Isolation and amino acid sequence of a short-chain neurotoxin from an Australian elapid snake, *Pseudechis australis*

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A short-chain neurotoxin *Pseudechis australis* a (toxin Pa a) was isolated from the venom of an Australian elapid snake *Pseudechis australis* (king brown snake) by sequential chromatography on CM-cellulose, Sephadex G-50 and CM-cellulose columns. Toxin Pa a has an LD₅₀ (intravenous) value of 76 µg/kg body wt. in mice and consists of 62 amino acid residues. The amino acid sequence of Pa a shows considerable homology with those of short-chain neurotoxins of elapid snakes, especially of true sea snakes.

INTRODUCTION

More than 40 post-synaptically acting short-chain neurotoxins have been isolated from the venoms of proteroglyphous snakes and their amino acid sequences determined. However, only one Australian elapid (*Acanthopis antarcticus*) toxin is included among them (Kim & Tamiya, 1981b). The present paper describes the isolation, some properties and amino acid sequence of a short-chain neurotoxin, *Pseudechis australis* a (toxin Pa a), from the venom of an elapid snake, *Pseudechis australis* (king brown snake), the largest venomous snake in Australia.

The venom of the same species is known to exhibit lethal activity (Leonardi *et al.*, 1979) and phospholipase A₂ and lesser lysophospholipase activities (Takasaki & Tamiya, 1982). Recently, a toxic phospholipase A₂ and a non-toxic phospholipase A₂ (named Pa-11 and Pa-13 respectively) were isolated (Nishida *et al.*, 1985a) from the venom and their amino acid sequences determined (Nishida *et al.*, 1985b).

MATERIALS AND METHODS

Snake venom

Desiccated *P. australis* venom was obtained from Australian Venom Supplies, Turramura, N.S.W., Australia.

M_r determination

M_r determination by gel filtration was carried out with the Pharmacia fast protein liquid chromatography system equipped with a column (1 cm × 30 cm) of Superose 12 (Pharmacia Fine Chemicals, Uppsala, Sweden). Sodium/potassium phosphate buffer, pH 7.0 (0.05 M), containing 5 M-guanidine hydrochloride and 0.05% (w/v) EDTA was used as a solvent. Protein samples (approx. 0.1 mg) were denatured and reduced in 0.05 M-sodium/potassium phosphate buffer, pH 7.0, containing 6 M-guanidine hydrochloride, 0.2% (w/v) dithiothreitol and 0.05% (w/v) EDTA (0.1 ml) for 4 h at 37 °C. The standard proteins (M_r values in parentheses) were ovalbumin (45000), myoglobin (17000), phospholipase Pa-11 (13000; Nishida *et al.*, 1985a), long-chain neurotoxin *Acanthopis antarcticus* b (8100; Kim & Tamiya, 1981a) and short-chain neurotoxin erabutoxin a (6800; Tamiya & Arai, 1966).

Amino acid analysis

Toxin Pa a (approx. 10 nmol) was hydrolysed *in vacuo* at 105 °C with 5.7 M-HCl (0.3 ml) for 24 h. The hydrolysates were dried *in vacuo* and analysed with an automatic amino acid analyser (JLC-10D; JEOL, Tokyo, Japan). For the determination of tryptophan content, 4 M-methanesulphonic acid (0.3 ml) containing 3-(2-aminoethyl)indole (0.2%, w/v; Pierce Chemical Co., Rockford, IL, U.S.A.) was used instead of 5.7 M-HCl.

Peptide samples (2–5 nmol) were hydrolysed with 5.7 M-HCl, similarly to toxin Pa a, and the hydrolysates were analysed with an automatic amino acid analyser (JLC-200A; JEOL, Tokyo, Japan).

