Cloning and characterization of the pseudonajatoxin b precursor

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An Australian common brown snake, *Pseudonaja textilis*, is known to contain highly lethal neurotoxins. Among them, a long-chain α -neurotoxin, pseudonajatoxin b, has been identified. In this report, while presenting evidence for the presence of at least four such long-chain α -neurotoxins in the venom of *P. textilis*, we describe the characteristics of both the mRNA and the gene responsible for the synthesis of these neurotoxins. A precursor toxin synthesized from the gene has been identified as being capable of producing the isoforms possibly by post-

translational modifications at its C-terminal end. Recombinant toxins corresponding to the precursor and its product have been found to possess similar binding affinities for muscular acetylcholine receptors ($IC_{50} = 3 \times 10^{-8}$ M) and a lethality, LD_{50} , of 0.15 μ g/g in mice.

Key words: gene cloning, long-chain α -neurotoxin, post-synaptic neurotoxin, *Pseudonaja textilis*.

INTRODUCTION

Post-synaptically acting α -neurotoxins from snake venom can be classified into two subgroups, termed short-chain α -neurotoxins (Sntxs) and long-chain α -neurotoxins (Lntxs). The Sntxs consist of 57–64 amino acid residues in a single polypeptide cross-linked by four disulphide bridges. The Lntxs are usually composed of 66–79 amino acid residues with five disulphide bridges and only in exceptional cases with four disulphide bridges [1]. Although both subgroups share a similar three-finger loop structure [2,3], they show significant differences in their primary structure. The most obvious variations are observed at the C-termini, as well as at the tips of loop 1 and loop 2 of these proteins.

The C-termini of Sntxs always end with CNX (X being N in most cases [4–7]). In contrast, Lntxs possess extra C-terminal residues varying from 2 to 15 amino acid residues beyond the C-terminus (CNX) of Sntxs. An important characteristic of Lntxs from different snakes is that the C-termini contain variable numbers of basic amino acids, which are partly responsible for the toxicity of Lntxs. Isoforms of Lntxs have been described based on their variable numbers at the C-termini and the substitutions of amino acids within the polypeptide chain. For example, the sea snake *Astrotia stokesii* [8] and an Australian elapid *Acanthophis antarcticus* [9–11], show two (As b and c) and three (Aa b, d and e) isoforms, respectively. As c differs from As b by possessing two additional C-terminal residues and substitutions at four amino acid residues. Similar observations can be made among the other isoforms, Aa b, d and e.

In addition to their longer and more diverse C-terminal ends, the Lntxs possess the fifth disulphide bridge formed at the tip of loop 2 which is responsible for the higher binding affinity to neuronal nicotinic acetylcholine receptors (nAChRs) than to muscular nAChRs [12,13]. However, the lack of this fifth disulphide bridge in Sntxs has been known to enhance and limit the binding of Sntxs only to muscular nAChRs [14,15].

Pseudonaja textilis is known to contain highly lethal neurotoxins. Among them, an Lntx known as pseudonajatoxin b (Pt-b) has been purified and characterized by Tyler et al. [16]. In our previous reports [17,18], we have described the cloning and characterization of six new isoforms of Sntx (Pt-Sntx 1–3 and 5–7) in *P. textilis*. In this report, we present the structure and properties of an extra long-chain precursor neurotoxin by gene cloning. This new Pt-b precursor (Pt-bp) contains an 82 amino acid polypeptide chain which is possibly post-translationally modified to 71 amino acid Pt-b [16] and other isoforms in the venom of *P. textilis*.

MATERIALS AND METHODS

Venom, venom glands and liver

An adult snake (*P. textilis*), kept undisturbed and unfed for 2 weeks, was milked for its venom and anaesthetized prior to collecting its venom glands and liver. The venom was lyophilized and stored at -20°C . The venom glands and liver were immediately frozen in liquid nitrogen and kept at -85°C .

