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The Amino Acid Sequence of Cytotoxin II from the Venom of the Indian Cobra (*Naja naja*)

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SUMMARY

Homogeneous cytotoxins I and II with high cytotoxicity to Yoshida sarcoma and hepatoma cells were isolated from the venom of the Indian cobra *Naja naja*. The amino acid sequence of cytotoxin II was determined with the aid of tryptic digestion and cleavage by cyanogen bromide. Although their pharmacological actions are quite different, the amino acid sequences of cytotoxins were similar to those of cobra neurotoxins.

INTRODUCTION

Chemical and pharmacological studies of snake venom indicate thre presence of many active principles, including neurotoxin, cardiotoxin, direct lytic factor, combramines, and phospholipase A. It is generally believed that the Elapidae, of which *Naja naja atra* is one species, contain a highly potent toxin, cobratoxin, which causes peripheral respiratory paralysis (1–4). The venom also contains cardiotoxin, which produces cardiovascular changes and is responsible for local necrotic lesions (5, 6). The cardiotoxin is pharmacologically indistinguishable from the direct lytic factor or combramine B (7, 8).

In the course of our studies on the purification of biologically active principles in the venom of *Naja naja*, two basic homogeneous proteins were isolated by gel filtration on Sephadex G-50 and G-75 and by chromatography on carboxymethyl cellulose (9).

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In 1967 Braganca *et al.* (10) reported that a basic protein isolated from $HClO_4$-treated cobra venom possessed cytotoxicity to Yoshida sarcoma cells. Accordingly, we examined the cytotoxicity of our basic protein according to their method. Two proteins were found to have high cytotoxicity to Yoshida sarcoma and ascites hepatoma cells (11), and were named cytotoxins I and II. In their pharmacological properties they resemble cardiotoxin.[2]

Cytotoxin II consists of 60 amino acid residues, cross-linked by four disulfide bridges. This paper describes the amino acid sequence of cytotoxin II and compares it with those of cardiotoxin (12) and cobratoxin (13).

MATERIALS AND METHODS

Cytotoxin II was prepared from the venom of the Indian cobra according to the method of Nakai *et al.* (9). Homogeneity was ascertained by disc gel electrophoresis and end group analysis. Amino acid analyses

[2] C. Y. Lee, personal communication.

of the toxin and peptides obtained by cyanogen bromide cleavage or tryptic digestion of the reduced, carboxymethylated cytotoxin II were carried out by standard methods. Sequential degradations were conducted by the modified Edman procedure (14, 15), and the phenylhydantoin amino acids were identified by thin-layer chromatography in several solvent systems (16–18). Carboxypeptidase digestions were performed according to standard procedures (19). Cyanogen bromide cleavage (20) of 20 μmoles of reduced, carboxymethylated cytotoxin II 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-voltage paper electrophoresis at pH 3.6, and by paper chromatography in a solvent system of 1-butanol–acetic acid–water, 4:1:5(v/v), or in water-saturated phenol.

RESULTS AND DISCUSSION

Cytotoxin II, which was homogeneous by disc gel electrophoresis, was obtained in a yield of 13.5% from crude venom. When injected intraperitoneally, cytotoxin II showed no lethal effects in mice within 2–3 hr at a dose of 10 μg per mouse, as compared with the LD_{50} of 0.7 μg in mice for crude venom. Cytotoxicity data, determined by the method of Braganca *et al.* (10), except that trypan blue was used instead of lissamine green, revealed that cytotoxins I and II are active at a concentration of 1 μg/ml on Yoshida sarcoma and ascites hepatoma cells (11).

The molecular weight of cytotoxin II was estimated by gel filtration to be 7000. Based on this value and on the amino acid analyses, 1 molecule of cytotoxin II contains 60 amino acid residues: Asp, 6; Thr, 3; Ser, 2; Glu, 0; Pro, 5; Gly, 2; Ala, 2: Cys, 8; Val, 7; Met, 2; Ile, 1; Leu, 6; Tyr, 4; Phe, 1; Trp, 0; Lys, 9; His, 0; Arg, 2.

