THE ISOLATION AND SEQUENCE DETERMINATION OF A CYTOTOXIN FROM THE VENOM OF THE MIDDLE-ASIAN COBRA *NAJA NAJA OXIANA*

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Received 30 August 1974

1. Introduction

Venom of snakes of the Elapid family contains, in addition to neurotoxins, a group of homologous basic peptides, characterized by the unfailing presence of methionine residues, by high contents of lysine and hydrophobic amino acid residues and by low contents of glycine and arginine residues [1]. Like the 60–4 neurotoxins [2] these peptides possess a total of 60–61 amino acid residues and have 4 intramolecular disulfide linkages. As a rule, they are of low toxicity, but exert a variety of biological effects, e.g. depolarize skeletal muscle, lyse erythrocytes, arrest the isolated frog's heart in systole, are cytotoxic etc. [3]. Presumably the underlying cause of these effects is the interaction of the peptides with cellular membranes so that they are, on good grounds, known as the 'membrane active components' of the venom or cytotoxins. In the present paper the isolation and total sequential determination of a cytotoxin of the Middle-Asian cobra venom is described.

2. Materials and methods

The whole venom of the Middle-Asian cobra *Naja naja oxiana* was obtained from the Kirghiz Serpentarium. It was fractionated on Sephadex G-75 followed by chromatography on CM-cellulose by a previously described procedure [4]. The cytotoxin was then further purified by chromatography on SE-Sephadex C-25 or on Bio-Rex 70 ion exchanger. Its amino acid composition was analyzed using a Bio-Cal BC-201 automatic analyser. Reduction and carboxymethylation were carried out according to Crestfield et al. [5]. Tryptic hydrolysis was performed by incubating a 1:50 w/w enzyme:substrate mixture for four hr at 37°C. The hydrolyzate of the carboxymethylated cytotoxin was chromatographed on Type P, Chromo-Beads resin (Technicon). Further purification was achieved by paper chromatography and electrophoresis. Determination of the amino acid sequence of the peptidic fragments was carried out by a method described earlier [6]. Cyanogen bromide cleavage of the carboxymethylated cytotoxin was done in 70% formic acid at room temperature for 22 hr, using a 100-fold excess of reagent. The products were chromatographed on Sephadex G-25 (fine) in 0.1 M NH_4HCO_3 buffer. The chymotryptic peptides of the cyanogen bromide fragment of the cytotoxin were isolated by paper chromatography. The N-terminal sequences of carboxymethylated cytotoxin and the cyanogen bromide fragment which had been shown to form the C-terminus of the original peptide were determined on a Beckman model 890C sequencer and the C-terminus sequence of the former, by means of carboxypeptidase A.

3. Results and discussion

A hemolytic, cardio-active and cytotoxic preparation ('3') was obtained by subjecting a whole *Naja naja oxiana* venom preparation to gel filtration on Sephadex G-75 and isolating it from the principal fraction by ion-exchange chromatography on carboxymethylcellulose CM-32 [4]. It was found by gel electrophoresis that '3' was a mixture; from it the pure cytotoxin could be isolated by chromatography on SE-Sephadex C-25 (fig.1) Only one of the components (S-1) displayed hemolytic activity, the strength of which depended linearly on its concentration. Components S-1 displayed cardio- and cytotoxic activity, as well as hemolytic action. Cytotoxin could also be isolated by chromatographing '3' on the Bio-Rex 70 ion exchanger (fig. 2) The component B-2 which resulted thereby reproduced all the aforementioned activities. Analysis of the amino acid composition of the cytotoxin showed it to be similar to that earlier isolated from the venoms of other cobra (table 1) [7–9]. The yield of cytotoxin isolated from the whole venom of the Middle-Asian cobra amounted to approx. 18%. From ultracentrifugation and gel filtration the mol. wt. was calculated to be about 7000 which was in good agreement with the results of the amino acid analysis.

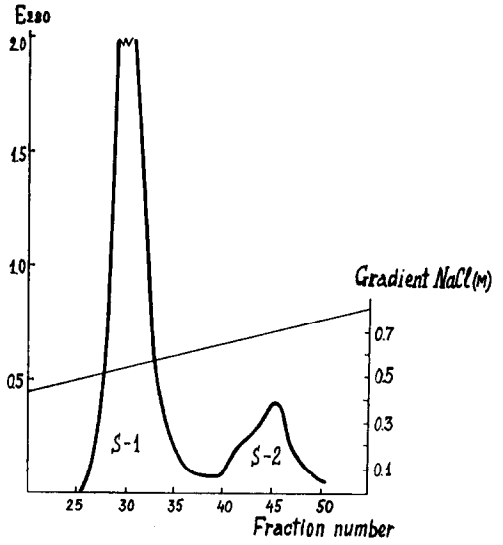

Fig. 1. Chromatography on SE-Sephadex C-25 of fraction '3'. 1.6 × 35 cm column in 0.05 M ammonium acetate, pH 5.0-Gradient elution to 0.8 M sodium chloride solution; flow rate, 30 ml/hr; fraction vol, 5 ml.