Purification and N-terminal amino acid sequencing of Lntxs

Lyophilized crude venom was reconstituted in 0.5 ml of water and subjected to reversed-phase HPLC (RP-HPLC; SMART system, Pharmacia) using a Sephasil C₁₈ μ Bore column. The buffer systems used were 0.1 % trifluoroacetic acid (buffer A) and 80 % acetonitrile in 0.1 % trifluoroacetic acid (buffer B). The RP-HPLC-purified proteins were subjected to N-terminal amino acid sequencing using a Procise-HT (model 494) protein sequencer attached to a 140C PTH analyser (Applied Biosystems, Foster City, CA, U.S.A.) after having been analysed by electrospray ionization MS (ESI-MS, Applied Biosystems). Protein concentrations were determined by the Bradford method [19].

Abbreviations used: Lntx, long-chain α -neurotoxin; Sntx, short-chain α -neurotoxin; Pt-b, pseudonajatoxin b; Pt-bp, Pt-b precursor; nAChR, nicotinic acetylcholine receptor; CAT, chloramphenicol acetyltransferase; RP-HPLC, reversed-phase HPLC; ESI-MS, electrospray ionization MS; RT-PCR, reverse-transcriptase PCR; GST, glutathione S-transferase; TIS, transcription-initiation site; CHO, Chinese hamster ovary; UTR, untranslated region; AP1, adaptor protein 1; MMTV, mouse mammary tumour virus.

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The cDNA and gene nucleotide sequence data reported here have been submitted to the Genbank Nucleotide Sequence Database under the accession numbers AF082982 and AY027493.

Isolation of total RNA and reverse-transcriptase PCR (RT-PCR)

Total RNA was isolated from the venom glands using the guanidine isothiocyanate method [20] and the integrity of the total RNA was analysed by denaturing formaldehyde agarose gel electrophoresis [21]. Oligonucleotide primers synthesized by the Oswel DNA Service (Southampton, U.K.) based on sequence at the 5' and 3' untranslated regions (UTRs) of the previously cloned cDNAs from a spitting cobra, *Naja naja sputatrix* [22,23], were used. The sense and antisense primers were: X133 (5'-TCCAGAAAAAGATCGCAAGATG-3') and X132 (5'-GAATTTAGACATTATCAGTTG-3'), respectively.

Total RNA (3 µg) was reverse transcribed [17] and used for the PCR. PCR was carried out in a PerkinElmer Cetus thermal cycler (model 480) according to the method of Gong et al. [17]. The reaction mixture contained 250 µmol each of dNTPs, 10 µmol each of sense and antisense primers in a final 50 µl reaction buffer (50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl₂ and 0.1 mg/ml gelatin) and 1 unit of *Taq* DNA polymerase.

Cloning and sequencing of cDNAs

The PCR products were cloned into pT7Blue(R) vector (Novagen, Madison, WI, U.S.A.). The ligated products were then transformed into *Escherichia coli* DH5α [*supE44 ΔlacU169 (Φ80lacZΔm15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and the recombinants were selected on a Luria–Bertani plate with 50 µg/ml ampicillin supplemented with isopropyl β-D-thiogalactoside and X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) as described by Sambrook et al. [24]. The plasmids isolated from the recombinant clones were then subjected to Sanger dideoxy DNA sequencing [25], using M13/pUC forward and reverse primers on an automated DNA sequencer (Applied Biosystems, model 373A). The DNA sequences and deduced amino acid sequences were analysed by the CLUSTALW program.

Expression of cDNAs encoding Lntxs

Primers containing *Bam*HI (sense) and *Hind*III (antisense) restriction sites were used to amplify the coding region from the cDNA by PCR. The primers for the amplification of cDNA encoding the precursor toxin Pt-bp (82 amino acids) were X472 (5'-CGCGGATCCATGAGGACATGCTTC-3') and X471 (5'-CAAGCTTTTATCAAGGATGGTCCT-3'). The primers for the production of cDNA corresponding to Pt-b (the first 71 amino acids of Pt-bp) were: X472 and X473 (5'-CAAGCTTTTATCAAGGACGTAAA-3'). The PCR products were subcloned into the pGEX-KG expression vector (Pharmacia) between the *Bam*HI and *Hind*III sites. Recombinant plasmids were sequenced to confirm the in-frame fusion of each of the neurotoxin cDNAs to the glutathione S-transferase (GST) sequence containing the thrombin endoprotease site.