TABLE 1

Amino acid composition of cytotoxin II, RCM-cytotoxin II, and peptides generated by CNBr cleavage

Numbers in parentheses represent nearest integers. In cytotoxin II, the value of phenylalanine was taken as 1.0; in fragments CB-I and CB-III, the value of glycine was taken as 1.0; and in fragment CB-II, the value of tyrosine was taken as 1.0.

Amino acid	Cytotoxin II	RCM-cytotoxin II	CNBr fragments		
			CB-I	CB-II	CB-III
CM-cysteine	7.6(8)	8	2.5(3)		3.7(5)
Aspartic acid	6.5(6)	6	1.9(2)		3.5(4)
Threonine	3.1(3)	3	0.9(1)		1.6(2)
Serine	1.9(2)	2	0		1.9(2)
Glutamic acid	0	0	0		0
Proline	5.3(5)	5	1.6(2)		1.9(3)
Glycine	2.2(2)	2	1.0(1)		1.0(1)
Alanine	2.2(2)	2	1.1(1)		0.8(1)
Valine	6.6(7)	7	1.2(1)		5.1(6)
Methionine	2.0(2)	2	trace		trace
Isoleucine	1.1(1)	1	0		0.7(1)
Leucine	6.0(6)	6	3.6(4)		2.0(2)
Tyrosine	4.0(4)	4	1.5(2)	1.0(1)	1.3(1)
Phenylalanine	1.0(1)	1	0.6(1)	trace	0
Tryptophan	0	0	0		0
Half-cystine	7.5(8)	0	0		0
Lysine	8.6(9)	9	4.2(5)		3.8(4)
Histidine	0	0	0		0
Homoserine	0	0	0.6(1)	1.1(1)	0
Arginine	2.0(2)	2	0		1.7(2)

TABLE 2

Amino Acid composition of tryptic peptides of CB-I

The values of the amino acids in boldface type were taken as 1.0. The numbers in parentheses represent the nearest integers.

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	CB-I
CM-cysteine			1.1(1)	1.0(1)	1.0(1)		3
Aspartic acid			1.1(1)		1.1(1)		2
Threonine				0.9(1)			1
Serine							0
Glutamic acid							0
Proline		1.0(1)		0.8(1)			2
Glycine				0.9(1)			1
Alanine				0.9(1)			1
Valine		0.8(1)					1
Methionine							0
Isoleucine							0
Leucine	1.2(1)	2.0(2)	1.1(1)				4
Tyrosine		0.9(1)	0.9(1)				2
Phenylalanine		0.8(1)					1
Tryptophan							0
Lysine	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)		5
Histidine							0
Homoserine						1.0(1)	1
Arginine							0

Twenty-eight stepwise Edman degradations of RCM-cytotoxin II[3] revealed the amino-terminal sequence to be: H-Leu-Lys-Cys-Asn-Lys-Leu-Val-Pro-Leu-Phe-Tyr-Lys-Thr-Cys-Pro-Ala-Gly-Lys-Asn-Leu-Cys-Tyr-Lys-Met-Tyr-Met-Val-Ala-. The carboxyl-terminal sequence was examined by the use of carboxypeptidases A and B, and by separation of the tryptic peptide derived from the carboxyl-terminal sequence. The amino acid composition of the peptide was found to be CM-Cys-Asp, 1.0:1.1. On the basis of these results the carboxyl-terminal sequence was determined to be -Asp-Arg-Cys-Asn-OH.

Next, RCM-cytotoxin II was cleaved by cyanogen bromide in 70% formic acid for 24 hr at 37° (20), and the resulting peptides were fractionated by gel filtration on a column of Sephadex G-25. Three major fragments, CB-I, CB-II, and CB-III, were further purified by chromatography on a column of DEAE-cellulose. The amino acid composition of these fragments is given in Table 1. The carboxyl-terminal fragment, CB-III, from which homoserine was absent, contained 34 amino acid residues. Stepwise Edman degradation gave the first 29 amino acid residues, viz. Val-Ala-Thr-Pro-Lys-Val-Pro-Val-Lys-Arg-Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-Ser-Ser-Leu-Val-Leu-Lys-Tyr-Val-Cys-Cys-Asn-. The central fragment, CB-II, located between the amino- and carboxyl-terminal fragments, was a dipeptide, tyrosylhomoserine.

