PURIFICATION OF ANIMAL NEUROTOXINS: ISOLATION AND CHARACTERIZATION OF THREE NEUROTOXINS FROM THE VENOM OF *NAJA NIGRICOLLIS MOSSAMBICA* PETERS

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1. Introduction

This work is part of a general study on snake neurotoxins. In previous papers [1,2], we had shown that the application to snake venoms of the general method of purification which was developed to isolate toxins from scorpion venoms [3–7] was very efficient. Four neurotoxins from two different sources of *Naja haje* venom [1] and two neurotoxins from *Naja nigricollis* venom [2] were purified when only one toxin had been previously isolated in a pure form from each of these venoms by others [8,9]. N-terminal sequences determination using automatic Edman degradation, was found to be the best method for rapid characterization of newly purified toxins [2]. We report, in this paper, on the application of these methods to the venom of *Naja nigricollis mossambica* Peters. The partial sequences of the neurotoxins show that these are more closely related to cobrotoxin (a toxin isolated from the venom of the formosan *Elapidae Naja naja atra*) than the toxins I and II isolated from the venom of *Naja nigricollis* living in Ethiopia [2]. The taxonomical importance of these results is discussed.

2. Experimental

2.1. *Purification of the toxins*

The venom (30 g) was obtained from D. Muller (Johannesburg, South-Africa). The crude venom, first dissolved in a 0.1 M ammonium acetate buffer (pH 8.5), was applied on Sephadex G-50 gel equilibrated in the same buffer. The gel filtration was performed using recycling (3 cycles) through four 1 meter columns of gel. The neurotoxic fraction was freeze dried and applied, for ion exchange chromatography, on a Bio-Rex 70 column equilibrated in a 0.2 M ammonium acetate buffer at pH 7.30. The elution with the same buffer led to the final purification of toxin I and toxin II. A linear gradient (from 0.2 M to 2 M ammonium acetate, pH 7.30) was then applied at the top of the column, eluting toxic fraction III. This fraction gave finally toxin III by equilibrium chromatography on Bio-Rex 70 (0.5 M ammonium acetate buffer, pH 7.30).

Qualitative and quantitative toxicity measurements were carried out, according to Behrens and Karber [10], using C_3H/He mice of 20 ± 3 g (C.S.E.A.L. – C.N.R.S., Orléans-la-Source, France) as previously described [5,7].

2.2. *Preparation of reduced and S-methylated toxins*

Preparation of reduced and S-methylated toxins (RM-toxins) was performed as previously described [11].

2.3. *Amino acid analysis*

Samples of native or RM-toxins were hydrolysed in 6 N HCl for 20 hr and 70 hr at 110°C in sealed evacuated tubes. For the determination of tryptophan, the hydrolyses were made in 0.3 M *p*-toluenesulfonic acid solution containing 0.2% of tryptamine [12]. The hydrolysates were analysed on a Spinco Automatic Amino Analyzer model 120 C.

Table 1
Purification of *Naja nigricollis mossambica* toxins

Purification step	Toxic fraction	Reference to fig. 1	Yield in toxicity from crude venom %		Specific toxicity
Crude venom			100		19
Gel filtration with recycling on Sephadex G-50	R	A	80		473
Chromatography on Bio-Rex 70 ($AcNH_4$ 0.2 M pH 7.30 then linear gradient to $AcNH_4$ 2 M pH 7.30)	T I	B	60.3	76.3	912
	T II		3.8		869
	F III		12.2		520
Chromatography on Bio-Rex 70 $AcNH_4$ 0.5 M pH 7.30	T III	C	12.1		526

The specific toxicity was arbitrarily defined as the number of LD_{50} per A_{280} unit.

2.4. *Edman degradation*

Automatic sequencing was performed on 0.4 μmole of RM-toxins using a Protein Sequencer PS 100 (Socosi, 94100-Saint-Maur, France) and procedures described earlier [2].

2.5. *Carboxypeptidase digestion*

The RM-toxins were dissolved (1%, w/v) in 0.4 M dimethylallylamine-trifluoroacetic acid buffer, pH 8.0. Carboxypeptidase A (diisopropylphosphofluoridate treated, Worthington) was used in an enzyme to substrate ratio of 2:100 (w/w). The digestion was carried out in a glass stoppered vial at 35°C. Aliquots of the solution (0.05 ml) were removed at various intervals (1, 5, 15, 30, 60, 180, 360 min), acidified with an equal volume of pure acetic acid, freeze dried and subjected to amino acid analysis.