The expression of the cloned genes was induced by isopropyl β-D-thiogalactoside (0.2 mM) at 37 °C for 5 h and the fusion proteins were analysed by 12 % Tris/Tricine SDS/PAGE [26]. The fusion proteins were passaged through the glutathione-agarose column and digested, while being bound to the column, with thrombin [27]. The recombinant neurotoxins were then eluted with 50 mM Tris/HCl (pH 7.4).

LD₅₀ and nAChR binding assays

Swiss albino mice (20 ± 1 g) were injected intravenously with 0.1 ml of saline as control and at least four different doses of

toxin. The mice were observed for up to 48 h, and the intravenous LD₅₀ values were calculated according to the Spearman–Karber method [28]. Experiments were carried out in accordance with the guidelines of the National Institutes of Health (Bethesda, MD, U.S.A.) regarding the care and use of animals for experimental procedures.

Both native and recombinant neurotoxins were tested for their ability to compete with ¹²⁵I-α-bungarotoxin for binding sites on the nAChRs from *Torpedo californica* prepared by the method of Ishikawa et al. [29]. *Torpedo* membrane (2.5 µg) suspension was incubated with 5 nM ¹²⁵I-α-bungarotoxin at room temperature (20–25 °C) and a range of concentrations of purified toxins in a total volume of 200 µl. After 1 h, the reaction was quenched on ice. The membranes were recovered by centrifugation, washed with 1 ml of buffer containing 0.1 % BSA and dried before being subjected to radioactive monitoring in a Packard COBRA Auto Gamma Counter (Packard Instruments, Downers Grove, IL, U.S.A.). The results were analysed and plotted using Slidewrite Plus 2 (AdvanceGraphic Software, Carlsbad, CA, U.S.A.).

Preparation of genomic DNA of *P. textilis*

The frozen liver from a single snake kept at –80 °C was ground gently into fine powder using a mortar and pestle. The method of Blin and Stafford [30] was employed to isolate high-molecular-mass genomic DNA from the powdered liver.

Genomic PCR and genome walking

The amplification of neurotoxin gene from the highly intact genomic DNA of the snake was carried out by using the AdvanTage Genomic PCR method (Clontech, Palo Alto, CA, U.S.A.). The primers used (X292, forward, 5'-CGTACATGCTTCATTACACC-3'; X293, reverse, 5'-GTGGGGCAGGTCTGCTGACA-3') were designed from the cDNA sequence of Pt-bp.

To determine the 5' and 3' ends of the neurotoxin gene, the Universal GenomeWalker method (Clontech) involving *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I libraries of the *P. textilis* DNA has been used. To amplify the 5' end of neurotoxin gene, adaptor primer 1 (AP1, 5'-GTAATACGACTCACTATAGGGC-3', forward) and a 25-mer gene-specific primer X294 (5'-CATCAGGTGTTATGAAGCATGTCCT-3', reverse) were used. Another set of primers, AP1 (forward) and X464 (reverse, 5'-AAGTCCAGGCACATGATTGTCACC-3'), were also used to amplify the 5' end of the gene. Amplification of the 3' end of the neurotoxin gene was carried out using a 24-mer gene-specific primer X295 (5'-AGCGAGTCGATTGGGATGTGCTG-3', forward) and AP1 as the reverse primer [18].

Primer-extension analysis

Primer-extension analysis was performed [18] to locate the transcription-initiation site (TIS) of the neurotoxin gene. The primer LPEXT (10 pmol; 5'-GGCACACGATTGTCACCAC-3') was used, labelled at the 5'-end with [γ-³²P]ATP. The results were analysed in an 8 % polyacrylamide gel containing 7 M urea.

