°C for 8 h. The digests were applied to a preparative high voltage paper electrophoresis using a solvent system of pyridine/acetic acid/water 1:10: 289, by volume, pH 3.6, at 30–35 V/cm for 2 h. Guide

Table 1
Amino acid composition of cardiotoxin-analogue IV and fragments generated by CNBr cleavage

Amino acid	Cardiotoxin--analogue IV	CNBr fragments		
		CB-I	CB-II	CB-III
CM-Cysteine	–	3.38 (3)		5.25 (5)
Aspartic acid	7.93 (8)	2.24 (2)		5.77 (6)
Threonine	2.61 (3)	1.00 (1)		1.89 (2)
Serine	2.17 (2)	0		1.77 (2)
Glutamic acid	0.12 (0)	0		0
Proline	4.15 (4)	1.85 (2)		1.98 (2)
Glycine	2.07 (2)	1.04 (1)		1.05 (1)
Alanine	1.80 (2)	1.00 (1)		1.00 (1)
Half-Cystine	7.20 (8)	0		0
Valine	5.83 (8)	1.15 (1)		5.28 (6)
Methionine	2.07 (2)	0		0
Isoleucine	1.00 (1)	0		1.21 (1)
Leucine	5.15 (6)	2.91 (3)		1.95 (2)
Tyrosine	2.73 (3)	1.87 (2)		0.96 (1)
Phenylalanine	1.63 (2)	1.27 (1)	1.00 (1)	0
Tryptophan	0	0		0
Lysine	6.93 (8)	5.95 (5)		2.86 (3)
Histidine	0	0		0
Homoserine	–	0.80 (1)	1.01 (1)	0
Arginine	2.76 (3)	0.96 (1)		1.95 (2)

Numbers in parentheses represent the values from the amino acid sequence. In cardiotoxin-analogue IV, the value of isoleucine was taken as 1.0, in CB-I and CB-III, the value of alanine was taken as 1.0, and in fragment CB-II, the value of phenylalanine was taken as 1.0.

strip (5 mm) was cut and stained with ninhydrin reagent. Each band was extracted with a solution of 0.1 M pyridine, adjusted to pH 5.0 with acetic acid. The purities of extracted peptides were examined by paper chromatography using a solvent system of (a) 1-butanol/acetic acid/pyridine/water 15:3:10:12, by volume, (b) 1-butanol/acetic acid/water 4:1:5, by volume, or (c) watersaturated phenol. When they did not show one spot, further purification was preparatively performed by paper chromatography using a solvent system described above. The peptide and the fragment numbers refer to the order of their location in the sequence starting from the amino-terminal end.

3. Results and discussion

CTX-analogue IV was obtained in a yield of about 8.1% from the crude venom. The LD_{50} in mice by subcutaneous injection was estimated to be 48 (42–53) μg/g body weight, and 50% lysis of Yoshida sarcoma cells (=cytotoxicity) was observed at a concentration of 13.5 μg protein/ml. Judging from the elution profile of the toxin on a Sephadex G-50 column, the amino acid composition, and the molecular weight of other known CTX-analogues, one molecule of CTX-analogue IV contains about 60 amino acid residues: Asp 7.93, Thr 2.61, Ser 2.17, Glu 0.12, Pro 4.15, Gly 2.07, Ala 1.80, Half-Cys 7.20, Val 5.83, Met 2.07, Ile 1.00, Leu 5.15, Tyr 2.73, Phe 1.63, Lys 6.93, Arg 2.76.

Direct Edman degradation of RCM-toxin revealed the first 29 amino acid sequence to be: H-Arg-Lys-Cys-Asn-Lys-Leu-Val-Pro-Leu-Phe-Tyr-Lys-Thr-Cys-Pro-Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Phe-Met-Val-Ser-Asn-. According to this sequence, two

Table 2
Amino acid composition of tryptic peptides of CB-I of cardiotoxin-analogue IV

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	CB-I
CM-Cysteine		1.00 (1)				1.13 (1)	1.19 (1)		3
Aspartic acid		1.10 (1)					1.13 (1)		2
Threonine						0.91 (1)			1
Serine									0
Glutamic acid									0
Proline			1.00 (1)		1.43 (1)	0.95 (1)			2
Glycine						1.11 (1)			1
Alanine						1.05 (1)			1
Half-Cysteine									0
Valine			0.84 (1)		0.93 (1)				1
Methionine									0
Isoleucine									0
Leucine			2.26 (2)		1.86 (2)		1.06 (1)		3
Tyrosine				0.75 (1)	0.64 (1)		0.83 (1)		2
Phenylalanine				0.75 (1)	0.71 (1)				1
Tryptophan									0
Lysine	1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)		5
Histidine									0
Homoserine								1.00 (1)	1
Arginine	1.07 (1)								1
Total	2	3	4	3	7	6	5	1	
Yield (%)	20	18	14	23	10	24	28	25	
Solvent[a]	(a)	(a,c)	(a)	(a)	(a)	(a,c)	(a)	(a)	

