ISOLATION OF A NON-NEUROTOXIC, NON-ENZYMATIC PHOSPHOLIPASE A HOMOLOGUE FROM THE VENOM OF THE AUSTRALIAN TIGER SNAKE *NOTECHIS SCUTATUS SCUTATUS*

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Received 23 September 1976

1. Introduction

Snake venom neurotoxins act on the peripheral nervous system, blocking transmission across the cholinergic neuromuscular junction by either a pre- or postsynaptic mode of action. Presynaptic neurotoxins interfere with the release of acetylcholine from the motor nerve terminals and might be useful tools for the investigation of the mechanism of transmitter release.

The most thoroughly characterized presynaptic venom neurotoxins either consist of, or contain as an indispensible part, a structural element which is both structurally and functionally very similar to non- or weakly neurotoxic phospholipases A of various origin. Furthermore, there is a fair amount of indirect evidence that the enzymatic activity might be involved somehow in the toxic action [1–4]. One should like to know which structural features render certain phospholipases A so highly neurotoxic.

The venom of the Australian tiger snake *Notechis scutatus scutatus* contains two highly potent basic phospholipases A, notexin [1, 5–8] and *notechis* II-5* [9], both of which exhibit presynaptic neurotoxic and myotoxic action. Treatment of notexin with *p*-bromophenacyl bromide leads to specific modification of His-48, abolishing by >99% both the phospholipase A and neurotoxic activity, and severely impairing the toxin's ability to bind calcium ions. Notexin and *notechis* II-5 differ in sequence in only seven of 119 positions and show nearly complete identity in the stretch between Lys-57 and Cys-90, which is the region in which they differ most drastically from a few homologous non- or weakly toxic phospholipases A [10]. If the enzymatic activity of notexin and *notechis* II-5 were merely an evolutionary relic and thus irrelevant to the neuro- and myotoxic actions, one might expect to find in the same venom a toxic protein devoid of catalytic activity [10,11]. Furthermore, comparison of the structure and function of homologous proteins from the same venom offers a means of localizing those structural features responsible for the various activities without the complications introduced by species variation.

Gel filtration of crude tiger snake venom on Sephadex G-75 followed by gradient ion-exchange chromatography on Bio-Rex 70 and elution chromatography on SP-Sephadex yielded a notexin homologue, *notechis* II-1, which is apparently devoid of catalytic, myo- and neurotoxic activities. *Notechis* II-1 binds calcium ions and can be modified with *p*-bromophenacyl bromide as effectively as notexin. Preliminary structural data indicate a high degree of homology with notexin, except for the stretch between Lys-57 and Cys-92.

2. Materials and methods

Gel filtration of the crude venom, ion-exchange chromatography on Bio-Rex 70, reduction and *S*-carboxymethylation, digestion with trypsin, purification of peptides, manual Edman degradation, identification of phenylthiohydantoin derivatives, and amino

* Previously designated toxin 5 or *notechis* 5.

acid analysis were performed as described previously in the elucidation of the notexin sequence [8]. Toxicity and phospholipase A assays, modification with *p*-bromophenacyl bromide, determination of molar extinction coefficients, and determination of the protein–calcium binding constant and number of binding sites were performed as described previously [1]. Myotoxic activity was assayed by determining the percentual weight increase of the extensor digitorum longus muscle of female Wistar rats weighing between 180–200 g upon subcutaneous injection in 0.9% w/v NaCl into the antero-lateral aspect of one hind limb [7]. Elution chromatography on SP-Sephadex (2 × 25 cm) was performed in 0.09 M ammonium bicarbonate, pH 8.0, at a flow rate of 20 ml/h and with 5 ml fractions.

3. Results and discussion

Gel filtration of the crude venom on Sephadex G-75 followed by ion-exchange chromatography on Bio-Rex 70 (fig.1) yielded *notechis* II-1 in 90% purity. The contaminants were removed by elution chromatography on SP-Sephadex. Six per cent of the ultra violet-absorbing material eluted at the breakthrough, 4% at one bed volume, and 90% at two bed volumes. The amino acid composition and properties of native *notechis* II-1 and one of its derivatives are compared with those of notexin in table 1.