Promoter-activity analysis

The activity of the Lntx gene promoter was determined by both chloramphenicol acetyltransferase (CAT) assay and Real-Time PCR (PerkinElmer Applied Biosystems; [18]).

The promoter region of the Lntx gene was amplified using primers X528 (forward, 5'-GGCCCATATGAAAAAAG-

GAAGG-3') and X529 (reverse, 5'-CTGGCTAGCCTTGCGA-TCTTTTCT-3') and subcloned in both the forward (LF-pMAMneo-CAT) and reverse (LR-pMAMneo-CAT) orientations. The primers used for cloning in the reverse orientation were X530 (forward, 5'-CTGGCTAGCAAAAAAAGGA-AGG-3') and X535 (reverse, 5'-CTGCATATGCTTGCGATC-TTTTCT-3'). The promoter activities were examined using a mammalian cell line, Chinese hamster ovary (CHO) cells, as described by Gong et al. [18].

Sequence and phylogenetic analysis

Nucleotide sequence homology searches of GenBank databases (National Center for Biotechnology Information) were performed using the BLAST program. DNA and amino acid sequence alignments and phylogenetic analysis based on nucleotide sequences of both Sntx and Lntx genes were carried out using the Dnastar software package (DNASTAR, Madison, WI, U.S.A.).

RESULTS

Purification and analysis of venom neurotoxins

Crude venom of *P. textilis* was fractionated using a Sephasil C18 μ Bore column on RP-HPLC (Figure 1) and fractions corresponding to the retention time for neurotoxins were subjected to N-terminal amino acid sequencing. Among them, six sequences with high homology to α -neurotoxins were obtained. Their sequences were as follows: Pt-N1, LTXYKGYRDTV; Pt-N2, LTXYKGYHDTVXKP; Pt-N3, RTXFITPDVSKXPX; Pt-N4, RTXFITPDVKKXPV; Pt-N5, RTXFITPDVK, and Pt-N6, RTXFITPDVK. Pt-N1 and Pt-N2 corresponded to Sntxs [17]. Pt-N3–Pt-N6 were considered as putative Lntxs, and were hence examined by ESI-MS analysis. ESI-MS showed that each of the purified proteins was homogenous and that they had molecular masses of: Pt-N3, 7362.77 Da; Pt-N4, 7892.91 Da; Pt-N5, 7509.83 Da, and Pt-N6, 7364.34 Da. Similar results were

obtained when LC-MS analysis was repeated with pooled venom samples obtained from several snakes.

cDNA cloning and sequencing

Total RNA prepared from the venom glands of a snake was used in RT-PCR. Two independent RT-PCRs using the primers X133 and X132 on total RNA gave a product of about 300 bp. In total, 15 putative clones were obtained from the PCR products. In another experiment using an adaptor-ligated cDNA library, five more clones were obtained. All clones were subjected to DNA sequencing on both strands of each cDNA and the results were analysed using the SeqEd program from Applied Biosystems. Only one type of cDNA (Pt-bp), encoding a new Lntx from *P. textilis*, was obtained in all cases (Figure 2). High sequence similarity with many Lntxs from other elapids could be observed in the deduced amino acid sequence of Pt-bp. It contains a 21-amino acid signal peptide that is identical with those of Ls III and α -bungarotoxin (Figure 3) and all the amino acid residues that are important for both structure and function of the toxin [31]. The structurally important residues included 10 cysteines at positions 3, 14, 21, 27, 31, 42, 46, 58, 59 and 64, Thr-45, Gly-17, -35, -41 and -53, Leu-40 and Ala-43 and -44. Functional residues included Asp-28, Arg-34, Trp-26 and Lys-51. Furthermore, Pt-bp contains Trp-26, Asp-28, Phe-30, Arg-34, Arg-37 and Phe-67, which could be involved in binding to both neuronal and *Torpedo* AChRs, and Lys-36, Cys-27 and Cys-31, which are able to selectively bind to α 7 AChRs, as well as Lys-24 and Lys-51, which can bind solely to *Torpedo* AChRs [32,33]. Apart from the similarity to other Lntxs, Pt-bp showed a major difference at the C-terminal end by possessing 11 extra amino acid residues, similar to the Lntx Aa e from another Australian elapid, *A. antarcticus* [11]. Compared with Pt-b (7762.0 Da) previously isolated from *P. textilis* [16], Pt-bp shows three amino acid substitutions (Figure 3) at Lys-24, Asp-39 and Ile-57, instead of Glu-24, Glu-39 and Gln-57. Except for these minor differences,

