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On the purification of notexin

Isolation of a single amino acid variant from the venom of *Notechis scutatus scutatus*

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Venom of the Australian tiger snake, *Notechis scutatus scutatus* was fractionated by conventional ion-exchange chromatography. The fraction containing notexin, a well-known single-chain toxic phospholipase A_2, was further purified by reverse-phase high-performance liquid chromatography. Two main components were isolated and the major one corresponded to notexin. The other component, designated as notechis N_s, was an isoform of notexin. Notechis N_s and notexin possessed similar in vitro esterase activity, in vitro neuromuscular activity and in vivo lethality. Amino acid composition and sequence of the *Staphylococcus aureus* V8-protease peptides demonstrated that primary structures of notechis N_s and notexin differed from each other by a single substitution amongst 119 amino acids: Lys→Arg at position 16.

Notexin; Isoform; Phospholipase A_2; Reverse-phase high-performance liquid chromatography

1. INTRODUCTION

Notexin, from venom of the Australian tiger snake, *Notechis scutatus scutatus*, is one of the most toxic single-chain phospholipase A_2s (EC 3.1.1.4) which has both presynaptic and myotoxic activities [1,2]. *N. s. scutatus* venom was previously reported to contain notechis II-5 [3] and notechis II-1 [4] which differ in sequence from notexin by 8 and 40 amino acids, respectively, and possess different toxic and enzymatic potencies. The primary structure of notexin was determined some years ago [5], using a protein which was purified by low-pressure gel filtration followed by cation-exchange chromatography [6]. Notexin has been extensively used in a variety of neurophysiological studies (for reviews, see [7-9]).

For use in studies involving chemical modifications, the protein of interest must be as pure as possible. In the course of a set of experiments devoted to the determination of the functional site of notexin [10], we reconsidered the initial purification procedure and observed that the ion-exchange fraction containing notexin [6] was in fact composed of several components which were separated from each other by RP-HPLC. This paper reports on the biochemical characterization of the two main ones.

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Abbreviations: RP-HPLC, reverse-phase high-performance liquid chromatography; LD_{50}, median lethal dose; EPPS, end-plate potentials; MEPPS, miniature end-plate potentials

2. MATERIALS AND METHODS

N. s. scutatus venom was collected in the Victoria region of Australia and purchased from the Australian Reptile Park (Gosford, Australia). It was chromatographed on a Biorex-70 column (Biorad, FRG), according to Karlsson et al. [6]. The fraction containing notexin was dialyzed three times against 0.05 M ammonium acetate and lyophilized. It was rechromatographed by RP-HPLC on a Nucleosil butyl large pore column 4.6 × 250 mm (SFCC, Neuilly-Plaisance, France) with a Waters 510 gradient system (Milford, MA, USA), using an acetonitrile linear gradient in 0.1% trifluoroacetic acid: 23.8-28.0% CH_3CN for 75 min at 1 ml/min.

Amino acid analysis of proteins and peptides was determined according to [11] using an LKB 4400 amino acid analyzer (LKB, Sweden).

Reduction, *S*-carboxymethylation, and enzymatic treatment of proteins were performed as previously described [10]. The reduced and blocked peptide samples were sequenced with a model 470A or 477A protein sequencer, and the resulting phenylthiohydantoin residues from each cycle were analyzed by HPLC using the on-line model 120 A phenylthiodantoin analyzer (Applied Biosystems, CA, USA).

The LD_{50}s were determined using the sequential method of Dixon and Mood [12]. The protein was injected into the vein of the tail of female BALB/c mice (b.wt. 20 ± 2 g). Injected mice were examined 24 h, 48 h, and one week after injection.

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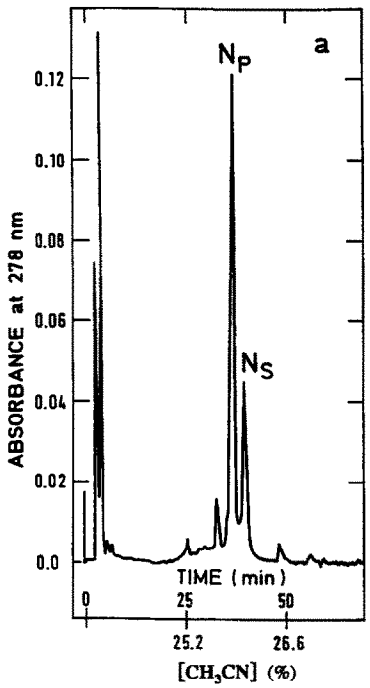

Fig.1. Elution profile of notexin by RP-HPLC on a butyl large pore column. Elution was performed with a linear gradient of CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The injected sample was the fraction containing notexin (400 μg) obtained after chromatography on a Biorex-70 column of *N. s. scutatus* crude venom. The venom was collected in the Victoria region of Australia.