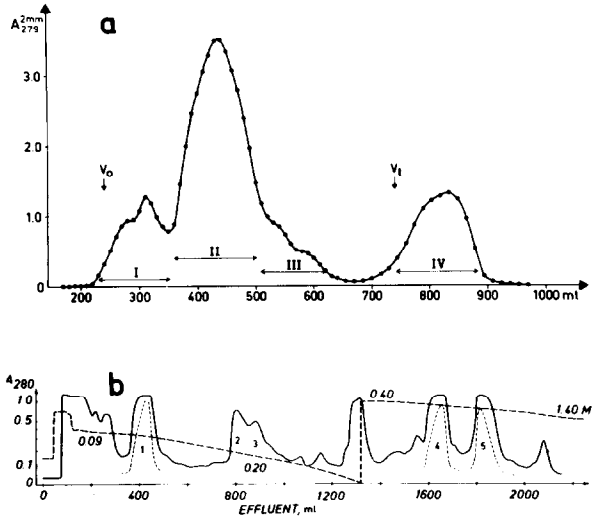

Fig.1. Fractionation of Tiger snake venom. (a) Gel filtration of 2.10 g venom on a 3.2 × 92 cm column of Sephadex G-75 in 0.1 M ammonium acetate. (b) Gradient chromatography of gel-filtration fraction II on a 3.2 × 27 cm column of Bio-Rex 70 as described previously [8]. *Notechis* II-1 corresponds to peak 1, notexin to peak 4, and *notechis* II-5 to peak 5.

The relative activities of five proteins having phospholipase A structure are summarized in table 2. The activity of notexin has been set at 100. Comparison of columns 1 and 2 suggests a close and semi-quantitative relationship between the lethal neurotoxicity and myotoxicity, possibly indicating a common underlying mechanism of action. Comparison of columns 1 and 3 suggests a qualitative relationship between the lethality and phospholipase A activity. The two proteins which are potent neurotoxins are also potent phospholipases, whereas the two poorly toxic proteins are also essentially inactive enzymatically. In this context we should point out, however, that the most potent presynaptic snake venom neurotoxin known, the complex glycoprotein taipoxin from the venom of *Oxyuranus scutellatus scutellatus*, is 30-fold more lethal than notexin but 100-fold less active enzymatically [12]. Taipoxin consist of three peptide chains, however, and may dissociate upon interaction with its target molecule, rendering somewhat speculative any comparison of the relative toxic and enzymatic activities of whole taipoxin with those of the single-chain phospholipase A homologues in table 2.

3.1. *Partial sequence of* Notechis *II-1*

Seventeen cycles of Edman degradation on the intact RCM-derivative of *p*-bromophenacyl *notechis* II-1 established the sequence

Asn–Leu–Val–Gln–Phe^{5}–Ser–Asn–Met–Ile–Gln^{10}–

Cys–Ala–Asn–His–Gly^{15}–Ser–Arg

which differs from the corresponding stretch in notexin by only three substitutions. Group fractionation of a 3 h tryptic digest by gel filtration on Sephadex G-50 followed by column zone electrophoresis of the fractions at pH 5.0 and/or pH 1.9 yielded pure peptides accounting for the whole protein. The peptide containing the *p*-bromophenacyl residue was localized by

Table 1
Amino acid composition and properties of tiger-snake phospholipase A homologues

Amino acid	*notechis* II-1	RCM-PBP-*notechis* II-1[a]	notexin	TRYP (47–54)
Cm-cysteine		14.33 (14)	14	0.89 (1)
Aspartic acid	14.96 (15)	15.73 (15)	18	2.07 (2)
Threonine	8.89 (9)	8.97 (9)	3	1.00 (1)
Serine	8.97 (9)	8.92 (9)	3	
Glutamic acid	8.28 (8)	8.32 (8)	7	
Proline	5.62 (6)	5.71 (6)	5	
Glycine	6.08 (6)	6.10 (6)	10	
Alanine	10.89 (11)	11.02 (11)	9	1.00 (1)
Half-cystine	14.33 (14)[b]			
Valine	3.09 (3)	2.84 (3)	4	
Methionine	1.22 (1)	0.86 (1)	2	
Isoleucine	3.12 (3)	3.05 (3)	4	
Leucine	4.17 (4)	4.05 (4)	4	
Tyrosine	9.20 (9)	8.92 (9)	10	1.01 (1)
Phenylalanine	2.97 (3)	2.59 (3)	5	
Histidine	1.98 (2)	1.12 (1)	3	(1)
Lysine	8.90 (9)	9.09 (9)	11	
Tryptophan[c]	(2)	(2)	2	
Arginine	4.90 (5)	5.03 (5)	5	1.01 (1)
Total	119	119	119	(8)
Formula weight	13 319		13 578	
Yield (%)	5		5	40
Molar extinction coefficient	25 300 (278 nm)	37 200 (272 nm)	27 000 (278 nm)	23 400 (262 nm)
Number *p*-bromophenacyl residues per mole		0.84 (1)		1.25 (1)
Specific phospholipase A activity (μeqv.OH/ min/mg protein)	0.4		840	
LD_{100} (mg/kg mouse)	>20		0.025	
Molar difference extinction coefficient upon calcium binding[d]	1400 (242 nm)		1200 (242 nm)	
Number of calcium binding sites[d]	0.9		1.1	
Protein–calcium dissociation constant (M)[d]	6×10^{-4}		1.4×10^{-4}	