3. Results

Table 1 summarises the data obtained concerning the purification of the three neurotoxins of *Naja nigricollis mossambica*. Although a third step was necessary to finally purify toxin III, the two first steps were sufficient to obtain in a pure form toxins I and II. The final yield in toxicity was 76.2% from the crude venom. Fig. 1 shows the elution patterns corresponding to the different chromatographic

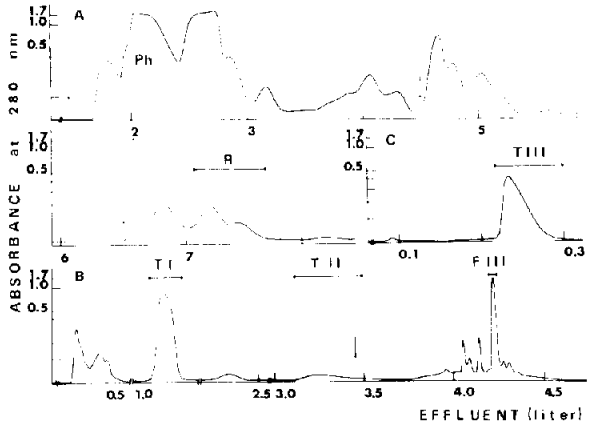

Fig. 1. Purification of *Naja nigricollis mossambica* neurotoxins. (A) Gel filtration with recycling on Sephadex G-50 of 6 g of venom. Four columns of 3.2 × 100 cm; 0.1 M ammonium acetate buffer pH 8.5; flow rate, 77 ml/hr. Fractions indicated by the full line are collected. Only the material marked by the dotted line is recycled. Vertical arrows indicate start of the 3 consecutive cycles. R = toxic fractions; Ph = phospholipase activity. (B) Chromatography on Bio-Rex 70 of fractions R obtained from 6 g of venom. 4 × 50 cm column; 0.2 M ammonium acetate buffer pH 7.30; vertical arrow indicates the start of the elution with a linear gradient of ammonium acetate buffer from 0.2 M pH 7.30 to 2 M pH 7.30 in a total volume of 1.24 litres; flow rate, 50 ml/hr. Double headed arrows indicate pooling of toxic fractions: T I (toxin I), T II (toxin II) and F III (toxic fraction III). (C) Chromatography on Bio-Rex 70 of fraction F III corresponding to 18 g of venom. 2 × 50 cm column; 0.5 M ammonium acetate buffer pH 7.30; flow rate 10 ml/hr. Double headed arrow indicates pooling of toxic fractions (T III = toxin III).

Table 2
Weight of the LD_{50} and purification yields

Material	Weight of the LD_{50} for the 20 g mouse (μg)	Coefficient of toxicity enhancement from crude venom (fold)	Yields of toxins from crude venom (% w/w)
Crude venom	40	–	–
Toxin I	0.80	50	1.17
Toxin II	0.77	52	0.07
Toxin III	1.01	40	0.38

Table 3
Amino-acid compositions of *Naja nigricollis mossambica* toxins

Amino acid	Toxin I	Toxin II	Toxin III
Tryptophan *	1.10 (1)	1.10 (1)	1.99 (2)
Lysine	4.05 (4)	4.04 (4)	4.92 (5)
Histidine	2.00 (2)	2.93 (3)	2.82 (3)
Arginine	7.01 (7)	6.21 (6)	5.96 (6)
Aspartic acid	7.11 (7)	8.10 (8)	8.19 (8)
Threonine	7.86 (8)	8.10 (8)	6.72 (7)
Serine	2.95 (3)	3.90 (4)	2.93 (3)
Glutamic acid	7.01 (7)	6.27 (6)	5.13 (5)
Proline	3.75 (4)	3.67 (4)	3.96 (4)
Glycine	5.92 (6)	5.09 (5)	3.97 (4)
Alanine	– (0)	– (0)	1.05 (1)
Half-cystine *	7.33 (8)	7.26 (8)	7.21 (8)
Valine	1.05 (1)	1.02 (1)	1.10 (1)
Methionine	– (0)	– (0)	1.00 (1)
Isoleucine	0.98 (1)	0.98 (1)	1.08 (1)
Leucine	2.03 (2)	1.86 (2)	1.96 (2)
Tyrosine	1.99 (2)	1.90 (2)	2.00 (2)
Phenylalanine	– (0)	– (0)	– (0)
Total	63	63	63
Molecular weight	7176.2	7173.1	7315.4
$A^{1\%}_{1\ cm}$ at 280 nm	13.70	14.46	21.48
$10^{-3} \times \epsilon$ (max) **	9.80 (280 nm)	10.37 (280 nm)	15.70 (280 nm)