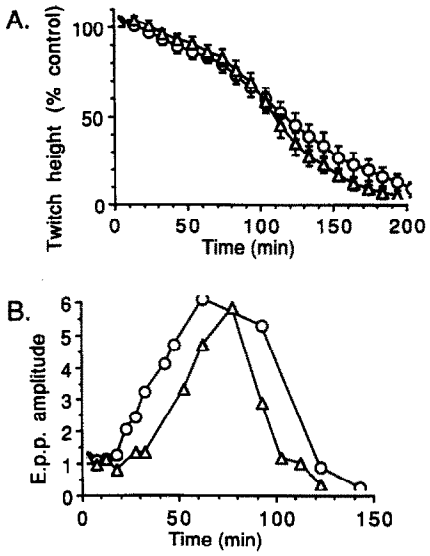

Fig.2. (A) In vitro effects of notexin (○) and notechis N_s (△) at 147 nM on indirectly elicited twitches of chick biventer cervicis preparations stimulated at 0.1 Hz. Points are mean ± SEM ($n=6$). (B) Effects of notexin (○) and notechis N_s (△) at 73 nM on endplate potential (E.p.p.) amplitude in frog cutaneous pectoris preparations. Values have been normalized to a control E.p.p. amplitude of 1. Experimental values were 1.9 mV (○) and 0.95 mV (△).

The effect of proteins (147 nM) on neuromuscular transmission was assessed in vitro by using the chick biventer cervicis nerve-muscle preparation, as described elsewhere [10]. Responses were obtained to indirect stimulation of the nerve at 0.1 Hz and the time-course of twitch blockade in the presence of proteins was monitored. Experiments were also performed with intracellular recording methods on frog (*Rana pipiens*) cutaneous pectoris preparations, as described elsewhere [13]. Muscles were prevented from contracting by reducing the release of acetylcholine by changing the Ca^{2+}:Mg^{2+} ratio. EPPS and MEPPS were recorded before and after the application of toxins. The effects of the toxins on nerve terminal action potentials were also determined, using the mouse triangularis sterni preparation [13].

Enzymatic assays were performed using a titrimetric method [14]. Titration of fatty acids released from egg yolk phosphatidylcholine

Table 1

Amino acid composition of notechis N_p and notechis N_s

Amino acid	Notexin	Notechis N_p	Notechis N_s
Aspartic acid	18	17.7 (18)	17.4 (18)
Threonine	3	2.99 (3)	3.06 (3)
Serine	3	3.13 (3)	3.11 (3)
Glutamic acid	7	6.97 (7)	6.96 (7)
Proline	5	5.20 (5)	5.15 (5)
Glycine	10	9.99 (10)	10.50 (10)
Alanine	9	9.04 (9)	8.50 (9)
Half-cystine	14	ND	ND
Valine	4	4.11 (4)	4.07 (4)
Methionine	2	2.07 (2)	1.97 (2)
Isoleucine	4	3.67 (4)	3.70 (4)
Leucine	4	4.20 (4)	4.06 (4)
Tyrosine	10	9.55 (10)	9.50 (10)
Phenylalanine	5	5.00 (5)	4.87 (5)
Histidine	3	2.82 (3)	2.87 (3)
Lysine	11	10.9 (11)	9.9 (10)
Tryptophan	2	ND	ND
Arginine	5	5.13 (5)	6.08 (6)

Relative molar quantities are given for each protein and the figures in parentheses indicate integral residue numbers. The amino acid composition of notexin, as deduced from primary structure [5], is given for comparison.
ND, not quantitatively determined

Table 2

Automated sequence analysis of the Biorex-70 fraction corresponding to notexin, HPLC-purified notechis N_p and notechis N_s and the peptides obtained after *S. aureus* V8-protease treatment of notechis N_s

Cycle number	Notechis N_p		Notechis N_s									
			Peptide 1 (1–40)		Peptide 2 (41–54)		Peptide 3 (55–73)		Peptide 4 (74–95)		Peptide 5 (96–119)	
	Residue assigned	Amount (pmol)	Residue assigned	Amount (pmol)	Residue assigned	Amount (pmol)	Residue assigned	Amount (pmol)	Residue assigned	Amount (pmol)	Residue assigned	Amount (pmol)
1	N	304	N	695	L	510	A	375	N	174	A	661
2	L	330	L	1061	D	380	G	275	G	170	A	598
3	V	290	V	1138	R	112	K	236	P	107	F	428
4	Q	284	Q	571	C	ND	K	172	Y	108	C	ND
5	F	312	F	947	C	ND	G	280	C	ND	F	624
6	S	160	S	231	K	140	C	ND	R	31	A	637
7	Y	223	Y	255	I	180	F	340	N	116	K	145
8	L	184	L	878	H	65	P	90	I	131	A	530
9	I	175	I	799	D	65	K	41	K	78	P	379
10	Q	150	Q	492	D	84	M	15	K	115	Y	226
11	C	ND	C	568	C	ND	S	42	K	118	N	392
12	A	162	A	749	Y	85	A	98	C	ND	N	296
13	N	114	N	261	D	60	Y	102	L	96	A	265
14	H	42	H	72	E	21	D	76	R	28	N	101
15	G	133	G	243			Y	39	F	71	W	30
16	K	140	R	72			Y	58	V	46	N	51
17	R	89	R	81			C	ND	C	ND	I	120
18	P	95	P	198			G	74	D	36	D	31
19	T	60	T	78			E	4	C	ND	T	46
20	W	35	W	40					D	24	K	15
21	H	32	H	37					V	21	K	35
22			Y	51					E	10	R	20
23			M	32							C	ND
24			D	30							Q	34
25			Y	51								
26			G	56								
27			C	ND								
28			Y	50								
29			C	ND								
30			G	57								
31			A	34								
32			G	60								
33			G	90								
34			S	17								
35			G	85								
36			T	26								
37			P	18								
38			V	8								
39			D	6								
40			E	4								