[a] PBP = *p*-bromophenacyl.
[b] Determined as cysteic acid.
[c] Determined spectrophotometrically.
[d] pH 7.4.

monitoring all separation runs at 262 nm (λ_{max} for the reagent) in addition to 276 nm and also at 570 nm after alkaline hydrolysis and ninhydrin reaction. The amino acid composition of the modified peptide TRYP (47–54) is given in table 1. Seven cycles of Edman degradation established the sequence

$$\overset{\text{PBB}}{\text{Thr}}\text{–His–Asp–}\overset{50}{\text{Asp}}\text{–Cys–Tyr–Ala–Arg}$$

which differs from the corresponding stretch in notexin at three positions.

The partial sequence of *notechis* II-1 is shown in figure 2. There appears to be a high degree of homology between *notechis* II-1 and notexin in the first half of the sequences. Of the 13 substitutions indicated in the first 57 positions, only Ser/Gly at position 26 involves a hitherto invariant residue in phospholipases

Table 2
Relative activities of snake venom phospholipase A homologues

	Lethality	Myotoxicity[a]	Phospholipase A
Notexin	100	100	100
Notechis II-5	42	20	165
Naja nigricollis	2.5	2	31
PBP-Notexin	0.25	1	0.21
Notechis II-1	< 0.10	0	0.05

[a]The myotoxicity assays were kindly performed by Dr John B. Harris.
[b]PBP = *p*-bromophenacyl.

In a previous communication [1] the myotoxicity of this notexin derivative was erroneously reported to be only somewhat less than that of the native.

A [10]. Furthermore, there is a high degree of identity in those two stretches, the integrity of which has been shown to be essential to enzymatic activity in porcine pancreatic phospholipase A; namely, the amino terminus, which is involved in interaction with micellar interfaces [13], and the stretch around His-48, which is involved in binding metal ions and monomeric substrates [14].

There is also a high degree of identity in the last fourth of the sequences, and none of the seven assumed substitutions in the last 28 positions involves hitherto invariant residues. The homology seems to break down, however, in the stretch between Lys-57 and Cys-92 (not shown), and it is impossible even to align, let alone infer the sequences of the tryptic peptides from this stretch in *notechis* II-1. It is tempting to conclude that some structural deficiency in the region 58–91 in the *notechis* II-1 sequence is responsible for the lack of both catalytic and toxic activity.

Acknowledgements

We thank Ragnar Thorzelius and Sven Centring for expert assistance with the amino acid analysis and Jan Fohlman for help with the Edman degradation. This investigation was supported by the Swedish Natural Science Research Council (dnr 2859-008).

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RESIDUES 1-57

NOTEXIN ASN-LEU-VAL-GLN-PHE-SER-TYR-LEU-ILE-GLN-CYS-ALA-ASN-HIS-GLY-LYS-ARG-PRO-THR-TRP-HIS-TYR-MET-ASP-TYR-GLY-CYS-TYR-CYS-GLY-
NOTECHIS II-1 - - - - - - -ASN-MET- - - - - - - - -SER- -(Ser,Pro,Ala,Ala, ,Leu, , ,Ser, , , , ,

ALA-GLY-GLY-SER-GLY-THR-PRO-VAL-ASP-GLU-LEU-ASP-ARG-CYS-CYS-LYS-ILE-HIS-ASP-ASP-CYS-TYR-ASP-GLU-ALA-GLY-LYS-
, , , , , , , , , , , , , X - - -X)THR- - - - - -ALA-ARGX ,Thr-)

RESIDUES 92-119

CYS-ASP-VAL-GLU-ALA-ALA-PHE-CYS-PHE-ALA-LYS-ALA-PRO-TYR-ASN-ASN-ALA-ASN-TRP-ASN-ILE-ASP-THR-LYS-LYS-ARG-CYS-GLN
(, , ,Asp, , ,Thr, , , - X , , , -Lys)(Asp, ,Tyr, , ,Glu,Pro- X , X ,)

Fig.2. Partial amino acid sequence of *notechis* II-1. Assignments set off by commas are inferred on the basis of the composition of tryptic peptides and homology with notexin. Residues 1–17 and 47–54 were determined by manual Edman degradation.

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