Reduction and S-carboxymethylation of toxin Pa a

The method of Crestfield *et al.* (1963) was used with a slight modification. Toxin Pa a (4 mg) was dissolved in 1 M-Tris/HCl buffer, pH 8.6 (3.5 ml), containing 6 M-guanidine hydrochloride and 10 mg of EDTA and denatured for 3 h at 37 °C. It was then reduced with dithiothreitol (4 mg) for 3 h at 37 °C and S-carboxymethylated with iodoacetic acid (200 mg) for 20 min at room temperature in the dark. Reduced and S-carboxymethylated toxin Pa a was desalted on a column (1.5 cm × 60 cm) of Sephadex G-25 (fine grade) with 0.05 M-NH₄HCO₃, pH 7.8, and freeze-dried.

Amino acid sequence determination

The amino acid sequences of reduced and S-carboxymethylated toxin Pa a and its chymotryptic peptides were determined with a gas-phase sequencer (model 470A; Applied Biosystems, Foster, CA, U.S.A.) operated essentially as described by Hewick *et al.* (1981). Amino acid phenylthiohydantoin derivatives were analysed by an h.p.l.c. system, SP8100, equipped with a column (0.46 cm × 30 cm) of absorbent C₈, a detector (SP8440) and an integrator (SP4270) (Spectra-Physics, Santa Clara, CA, U.S.A.).

RESULTS

Purification of toxin Pa a

The preliminary fractionation of *Pseudechis australis* venom components by CM-cellulose CM-52 column chromatography was described previously (Nishida *et al.*, 1985a). Fraction X (total 681 A₂₈₀ units) was freeze-

Fig. 1. Purification of toxin Pa a by rechromatography on CM-cellulose

Gel-filtered and freeze-dried residue (22 mg) from fraction X (see the text) was dissolved in 5 ml of 0.05 M-sodium borate buffer, pH 8.3, and applied to a column (1.5 cm $\times$ 14 cm) of CM-cellulose CM-52 equilibrated with the same buffer containing 0.03 M-NaCl. The elution was performed with the same buffer containing 0.03 M-NaCl (25 ml), followed by a linear-concentration-gradient elution from 0.03 M- to 0.12 M-NaCl in the same buffer (total 240 ml), and then with 0.3 M-NaCl in the same buffer (arrow). Fractions of volume size 5.0 ml were collected. The protein-containing fractions (CM-I, CM-II and CM-III), indicated by the bars, were collected separately. —, A_{280} ; - - - - -, NaCl concentration.

dried and the residue gel-filtered through a column (1.8 cm $\times$ 144 cm) of Sephadex G-50 (fine grade) in 10% (v/v) acetic acid in one-fifth portions. Most of the components were eluted at 200–235 ml, and this fraction showed phospholipase A_2 activity. The eluate from 235 ml to 250 ml was pooled and freeze-dried (yield 22 mg). The residue was rechromatographed on a column of CM-cellulose CM-52 (Whatman, Maidstone, Kent, U.K.) (Fig. 1). The main component in fraction CM-II was lethal to mice and gave a single band on polyacrylamide-disc-gel electrophoresis at pH 4.0. The component was named 'toxin *Pseudechis australis* a' (toxin Pa a). A yield of 18 mg of purified toxin Pa a was obtained from 5 g of desiccated *P. australis* venom.

The component in fraction CM-I also showed lethal activity, but was not purified further. The component in fraction CM-III was pure on disc electrophoresis, but was not lethal to mice.

Some properties of toxin Pa a

The lethal activity (LD_{50}) of toxin Pa a by intravenous injection in mice was 76 μ g/kg body wt.

The M_r was estimated to be 7100 by gel filtration in the presence of 5 M-guanidine hydrochloride.

The amino acid composition of toxin Pa a is given in Table 1. Toxin Pa a consists of 62 amino acid residues including nine half-cystine, an alanine, a methionine and no phenylalanine residues. The amino acid composition

Table 1. Amino acid compositions of toxin Pa a and its chymotryptic peptides

The composition of each peptide is given as the molar proportions of amino acids determined. The numbers in parentheses represent the values confirmed by the sequence study. Amino acids detected to an extent less than 0.10 mol/mol are ignored.