In each column the values of the amino acids underlined were taken as 1.0.
The numbers in parentheses represent the values from the amino acid sequences.

[a] Solvent represents the solvent system used for the separation of tryptic peptides by paper chromatography (see Materials and methods).

methionine residues were found in the central part of the toxin molecule. Then, the RCM-toxin was cleaved with cyanogen bromide. The resulting fragments were separated with a Sephadex G-50 column using a 0.2% acetic acid as an eluent. The amino acid compositions of the fragments obtained are given in the table 1.

Using the carboxy-terminal fragment CB-III, from which homoserine was absent, stepwise Edman degradation was performed to demonstrate all of the amino acid sequence, viz: -Val-Ser-Asn-Leu-Thr-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Asn-Ser-Ala-Leu-Val-Lys-Tyr-Val-Cys-Cys-Asn-Thr-Asp-Arg-Cys-Asn-OH. The central fragment CB-II which was eluted at the column volume in the gel filtration was determined to be phenylalanylhomoserine. On the basis of the above results, the amino acid sequence of CTX-analogue IV was deduced to be CB-I-II-III.

This sequence of CTX-analogue IV was further ascertained by analyzing the peptides obtained by the trypsin digestion of the CB-I and CB-III. After the digestion of each fragment by trypsin, the resulting peptides were separated by a combination of high-voltage paper electrophoresis and paper chromatography. The amino acid compositions of the peptides were determined by amino acid analyses and are shown in tables 2 and 3.

The complete amino acid sequence of CTX-analogue IV from the present study is now shown in fig.1 and indicates that the molecular weight is 6 801. This value is almost the same as those of other CTX-analogues.

There is a remarkable similarity between CTX-

Table 3
Amino acid composition of tryptic peptides of CB-III of cardiotoxin-analogue IV

Amino acid	T'-1	T'-2	T'-3	T'-4	T'-5	T'-6	CB-III
CM-Cysteine			1.89 (2)		1.98 (2)	0.97 (1)	5
Aspartic acid	1.50 (1)		1.20 (1)	1.36 (1)	2.19 (2)	1.00 (1)	6
Threonine	0.86 (1)				0.96 (1)		2
Serine	1.30 (1)			1.30 (1)			2
Glutamic acid							0
Proline	1.30 (1)		1.24 (1)				2
Glycine			1.11 (1)				1
Alanine				1.19 (1)			1
Half-Cysteine							0
Valine	3.36 (3)		0.81 (1)	1.36 (1)	0.64 (1)		6
Methionine							0
Isoleucine			1.00 (1)				1
Leucine	1.44 (1)			1.22 (1)			2
Tyrosine					0.68 (1)		1
Phenylalanine							0
Tryptophan							0
Lysine	1.00 (1)		1.00 (1)	1.00 (1)			3
Histidine							0
Homoserine							0
Arginine		1.00 (1)			1.00 (1)		2
Total	9	1	8	6	8	2	
Yield (%)	27	38	29	35	25	60	
Solvent[a]	(a)	(a)	(b)	(a)	(b)	(a)	

In each column the values of the amino acid underlined were taken as 1.0.
The numbers in parentheses represent the values from the amino acid sequences.

[a] Solvent represents the solvent system used for the separation of tryptic peptides by paper chromatography (see Materials and methods).