Fragments CB-I and CB-III 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 standard methods and is shown in Tables 2 and 3.

On the basis of these results the primary structure of cytotoxin II can now be expressed as shown in Fig. 1. There is a remarkable similarity with cytotoxin I (21) and cardiotoxin isolated from the venom of the Formosan cobra (*Naja naja atra*) (12). In particular, cytotoxin II differs from cardiotoxin only in the presence of a tyrosine in place of a phenylalanine residue. Although the amino acid composition differs from that

[3] The abbreviations used are: RCM, reduced, carboxymethylated; CM-, carboxymethyl-.

TABLE 3

Amino acid composition of tryptic peptides of CB-III

The values of the amino acids in boldface type were taken as 1.0. The numbers in parentheses represent the nearest integers.

Amino acid	T'-1	T'-2	T'-3	T'-4	T'-5	CB-III
CM-cysteine				2.0(2)	3.4(3)	5
Aspartic acid				1.2(1)	3.6(3)	4
Threonine		1.0(1)			1.2(1)	2
Serine			1.8(2)			2
Glutamic acid						0
Proline	1.0(1)	1.1(1)		0.9(1)		3
Glycine				1.0(1)		1
Alanine		1.0(1)				1
Valine	1.8(2)	1.1(1)	1.0(1)	1.0(1)	1.1(1)	6
Methionine						0
Isoleucine				0.9(1)		1
Leucine			2.2(2)			2
Tyrosine					0.9(1)	0
Phenylalanine						0
Tryptophan						0
Lysine	1.0(1)	1.0(1)	1.0(1)	1.0(1)		4
Histidine						0
Homoserine						0
Arginine	1.1(1)				1.0(1)	2

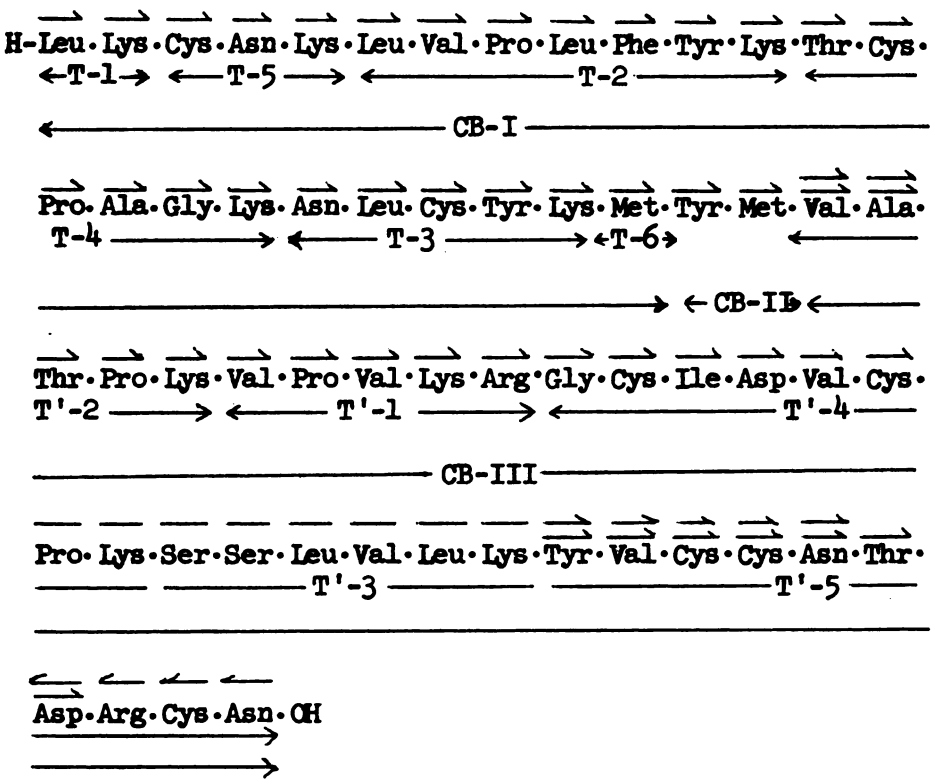

FIG. 1. *Amino acid sequence of cytotoxin II from Indian cobra (Naja naja) venom*

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-III, respectively.

of the major neurotoxin, "toxin A," of the venom of the Indian cobra (22), there is still considerable overlap in the amino acid sequences of the two venoms. Interestingly enough, the cysteine residues in the primary structure of cytotoxins and neurotoxins are located in similar positions (12, 13, 21–27). Figure 2 shows the sequences of cytotoxins I, II, cardiotoxin, and a neurotoxin ("cobratoxin") of the venom of the Formosan cobra.