Amino acid	Amino acid composition (mol/mol)					
	Toxin Pa a	Residues...	Peptide C-Ia 30–52	Peptide C-Ib 26–29	Peptide C-II 30–62	Peptide C-III 1–25
Asp	5.00 (5)*		1.00 (1)*		4.00 (4)*	1.00 (1)*
Thr†	7.48 (8)		0.98 (1)	0.94 (1)	2.94 (3)	3.70 (4)
Ser†	5.46 (6)		1.88 (2)		1.86 (2)	3.69 (4)
Glu	6.06 (6)		1.06 (1)		2.13 (2)	3.92 (4)
Pro	3.03 (3)		2.62 (2)		2.05 (2)	1.01 (1)
Gly	5.97 (6)		4.11 (4)		4.13 (4)	2.10 (2)
Ala	1.04 (1)				0.13	0.99 (1)
Cys†	6.89 (9)		1.75 (2)		3.82 (5)	3.23 (4)
Val	1.28 (1)		0.91 (1)		1.08 (1)	
Met‡	0.99 (1)					0.70 (1)
Ile	1.74 (2)		0.92 (1)		0.92 (1)	0.98 (1)
Leu	0.96 (1)		1.00 (1)		1.01 (1)	
Tyr	1.07 (1)					0.90 (1)
Phe	(0)					
Lys	6.14 (6)		1.95 (2)	2.00 (2)*	2.93 (3)	1.08 (1)
His	1.91 (2)		1.94 (2)		1.78 (2)	
Arg	3.14 (3)		3.01 (3)		2.90 (3)	
Trp	0.90 (1)§			(1)¶		
Total	(62)		(23)	(4)	(33)	(25)
Yield			15%	74%	38%	59%

* The amounts of these amino acids are taken as standards.

† Cystine residues in peptides are determined as *S*-carboxymethylcysteine. Cystine and *S*-carboxymethylcysteine tend to give low values.

‡ These residues tend to give low values owing to destruction during hydrolysis.

§ Measured on 4 M-methanesulphonic acid hydrolysate.

¶ Detected by u.v.

Fig. 2. Elution profile of chymotryptic peptides

A chymotryptic digest of reduced and *S*-carboxymethylated toxin Pa a (460 nmol) was diluted with water (4.5 ml) and applied to a column (1.5 cm × 8 cm) of DEAE-cellulose DE-52 equilibrated with 0.02 M-NH₄HCO₃, pH 7.6. A linear-concentration-gradient elution was carried out from 0.02 M- to 0.4 M-NH₄HCO₃ (total 400 ml). The arrow indicates the starting point of the gradient. Elution was monitored by measuring the absorbance at 230 nm and 280 nm, and 5.0 ml fractions were collected. The peptide fractions (I–IV), indicated by the bars, were collected separately. —, *A*₂₃₀; ·····, *A*₂₈₀.

of toxin Pa a is very similar to those of short-chain neurotoxins from snakes belonging to true sea snakes.

Separation of chymotryptic peptides

Reduced and *S*-carboxymethylated Pa a (3.2 mg, 460 nmol) was dissolved in 0.2 M-NH₄HCO₃, pH 7.8 (0.5 ml), and digested with α-chymotrypsin (three-times crystallized; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) at 37 °C for 1 h. The enzyme/substrate ratio was 1:110 (w/w).

The digest was applied to a column of DEAE-cellulose DE-52 (Whatman) (Fig. 2). Freeze-dried fraction I gave two peptides, C-Ia and C-Ib, on chromatography on a CM-cellulose CM-52 column (1.0 cm × 8 cm) equilibrated with 0.01 M-NH₄HCO₃, pH 7.2. The elution was performed with 0.07 M-NH₄HCO₃ for peptide C-Ia and with 0.15 M-NH₄HCO₃ for peptide C-Ib. Peptides C-II, C-III and C-IV were purified separately from freeze-dried fractions II, III and IV respectively by gel filtration on a Sephadex G-25 (fine grade) column (1.5 cm × 76 cm) in 25 mM-ammonium acetate buffer, pH 8.3.

The amino acid compositions and yields of the peptides are given in Table 1. Amino acid analysis showed that peptides C-Ia and C-IV were partial-digestion products from peptide C-II.