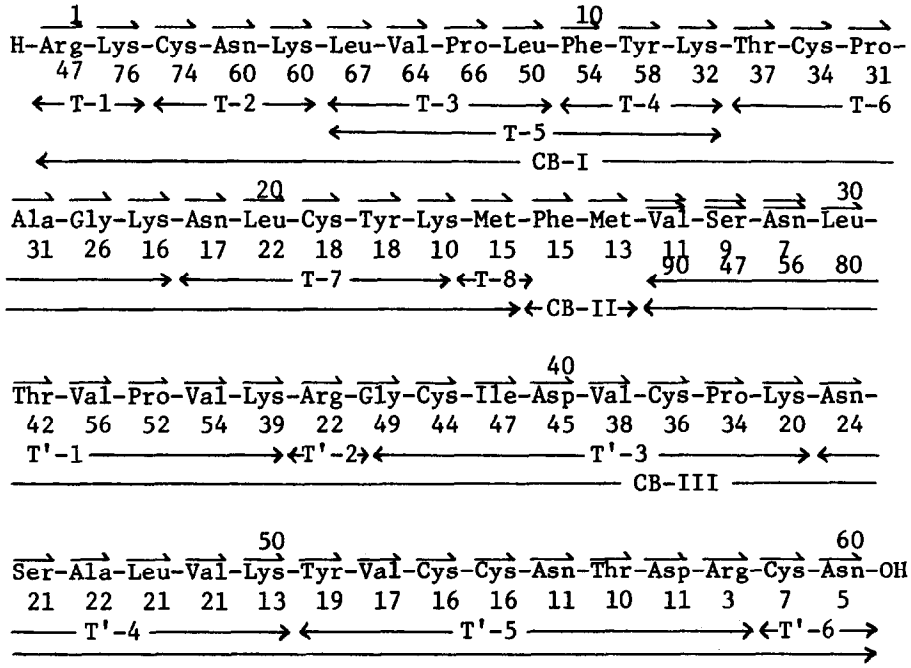

Fig.1. Amino acid sequence of cardiotoxin-analogue IV from formosan cobra venom (*Naja naja atra*). Horizontal arrows below amino acid residues denote the sequence of CNBr fragments derived from RCM-toxin and tryptic peptides. Right-handed arrows show that the sequence was elucidated by Edman degradation. T and T' represent the peptides produced by tryptic digestion of fragments CB-I and CB-III, respectively. Numbers below amino acid residues show the yield (%) of phenylthiohydantoin amino acids estimated from the molar absorbance coefficient at 269 nm and based on the amounts of samples used in the Edman degradation.

analogue II and CTX-analogue IV. CTX-analogue IV differs from CTX-analogue II only in the presence of arginine residue in place of leucine residue at position 1. CTX-Analogue IV is the first toxin found with an arginine residue at amino terminus of the toxin molecule among cardiotoxins, although there are some neurotoxins having an arginine residue in the position [16,17].

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References

[1] Venomous Animals and Their Venoms (1968) (Bücherl, W., Buckley, E. and Deulofeu, V. eds) Vol. 1 Academic Press, New York.
[2] Lee, C. Y. and Chang, C. C. (1966) Mem. Inst. Butantan Simp. Internac. 33, 555–572.
[3] Ohta, M. and Hayashi, K. (1975) Tampakushitsu Kakusan Koso (in Japanese) 20, 53–69.
[4] Takechi, M., Sasaki, T., Kaneda, N. and Hayashi, K. (1976) FEBS Lett. 66, 210–214.
[5] Hayashi, K., Takechi, M., Sasaki, T. and Lee, C. Y. (1975) Biochem. Biophys. Res. Commun. 64, 360–366.
[6] Kaneda, N., Sasaki, T. and Hayashi, K. (1976) Biochem. Biophys. Res. Commun. To be published.
[7] Narita, K. and Lee, C. Y. (1970) Biochem. Biophys. Res. Commun. 41, 339–343.
[8] Williams, D. E. and Reisfeld, R. A. (1964) Annals New York Acad. Sci. 121, 373–381.
[9] Litchfield, J. T. and Wilcoxon, F. (1948) J. Pharmacol. Exp. Ther. 96, 99–113.

[10] Braganca, B. M., Patel, N. T. and Badrinath, P. G. (1967) Biochim. Biophys. Acta 136, 508–520.
[11] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627.
[12] Gross, E. and Witkop, B. (1962) J. Biol. Chem. 237, 1856–1860.
[13] Iwanaga, S., Wallen, P., Gröndahl, N. J., Henschen, A. and Blombäck, B. (1969) Eur. J. Biochem. 8, 189–199.
[14] Jeppsson, J. O. and Sjöquist, J. (1967) Anal. Biochem. 18, 264–269.
[15] Summers, M. R., Smythers, G. W. and Oroszlan, S. (1973) Anal. Biochem. 53, 624–628.
[16] Strydom, A. J. C. (1973) Biochim. Biophys. Acta 328, 491–509.
[17] Strydom, D. J. (1972) J. Biol. Chem. 247, 4029–4042.