* Half-cystine and tryptophan residues were quantified after *p* - toluene - sulfonic acid hydrolysis

** $M^{-1} \times cm^{-1}$.

steps. Table 2 gives the purification yields and the weight of the three toxins corresponding to their LD_{50} for the 20 g mouse.

The amino acid compositions of the three toxins are given in table 3 together with their molecular weights and their molecular extinction coefficients at 280 nm. The N-terminal and C-terminal sequences, as they were determined by Edman automatic sequencing and carboxypeptidase A hydrolysis on reduced and methylated toxins, are given in fig. 2 and compared to the sequences of *Naja naja atra* cobrotoxin [13] and *Naja nigricollis* toxins I and II [2,14].

4. Discussion

In the case of *Naja nigricollis mossambica* venom it has been possible to shorten the general method of purification used in our laboratory [1, 2, 7]. The first two steps: extraction by water and dialysis, found to be absolutely necessary for the purification of scorpion venoms [15], could be avoided and the venom dissolved in an ammonium acetate buffer was applied straight on a gel filtration column for recycling. Contrary to what we observed with scorpion toxins (unpublished results) Bio-Rex 70 was found to be more suitable than Amberlite CG-50 as it was noticed for other snake toxins [16]. Fraction III which was eluted using an ionic strength linear gradient had to be finally purified by equilibrium chromatography which, obviously, gives a better resolution for proteins having very similar pH_i. It is the first toxin for which we found necessary to use a gradient. This can be due to its relatively high content in tryptophan added to its strong basicity. The three purified toxins account all together for 76.2% of the toxicity of the venom. As 20% of the venom toxicity, corresponding to the phospholipase activity, was recovered in the second protein fraction eluted