Amino acid sequence of toxin Pa a

Sequence analysis was performed on reduced and *S*-carboxymethylated toxin Pa a, peptide C-II and peptide C-IV up to 46th, 33rd and 10th steps respectively as shown in Table 2. The repetitive yields were 92, 93 and 88% respectively. From the overlappings of sequences (30th–33rd steps of reduced and *S*-carboxymethylated toxin Pa a and 1st–4th steps of peptide C-II, and 24th–29th

Table 2. Sequence data on reduced and *S*-carboxymethylated toxin Pa a, peptide C-II and peptide C-IV

The values are in pmol. Repetitive yields are determined by linear-regression analysis of reliable residues. The amount loaded is given as ‘cycle 0’. Abbreviations: RCM-Pa a, reduced and *S*-carboxymethylated toxin Pa a; Cm, carboxymethyl; R.Y., repetitive yield; N.D., not determined; N.Q., not quantified.

Cycle	RCM-Pa a	Peptide C-II	Peptide C-IV
0	6000	2000	1000
1	Met 2640	Ser N.Q.	Thr N.Q.
2	Thr N.Q.	Asp 863	Cm-Cys 237
3	Cm-Cys 1680	His 335	Cm-Cys 200
4	Cm-Cys 1620	Arg 295	Lys 358
5	Asn 1440	Gly 829	Thr N.Q.
6	Gln 1400	Ser N.Q.	Asp 172
7	Gln 1420	Arg 242	Glu 149
8	Ser N.Q.	Thr N.Q.	Cm-Cys 112
9	Ser N.Q.	Glu 611	Asn 118
10	Gln 820	Arg 262	Asn 71
11	Pro 370	Gly 690	R.Y. 88%
12	Lys 695	Cm-Cys 665	
13	Thr N.Q.	Gly 436	
14	Thr N.Q.	Cm-Cys 603	
15	Thr N.Q.	Pro 475	
16	Ile 530	His 62	
17	Cm-Cys 485	Val 271	
18	Ala 465	Lys 384	
19	Gly 285	Pro 248	
20	Gly 320	Gly 198	
21	Glu 380	Ile 221	
22	Ser N.Q.	Lys 240	
23	Ser N.Q.	Leu 212	
24	Cm-Cys 1055	Thr N.Q.	
25	Tyr 560	Cm-Cys 202	
26	Lys 370	Cm-Cys 249	
27	Lys 825	Lys 177	
28	Thr N.Q.	Thr N.Q.	
29	Trp 195	N.D.	
30	Ser N.Q.	Glu 152	
31	Asp 690	Cm-Cys 160	
32	His 125	Asn 70	
33	Arg 180	Asn 101	
34	N.D.	R.Y. 93%	
35	N.D.		
36	N.D.		
37	Thr N.Q.		
38	Glu 222		
39	N.D.		
40	N.D.		
41	Cm-Cys 453		
42	N.D.		
43	N.D.		
44	Pro 94		
45	N.D.		
46	Val 124		
	R.Y. 92%		

steps of peptide C-II and 1st–6th steps of peptide C-IV), the alignment of the peptides was deduced as shown in Fig. 3. The complete amino acid sequence of toxin Pa a was thus determined by single run of proteolytic digestion and by three runs of the gas-phase sequencer operation.