Figure 1 Purification of Pt-N3–Pt-N6 from venom by RP-HPLC

Pt-N3–Pt-N6 represent the native Lntxs in *P. textilis*. Pt-N1 and Pt-N2 represent the native Sntxs. PLA₂, phospholipase A₂.

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	-1	10	20	30	40	50	60	70	80	89
	/	Loop I	/	/	Loop II	/	Loop III	/		
Pt-bp	RTCFITPD--	-VKSPPCPG	QEVCTKTC	DGFCGIRGR	VDLGCAATCP	TPKKTGDI	CCSTDDCNWF	PLRP----	RG	RLSSIKDHP
Pt-b	RTCFITPD--	-VKSPPCPG	QEVCTKTC	DGFCGIRGR	VDLGCAATCP	TPKKTGDI	CCSTDDCNWF	PLRP----		
α-Bgt	IVCHTATSP	-ISAATCPPG	ENLCYKMW	DGFCSSRGV	IIMGCAATCP	S-KKPYEVT	CCSTDKCNPH	PK-----	QR	PG-----
As b	LSCYLGYKH	-S--QTCPG	ENLCYKMW	DGFCSSRGV	IIMGCAATCP	T-AKSGVHIA	CCSTDKCNPH	AKWG-----		
As c	LSCYLGYKH	-S--QTCPG	ENLCYKMW	DGFCSSRGV	IIMGCAATCP	T-AKSGVHIA	CCSTDKCNPH	TKWG-----	S	R-----
Aa b	VICRYGNN	-P--QTCPG	ENLCYKMW	DGFCSSRGV	VELGCAATCP	I-VKSYNEVK	CCSTDKCNPH	PV-----	RP	RRPP-----
Aa d	VICRYKYTN	-V--KTCPG	ENLCYKMW	DGFCSSRGV	VELGCAATCP	I-RKPGNEVK	CCSTDKCNPH	PK-----	RK	KRRP-----
Aa e	VICRYGNN	-P--QTCPG	ENLCYKMW	DGFCSSRGV	VELGCAATCP	I-RKPGNEVK	CCSTDKCNPH	PK-----	RK	KRRP-----
α-Cbt	IRCFITPD--	-ITSKDCPPG	-HVCYTKTC	DARFCHQGR	VDLGCAATCP	T-VKRGVDIK	CCSTDKCNPH	PK-----	RP	
Nns-lntx	IRCFITPD--	-VTSDDCPG	-HVCYTKTC	DGFCSSRGV	VELGCAATCP	T-VKPGVDIQ	CCSTDKCNPH	PTR-----	P	
Lc a	RICYLAPR--	-DTQICAPG	QECYLYKSWD	DGTFGLKGR	LEFGCAATCP	T-VKPGDIK	CCSTDKCNPH	PKLA-----		
LsIII	MKTLLTLVVVTIVCLDLGYT	RECYNLPH--	--DTQICPSG	QECYLYKSWC	NAMCSSRGV	LEFGCAATCP	CCSADKCNTP	P-----		
Pt-sntx1	MKTLLTLVVVTIVCLDLGYT	LTCYKGYHDT	-----VUCKPH	ETICYEYFIP	ATHG---NAI	LARGCGTSCP	G-G---IRPV	CCRTDLCKN-		
Pt-sntx2	MKTLLTLVVVTIVCLDLGYT	LECHNQSSQ	APTPTGCSGG	ETICYKKSQR	D-----HRGYR	IERGCG--CP	S-VKKGIEIN	CCRTDLCKN-		
Cbt	MKTLLTLVVVTIVCLDLGYT	LECHNQSSQ	TPPTTGCSGG	ETICYKKSQR	D-----HRGYR	TERGCG--CP	S-VKKGIEIN	CCRTDLCKN-		
Ec	MKTLLTLVVVTIVCLDLGYT	RICFNHQSSQ	PQTTKTCSPG	ESSCYHKQWS	D-----FRGTI	IERGCG--CP	T-VKPGINLS	CCSEVCCNN-		