Analysis was carried out with 200–500 pmol of S-carboxymethylated proteins. ND, not quantitatively determined

was carried out under a nitrogen atmosphere with 10 mM NaOH on a Methrom autoburette at pH 8.0 and 40°C. Micelles of sonicated egg yolk phosphatidylcholine (type IX-E, Sigma) mixed with an equal molar concentration of taurodeoxycholate were used as substrate, in the presence of 5 mM $Ca(NO_3)_2$. Final concentrations of phosphatidylcholine and taurodeoxycholate were equal to 100 mM in a 2-ml reaction volume.

3. RESULTS AND DISCUSSION

Chromatography by RP-HPLC on a Nucleosil butyl large pore column of the Bio-rex 70 fraction containing notexin [6] revealed the presence of several components (fig.1). The two main components were designated as notechis N_p and notechis N_s.

3.1. *Functional characterization of notechis N_p and notechis N_s*

Notechis N_p and notechis N_s behaved similarly in three different types of biological assays, suggesting that notechis N_p and notechis N_s were exceedingly similar proteins. First, they have indistinguishable in vitro esterase properties, when measured in a pH-stat apparatus on micelles of phosphatidylcholine mixed with taurodeoxycholate (1/1 mol/mol). Thus, using seven identical concentrations varying between 0 and 16.5 nM, the apparent maximal velocities to hydrolyze 100 mM varied identically between 60 and 310 ± 10 μM NaOH/min, for both notechis N_p and notechis N_s. Secondly, the two proteins possessed the same in vivo toxicity, with a LD_{50} value of 0.34 ± 0.02 μg per 20 ± 2 g female BALB/c mouse, which was also equal to the LD_{50} previously reported for notexin [6]. Thirdly, both toxins had very similar effects on neuromuscular transmission. They produced superimposable time-courses of twitch blockade of the biventer cervicis preparation in response to nerve stimulation (fig.2A). In frog cutaneous pectoris nerve muscle preparations, they both produced an initial increase in the evoked release of acetylcholine followed by a blockade of release (fig.2B). They did not change the amplitude or time-course of MEPPS or affect the resting membrane potential; i.e. both proteins produced a prejunctional block of acetylcholine release. In mouse triangularis sterni preparations, both toxins (220 nM) produced similar selective reductions in the late phase of the perineural waveform, which is associated with potassium currents in nerve terminals (data not shown). Such an effect was previously noted for the mixture of notexin isoforms, β-bungarotoxin and other phospholipase A_2 presynaptic toxins [13].

3.2. *Biochemical characterization of notechis N_p and notechis N_s*

The amino acid composition of notechis N_p (table 1) showed no difference with that of notexin [6]. In addition, we previously showed [10] that enzymatic treatment of notechis N_p with V8-protease from *Staphylococcus aureus* led to a set of peptides which correspond to those expected for notexin whose primary structure is known [5]. Notechis N_p and notexin are therefore identical compounds. The amino acid compositions of notechis N_s and notexin were similar although not identical: notechis N_s has 10 lysine and 6 arginine residues instead of 11 lysine and 5 arginine residues in notexin. Twenty cycle runs from the NH_2-terminus revealed a difference at position 16 (table 2) where lysine in notechis N_p (i.e. notexin) was replaced by an arginine residue in notechis N_s. Enzymatic digestion of the *S*-carboxymethylated notechis N_s using V8-protease from *S. aureus* led to a set of peptides which were fractionated as described [10] and which encompassed the whole protein chain (table 2). Automated Edman degradation of these peptides revealed no other amino acid modification when compared to notexin [5]. We concluded that notechis N_s constitutes an isoform that differs from notexin by a single amino acid (Lys→Arg) at position 16.

Since lysine and arginine are both basic residues, the isoelectric point of notechis N_s does not differ significantly from that of notechis N_p. This explains why notechis N_s was not initially separated by ion-exchange chromatography [6]. As shown in the present paper this, however, can be achieved using RP-HPLC. Since then, notechis N_s has been isolated from *N. s. scutatus* venom samples collected in different regions of Australia. Depending on the geographic origin of the snakes, the relative quantity of notechis N_s varied between 38% and 61% of notexin [15]. Interestingly, the existence of notechis N_s may be related to the mutation of a single base in the gene (AAA→AGA or AAG→AGG for Lys→Arg). By contrast with the other isoforms notechis II-5 and notechis II-1, notechis N_s is a single amino acid variant of notexin which furthermore possesses the same biological activities as notexin.

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