The N-terminal amino acid sequence of the carboxymethylated cytotoxin as determined by a sequencer using the standard fast protein-quadrole program

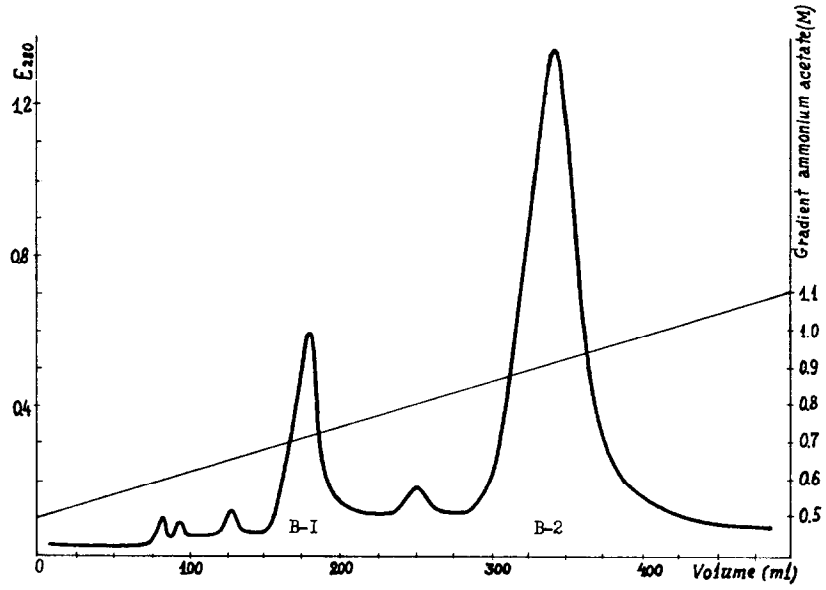

Fig.2. Chromatography on Bio-Rex 70 of fraction '3'. 1.5 × 28 cm column in 0.2 M ammonium acetate pH 6.5. Gradient elution from 0.5 M to 1.1 M ammonium acetate solution; flow rate, 45 ml/hr.

Table 1
Amino acid composition of cytotoxins from the venoms of cobras and cyanogen bromide peptides of cytotoxin from *Naja naja oxiana* venom

Amino acid	*Haemachatus haemachates* 'direct lytic factor'	*Naja naja atra* cardiotoxin	*Naja naja* cytotoxin II	*Naja naja oxiana* cytotoxin			
					CB-1	CB-2	CB-3
Lys	10	9	9	10	5.6 (6)	-	4.0 (4)
His	1	—	—	1	-	-	1.0 (1)
Arg	1	2	2	1	-	-	0.9 (1)
Asp	6	6	6	5	1.2 (1)	-	3.8 (4)
Thr	3	3	3	2	1.0 (1)	-	1.0 (1)
Ser	3	2	2	3	1.1 (1)	-	2.0 (2)
Glu	1	—	—	—	-	-	-
Pro	5	5	5	5	2.2 (2)	-	2.8 (3)
Gly	2	2	2	2	1.3 (1)	-	1.1 (1)
Ala	1	2	2	3	1.0 (1)	-	2.1 (2)
½Cys	8	8	8	8	3.0 (3)	-	5.0 (5)
Val	4	7	7	7	1.0 (1)	-	5.6 (6)
Met	2	2	2	2	(1)	(1)	-
Ile	2	1	1	1	-	-	1.0 (1)
Leu	6	6	6	6	3.7 (4)	-	1.9 (2)
Tyr	1	3	4	2	0.8 (1)	-	1.0 (1)
Phe	1	2	1	2	1.0 (1)	(1)	-
Total	57	60	60	60	24	2	34
N-terminus	Leu	Leu	Leu	Leu	Leu	Phe	Val
C-terminus	Ser	Asn	Asn	Asn	Met	Met	Asn

(072172C) turned out to be as follows: Leu–Lys–Cys–Lys–Lys–Leu–Val–Pro–Leu–Phe–X–X–X–Cys–Pro–Ala–Gly–X–X–Leu–X–Tyr–X–Met–Phe–Met . . .

Edman degradation of the carboxymethylated cytotoxin made it possible to determine the sequence of the first seven amino acid residues: Leu–Lys–Cys–Lys–Lys–Leu–Val. . .