Figure 3 Comparison of amino acid sequences of Lntxs and Sntxs

The Lntxs from *P. textilis* are compared with those from other snakes using the CLUSTALW program. The variant amino acid and conserved cysteine residues of Pt-bp and Pt-b are highlighted. Abbreviations used for the sequences are the same as in Figure 2 with the following additions: α-Cbt (cobratoxin; P01391), Lntx from *Naja kaouthia*; Cbt (cobratoxin; U42582), *Naja atra* Sntx; Aa b (P01385) and Aa d (A60518), *A. antarctica* Lntxs; As b (P01380) and As c (P01381), Lntxs from *Astrota stokesi*; Lc a, *Laticauda colubrina* Lntx (0901189A); Ls III (AB015513) and Ec (X51410), *Laticauda semifasciata* Lntx and Sntx.

that of Pt-bp, it is possible that Pt-N3–Pt-N6 differ at the C-terminal ends. Pt-N3–Pt-N6 can be shorter than the deduced sequence for Pt-bp by 10–14 residues. Based on the determined molecular masses of Pt-N4 (7892.91 Da) and Pt-b (7762 Da [16]), it could be deduced that Pt-N4 is similar to Pt-b. N-terminal amino acid sequencing of the first 60 amino acid residues of Pt-N4 showed that Pt-b contains a similar sequence except for three amino acids (Lys-24, Asp-39 and Ile-57). These differences could be due to variation in samples used for the two independent experiments conducted with a time lag of 14 years. Further studies involving many random samples are required to confirm these observations.

Structure and organization of *P. textilis* Lntx gene

PCR was used to amplify the neurotoxin genes. The strategies used in the cloning are outlined schematically in Figure 4(a). The primers were designed based on the *P. textilis* Lntx as well as its corresponding cDNA. In order to clone a part of the protein-coding region, primers X292 and X293 were used, which corresponded to the very beginning and the middle of the amino acid sequence of the mature protein-coding region of Pt-bp cDNA. Amplification by primers X292 and X293 gave a fragment of about 0.7 kb (Figure 4a) on agarose gel. PCR products were then subcloned into the pT-Adv vector. We sequenced 20 such genomic clones and all of them gave a sequence identical with that of Pt-bp cDNA.