Table 3. Amino acid sequences of some short-chain neurotoxins

No.	Species	Component	-----1-----2-----3-----4-----5-----6-----	Different†	Reference
1.	<i>Laticauda laticaudata</i> (Am)*	Ll b(Am)	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTITERGGCGPQVKSGIKLTCCQSDDCNN	22	Tamiya <i>et al.</i> (1983)
2.	<i>Laticauda semifasciata</i>	Ls Ec	RICFNHQSSQPQTTKTCSPESSCYHKQWSDFRGTIIERGCGPQVKPGINLSCCESEVCNN	22	Tamiya & Abe (1972)†
3.	<i>Enhydryna schistosa</i>	Es 5	MTCCNQSSSQPKTTTNCA ESSCYKKTWSDHRGTIERGGCGPQVKSGIKLECCHTNECNN	10	Fryklund <i>et al.</i> (1972)
4.	<i>Aipysurus laevis</i>	Al b	LTCCNQSSSQPKTTTDCAN DSCYKMTWRDHRGTIERGGCGPQVKPGIKLECCCKTNECNN	13	Maeda & Tamiya (1976)
5.	<i>Pseudechis australis</i>	Pa a	MTCCNQSSSQPKTTTICAGGESSCYKKTWSDHRGSRTERGGCGPHVKPGIKLTCCCKTDECNN	0	This paper
6.	<i>Acanthophis antarcticus</i>	Aa c	MQCCNQSSSQPKTTTTCPPGVSSCYKKTWRDHRGTIIERGCGPQVKPGIRLICKCKTDECNN	11	Kim & Tamiya (1981b)
7.	<i>Dendroaspis jamesoni kaimosae</i>	Vn-II	RICYNHQSTTPATTKSC GENSCYKKTWSDHRGTIIERGCGCPQVKQGIHLHCCQSDKCNN	22	Strydom (1973)
8.	<i>Dendroaspis polylepis</i>	Dp a	RICYNHQSTTRATTKSC EENSICYKYWRDHRGTIIERGCGCPQVKPGVGIHCCQSDKCN	28	Strydom (1972)
9.	<i>Naja nigricollis</i>	Toxin α	LECHNQSSSQPPTTKTCP GETNICYKVVWRDHRGTIIERGCGCPQVKPGIKLNCCTTDDKCNN	19	Eaker & Porath (1967)
10.	<i>Naja mossambica mossambica</i>	NmIII	LNCHNQMSAQPPPTTRCSRWETNICYKRWDRDHRGYKTERGGCGPQVKKGILHCCCTSDNCNN	23	Gregoire & Rochet (1977)

* From Amami Island, Okinawa Islands.

† Number of residues different from those of toxin Pa a. Deletion is counted as 21st amino acid residue.

‡ Revised by Nishida *et al.* (1985c).

Underlines indicate amino acid residues detected by Edman degradation.

A neurotoxin, toxin Pa a, was isolated from the venom of an Australian elapid snake, *Pseudechis australis*, and its amino acid sequence determined. Toxin Pa a belongs to the short-chain type, consisting of 62 amino acid residues with eight half-cystine residues. This is the second short-chain neurotoxin from the Australian elapid snakes to be sequenced. All the 22 residues that are conserved in short-chain neurotoxins with so-far known sequences are present in toxin Pa a.

In Table 3, the amino acid sequences of two toxins each of representatives of sea kraits (*Laticauda*, nos. 1 and 2), true sea snakes (*Enhydrina* and *Aipysurus*, nos. 3 and 4), mambas (*Dendroaspis*, nos. 7 and 8) and cobras (*Naja*, nos. 9 and 10) are compared with those of toxins of Australian elapids (*Pseudechis* and *Acanthophis*, nos. 5 and 6). These toxins are so chosen that their sequence differences are the largest among toxins with established sequences from snakes belonging to each group.

As shown in Table 3, there are 11 amino acid residue replacements between the amino acid sequence of toxin Pa a (no. 5) and that of toxin *Acanthophis antarcticus* c (Aa c, no. 6), a toxin from another Australian elapid snake, *Acanthophis antarcticus*. The residue replacement numbers of toxin Pa a (10 and 13) are about the same for the toxins of true sea snakes (Table 3, nos. 3 and 4), whereas they are larger than 19 for the toxins from other snakes (Table 3, column 'Difference'). The presence of a cystein residue, which was proved to be in the free thiol state (Kim & Tamiya, 1981*b*), at position 4 and that of a glutamic acid residue at position 59 are especially characteristic to Australian elapid and true sea-snake neurotoxins. These results show a close relationship between the Australian elapids and true sea snakes.

The inclusion of Australian elapids as subfamily Oxyuraninae together with subfamily Hydrophiinae (true

sea snakes) in family Hydrophiidae is proposed by Smith *et al.* (1977) on morphological data. The present observation agrees with this classification.

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