The C-terminal sequence of carboxymethylated cytotoxin determined by means of carboxypeptidase A turned out to be: . . .Lys–Cys–Asn.

According to the results of the amino acid analysis, tryptic cleavage of carboxymethylated cytotoxin should have given 12 relatively small tryptic peptides. These were separated with the aid of the Chromo-Beads, P4 ion exchanger (fig. 3). In the combined fractions III, VIII, IX and XIII, no analytically significant amounts of peptide were found. From the remaining fractions 11 peptides were isolated; their amino acid compositions are given in table 2, and their sequences in table 3.

In order to obtain large blocks, the carboxymethylated cytotoxin was cleaved by cyanogen bromide and the resulting peptide fragments separated by gel filtration on Sephadex G-25. Their amino acid compositions are given in table 1. The N-terminal amino acid residue of peptide CB-1 proved to be Leu. Hence this peptide is the N-terminus of the cytotoxin molecule, which is confirmed by analysis of its amino acid composition. The N-terminal amino acid residue of CB-2 is phenylalanine; thus CB-2 must be Phe–Met. In order to determine the N-terminal amino acid sequence of peptide CB-3 the *NN*-dimethylallylamine slow peptide program (071472) was used in the sequencer. Thus the N-terminal amino acid sequence of peptide CB-3 was determined as: Val–Ala–Ala–Pro–His–Val–Pro–Val–Lys–Arg–Gly–Cys–X–Asp–Val–Cys–Pro. . .

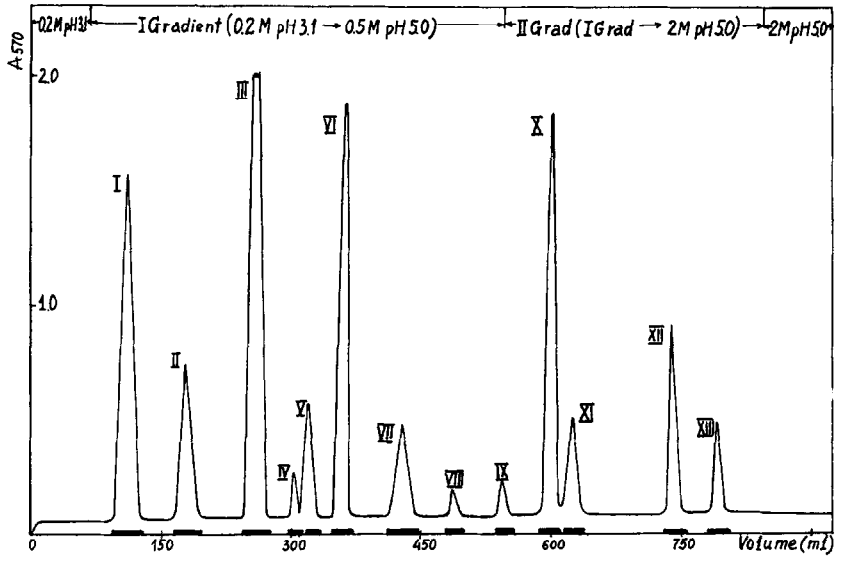

Fig.3. Fractionation on Chromo-Beads resin P4 of tryptic digest of *S*-carboxymethylated cytotoxin. 0.9 × 100 cm column in 0.2 M pyridine acetate pH 3.1. Gradient of concentration and pH from 0.2 M pH 3.1 to 2.0 M pH 5.0 pyridine acetate buffer; flow rate, 30 ml/hr; fraction vol, 6ml.

Table 2
Amino acid composition of tryptic peptides from carboxymethylated cytotoxin (*Naja naja oxiana*)