Two DNA fragments corresponding to the 5' end of the Lntx gene were obtained by genome walking. The 1.0 kb fragment (Figure 4a, part 2) was obtained using an *EcoRV* library of the *P. textilis* genome and primers AP1 (forward) and X294 for primary PCR followed by primers AP2 and X294 for secondary PCR. Likewise, the 0.5 kb fragment (Figure 4a, part 3) was obtained using a *DraI* library of the *P. textilis* genome and primers AP1 (forward) and X464 for primary PCR followed by primers AP2 and X464 for secondary PCR. Both DNA fragments were subcloned and sequenced. A total of 16 such clones were sequenced. From these sequences the nucleotide sequence of the 5' end of the gene was determined. The 3' end of the neurotoxin gene was elucidated similarly by sequencing of a 0.5 kb PCR fragment (Figure 4a, part 4) obtained from a *EcoRV* library using primers X295 and AP1 (reverse) followed by nested PCR involving primers X295 and AP2. Once the complete nucleotide sequence of the Lntx gene of *P. textilis* was established, two new primers, LPTF (5'-GGAAGGTGCTGAACCTTTGCAGAGA-3') and LPTR (5'-CCTGCCATCCTTCCCCCAAA-3'), corresponding to the 5' and 3' ends of the gene respectively, were synthesized and the Lntx gene (2.6 kb) was amplified by PCR from the genomic DNA of *P. textilis*. (Figure 4a, part 5, and Figure 5). After subcloning the fragment into the pT-Adv vector, 18 positive clones were sequenced by using vector-specific primers and appropriate gene-specific primers. All of them were identical with each other. Hence, only one Lntx gene exists in *P. textilis*. This is consistent with our cDNA cloning, which also gave a single cDNA encoding Lntx. Comparison of the cDNA and the gene sequences showed that the exon–intron junctions follow the universal GT/AG rule (Figure 5).

The *P. textilis* Lntx gene was found to contain two introns of 885 bp (nt 87–971) and 539 bp (nt 1071–1609) and three exons (Figure 4b and Figure 5). The first intron interrupts the neurotoxin leader-sequence region (Figure 5) at the same position as in α-bungarotoxin [34] and Sntxs Ec [5], Cbt [4], Cbt b [35] and Ntx1 from *N. naja sputatrix* [36]. The second intron was found to interrupt the coding region (Figure 5) at a cysteine codon. The

Figure 4 Cloning strategies and general organization of the *Lntx* gene of *P. textilis*

(a) Strategies for PCR. Primers used and their relative positions are shown. Part 1, genomic PCR of the coding region; parts 2 and 3, genome walking for the 5' end; part 4, genomic PCR for the 3' end; part 5, amplification of the entire gene. The agarose gel electrophoresis of the PCR products are shown in the insert. Lane a1, 500 bp of 5' end; lane a2, 1 kb of 5' end; lane a3, 700 bp of 5' coding region; lane a4, 500 bp of 3' end; lane a5, whole gene; lanes M1 and M2 contain DNA markers. (b) Organization of the *Lntx* gene (Pt-bp) in *P. textilis*. The exons are shown as boxes and introns as thin lines. The signal-peptide-coding region is represented by open boxes while the mature-protein-coding region is represented by closed boxes. Comparisons with similar genes are also shown (see Figures 2 and 3 for abbreviations).

first exon (86 bp) contains the TIS, 5' UTR and the nucleotide sequence encoding most of the signal peptide (18 amino acid residues). The second exon (99 bp) encodes the C-terminus of the signal peptide (three amino acid residues) and the N-terminal

half of the mature neurotoxin, and the third exon contains the remainder of the mature neurotoxin, which also includes the extra 11 amino acid residues (compared with that of Pt-b) and the 3' UTR.