Moreover, the circular dichroism spectrum of cytotoxins I and II closely resembled that of cobratoxin, which was quite different from that of ordinary proteins (28). However, their pharmacological properties differed markedly: the mode of neuromuscular blocking action of cobra neurotoxin is similar to that of curare, although the former acts much more slowly and less reversibly than the latter (1–4). On the other hand, cardio-

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                    1                                                  10
Cytotoxin I    H-Leu·Lys·Cys·Asn·Lys·Leu·Ile·Pro·Leu·Ala·Tyr·Lys·
Cytotoxin II   H-Leu·Lys·Cys·Asn·Lys·Leu·Val·Pro·Leu·Phe·Tyr·Lys·
Cardiotoxin    H-Leu·Lys·Cys·Asn·Lys·Leu·Val·Pro·Leu·Phe·Tyr·Lys·
Cobratoxin     H-Leu·Glu·Cys·His·Asn·Gln·Gln·Ser·Ser·Gln·Thr·Pro·

                                                  20
Thr·                ·Cys·Pro·Ala·Gly·Lys·Asn·Leu·Cys·Tyr·Lys·Met·Tyr·
Thr·                ·Cys·Pro·Ala·Gly·Lys·Asn·Leu·Cys·Tyr·Lys·Met·Tyr·
Thr·                ·Cys·Pro·Ala·Gly·Lys·Asn·Leu·Cys·Tyr·Lys·Met·Phe·
Thr·Thr·Thr·Gly·Cys·Ser·Gly·Gly·Glu·Thr·Asn·Cys·Tyr·Lys·Lys·Arg·

                    30                                                 40
Met·Val·Ser·Asn·Lys·Thr·Val·Pro·Val·Lys·Arg·Gly·Cys·Ile·Asp·Val·
Met·Val·Ala·Thr·Pro·Lys·Val·Pro·Val·Lys·Arg·Gly·Cys·Ile·Asp·Val·
Met·Val·Ala·Thr·Pro·Lys·Val·Pro·Val·Lys·Arg·Gly·Cys·Ile·Asp·Val·
Trp·Arg·Asp·His·Arg·Gly·Tyr·Arg·Thr·Glu·Arg·Gly·Cys·Gly·

                                                  50
Cys·Pro·Lys·Asn·Ser·Leu·Val·Leu·Lys·Tyr·Glu·Cys·Cys·Asn·Thr·Asp·
Cys·Pro·Lys·Ser·Ser·Leu·Val·Leu·Lys·Tyr·Val·Cys·Cys·Asn·Thr·Asp·
Cys·Pro·Lys·Ser·Ser·Leu·Val·Leu·Lys·Tyr·Val·Cys·Cys·Asn·Thr·Asp·
Cys·Pro·Ser·Val·Lys·Asn·Gly·Ile·Glu·Ile·Asn·Cys·Cys·Thr·Thr·Asp·

          60
Arg·Cys·Asn·OH
Arg·Cys·Asn·OH
Arg·Cys·Asn·OH
Arg·Cys·Asn·Asn·OH
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FIG. 2. *Structures of cytotoxin I (22), cytotoxin II (Naja naja), cardiotoxin (Naja naja atra) (10, 23), and the neurotoxin "cobratoxin" (Naja naja atra) (14)*

toxin causes muscular contraction as well as neuromuscular blockade by depolarizing both muscle and nerve fibers (5, 6). Also, the pharmacological properties of the cytotoxins resembled those of cardiotoxin.[2] The pharmacological differences among cobratoxin, cardiotoxin, and cytotoxins may depend on the replacement of lysine residues 2, 18, 35, and 50 by glutamic acid residues.

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