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10	T-11
Lys	1.0 (1)	1.8 (2)	2.0 (2)	1.0 (1)	1.2 (1)	1.0 (1)	0.9 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)
His	-	-	-	-	-	-	0.8 (1)	-	-	-	-
Arg	-	-	-	-	-	-	-	0.9 (1)	-	-	-
Asp	-	-	-	-	-	1.0 (1)	-	1.1 (1)	1.0 (1)	-	2.9 (3)
Thr	-	-	-	-	0.9 (1)	-	-	-	-	-	0.9 (1)
Ser	-	-	1.0 (1)	1.0 (1)	-	-	-	-	-	1.7 (2)	-
Pro	-	-	0.8 (1)	1.0 (1)	1.0 (1)	-	1.8 (2)	0.8 (1)	0.9 (1)	-	-
Gly	-	-	-	-	1.1 (1)	-	-	0.9 (1)	1.1 (1)	-	-
CM-Cys	-	0.8 (1)		-	0.8 (1)	0.8 (1)	-	1.8 (2)	1.8 (2)	-	2.7 (3)
Val	-	-	1.1 (1)	1.1 (1)	-	-	2.8 (3)	1.0 (1)	0.8 (1)	1.0 (1)	0.7 (1)
Ala	-	-	-	-	1.0 (1)	-	2.0 (2)	-	-	-	-
Met	-	-	-	-	-	-	1.5 (2)	-	-	-	-
Ile	-	-	-	-	-	-	-	1.0 (1)	0.9 (1)	-	-
Leu	1.0 (1)	-	1.9 (2)	1.8 (2)	-	1.0 (1)	-	-	-	2.0 (2)	-
Tyr	-	-	-	-	-	0.8 (1)	-	-	-	-	0.8 (1)
Phe	-	-	1.0 (1)	0.9 (1)	-	-	0.9 (1)	-	-	-	-
Total	2	3	8	7	6	5	12	9	8	6	10
N-terminus	Leu	CM-Cys	Lys	Leu	Thr	Asn	Met	Arg	Gly	Ser	Tyr

Table 3
The structure of tryptic peptides of cytotoxin from *Naja naja oxiana* venom

T- 1	Leu–Lys
T- 2	Cys–Lys–Lys
T- 3	Lys–Leu–Val–Pro–Leu–Phe–Ser–Lys
T- 4	Leu–Val–Pro–Leu–Phe–Ser–Lys
T- 5	Thr–Cys–Pro–Ala–Gly–Lys
T- 6	Asn–Leu–Cys–Tyr–Lys
T- 7	Met–Phe–Met–Val–Ala–Ala–Pro–His–Val–Pro–Val–Lys
T- 8	Arg–Gly–Cys–Ile–Asp–Val–Cys–Pro–Lys
T- 9	Gly–Cys–Ile–Asp–Val–Cys–Pro–Lys
T-10	Ser–Ser–Leu–Leu–Val–Lys
T-11	Tyr–Val–Cys–Cys–Asn–Thr–Asp–Lys–Cys–Asn

The total amino acid sequence of CB-3 was established from the sum total of its chymotryptic fragments. Chromatography and electrophoresis of the chymotryptic hydrolysate of CB-3 led to the isolation of 3 peptides for which the partial structures followed from amino acid analysis and elucidation of the N- and C-termini.

Ch-1 Val–Ala–(Ala,Pro,His,Val,Pro,Val,Lys,Arg,Gly, Cys,Ile,Asx,Val,Cys,Pro,Lys,Ser,Ser,Leu)–Leu

Ch-2 Val–Lys–Tyr

Ch-3 Val–Cys–Cys–Asn–Thr–Asp–(Lys,Cys,Asx)

The results obtained thus enabled us to formulate the total amino acid sequence of cytotoxin from the Middle-Asian cobra *Naja naja oxiana* (fig.4).

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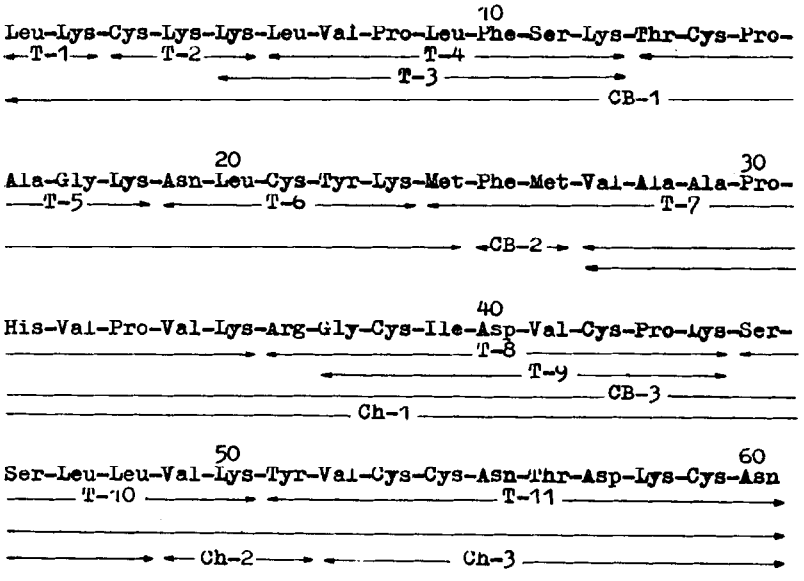

Fig.4. The amino acid sequence of cytotoxin from *Naja naja oxiana* venom: T, tryptic peptides; CB, cyanogen bromide peptides; Ch, chymotryptic peptides of the CB-3.