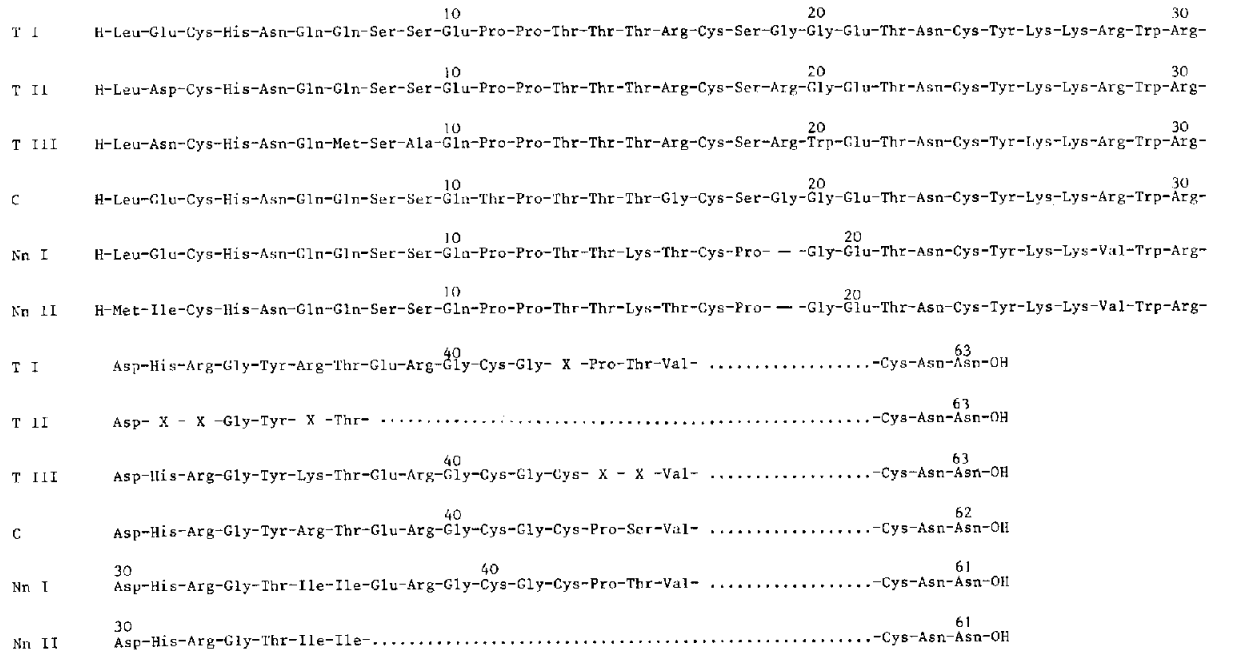

Fig. 2. N- and C-terminal sequences of *Naja nigricollis mossambica* neurotoxins (T I, T II and T III). C: cobrotoxin; Nn I and Nn II: toxins I and II of *Naja nigricollis*. X: not determined. –: deletion.

from Sephadex G-50 (see fig. 1.A), one can assume that the yield of neurotoxicity (95% from the crude venom) is excellent.

Elapidae and *Hydrophidae* snake neurotoxins may be divided into two groups according to the number of constitutive amino acids [1]. The first group consisted of the so called 'short' toxins (60–62 residues) [17]. The three toxins of *Naja nigricollis mossambica* belong obviously to this first group although they are made of 63 amino acid residues. As the three 'short' toxins account for 95% of neurotoxicity, one can assume that this venom does not contain toxin belonging to the second group consisted of the so called 'long' toxins (71–74 residues). Toxin III is the first newly discovered 'short' toxin containing two tryptophan residues and one alanine residue; in addition the only methionine residue is not at the N-terminal position as found until now for 'short' toxins containing methionine. In the second position from the N-terminal end, Glu, Asp and Asn residues have been found respectively in toxins I, II and III where generally Glu is present. In the positions 19 and 20 the sequence –Gly–Gly– found in toxin I, just as in cobrotoxin, is replaced by the sequences –Arg–Gly– (toxin II) or –Arg–Trp– (toxin II). This is of interest as Gly 20 was till now considered to be an invariant residue not only in 'short' and 'long' toxins [17] but also in cytotoxins [18,19].

If Gln was found in position 10 in toxin III as in all other 'short'toxins but *Dendroaspis polylepis* toxin α [17], a Glu residue was shown to exist in this position in the toxins I and II.

According to amino acid sequence the three toxins of *Naja nigricollis mossambica* are more similar to cobrotoxin from *Naja naja atra* [13] than to the toxins I and II of *Naja nigricollis* [2,14]. Considering the amino acid compositions of cobrotoxin [13] and of *Naja nigricollis mossambica* toxins I, II and III (table 3), as also the available sequence data (fig. 2), one can assume that the sequences still remaining to be determined, will be very close to that of cobrotoxin. These results may show to be of some taxonomical importance as it has been found that *Naja nigricollis* toxin I and II show amino acid sequences very close to those of toxins I and II of *Naja haje* [2]. However the obvious problem of the

authenticity of the starting material exists (for example in our case the presence of toxin II in a very low amount might well be taken as one indication of contamination in spite of the real competence of the snake catcher): the venom of *Naja haje* and *Naja nigricollis* were both from Ethiopia [2] whereas *Naja naja atra* and *Naja nigricollis mossambica* venoms were collected respectively in China and in South-Africa which made impossible, in that case, any possible confusion. For these reasons, the rapid characterization of the proteins isolated from snake venoms has to be taken as a very important problem which must be urgently solved. The way we choose does not use a big amount of protein and is not time consuming like the determination of a complete amino acid sequence which is, till now, the best chemical test. As a matter of fact it will allow a quite better classification than to take into consideration the antigenic characteristics of these molecules [20]. Moreover, using such chemical criteria, people would never have published hypothesis based on pharmacological and immunological results [21–23] obtained with a venom which as obviously [2] a mixture of *Echis carinatus* (*Viperidae*) and *Naja nigricollis* (*Elapidae*) venoms.

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