aaaaaaaaaggaaggtgctgaactttgcagagagatatattttgtttaattttgcattatgt	-281
ataatcttgtataaatacaggaagagatgccagttgtccaggtgccaggtttttggatgc	-221
agaagagaagggctcttccttgaagtcggggaatgggtggtccttcacctgatctgagctg	-161
AP-2 GC box (-)	
gcttccggttttgggtgctggggcaaaaagggccacacccctggcctccgccttttctcctg	-101
GATA-2 GC box (-)	
aatcttctctaggtcttctctccagatatccataaatatacagccccacccccacccccatc	-41
+1 (TIS)	
tcaaaggggtataaataagtgtctgcttccaccctgggctACACTTTGCAGGCTGCAGAA	20
AAGATCGCAAGATGAAAACCTCTGCTGCTGACCTGGTGGTGGTGACAATCGTGTGCTGG	80
M K T L L L T L V V V T I V C L	
ACTTAGGtaaggccaccaaaccttgatcttgggtagcaatcaaaccccccaaaaggggc	140
D L	
agagtaaggggtggtatgaaaactctactgctgaccttgggtgggtgacaatcgtgtgc	200
ctggacttaggtaagggccaccaaaccttgatcttgggtggcaatcaaacccccgcaaaag	260
gggcagagtaaggggggggtgtattcgaggcagttgtggtccgtttttgggtgggacgtt	320
ctcactgttcccggtctgcacaaggaggaagcttggggggagaaaaagaggtctccgtttg	380
gagagaagcccatggctgagacaggggctgttccaggtggcgtttctgggtagactgc	440
ttctgaaagtagaggttcccttgcgtagtgagctctgcccgtgggattactccagggaaag	500
aatgcgatgaaaaaggtttccgctcacctgtctctggccttggccaccctcagtatattgg	560
gatggtacggccaacggggaccagtgatgcctccttcaagtcaagcaggagattcctca	620
acatcagccggtgaagaggcataaaacccgaagtgttatagctgtttgtaatgaccaag	680
gaagcgaagaaacctaatactcctttctcccatcttccccacagcaacaacctgtgagg	740
taggatgggcagagagaggggtgaatagtcctaaagttgctaagttgccttttatgcctaaa	800
gtgggacgagaactcaggtttcctattttctagaacctttgatctttctagggaaattt	860
tcacagctgaccttagtgtgaaaagatctatcgtctaataagtaataagattagaagatc	920
agaaaaataaatatcaaagtaataatataatattatctttcatctagGATACACCA	980
G Y T	
GGACATGCTTCATAACACCTGATGTTAAATCTAAGCCTTGTCACCTGGGCAGGAGGTAT	1040
R T C F I T P D V K S K P C P P G Q E V	
GCTACACAAAGACTTGGTGTGATGGTTTTTgtaagtacagttcttcttccactcccagt	1100
C Y T K T W C D G F	
tttttttcttcttctgatcccagactagagctcccaaatgcacctgagaactggaggtcacc	1160
gaggacctgtctgtgcccctgcctgggttccctcaggtcacatcctgttgccggacaccc	1220
tgagcaatgcaaggaaaggggggtcttggactggggtgccccatctttctcatcaacagc	1280
ccacacgggagggggaaaccttctctatagaaagggggagggaaatgctgggagagttgc	1340
cggtttgttccagaaatttagggaaacccgacaaacctgaaacatccccgaaaagtgca	1400
ttgcccctcactcttgtctaaacacgagactccccagaaatctcttcttcatcattta	1460
ctcttgagttttgagtagaggaggtggaggtctncttccctcatgggacagggagcgtg	1520
aatgtgttcagtggtgggtttaccagtttgttcagcaacacccacaggtctgttcttctgt	1580
attttttaataatcattctgcttctgtagGTGGCATCAGAGGAAAGCGAGTCGATTGGG	1640
C G I R G K R V D L G	
ATGTGCTGCTACTTGCCCTACTCCAAAGAAGACCGGTATAGATATTATCTGTTGCTCCAC	1700
C A A T C P T P K K T G I D I I C C S T	
AGACGATTGCAACACATTTCCATTACGTCCTCGAGGGAGGCTCTCATCCATCAAGGACCA	1760
D D C N T F P L R P R G R L S S I K D H	
TCCTTGAaaatttatgcttgggtggcctttaccgcagatgggtccatccctgctgtctt	1820
P ***	
tgacatctcgacatctttcccttttctcgcgttctgtcagtttcttctgctagttctgt	1880
agtttgagaatcgaataaaactcagcatttaattcaagtgtttttgatttttaattcccc	1940
ccccctcaaaaacattccctttcacgaaaacccacaggtaaagagtttttctccaggtt	2000
tcagcaggcagtgaggctgggatgagcaggattcgggtccgatccctgttgaatcatggct	2060
tggattgcctaacgggtaataatctgacattcgggcagggttaaagaaagaggggaaaccc	2120
acaaccgagtccttctcaggaaaaccttccacttcaaagcctcccgttcttctctctgg	2180
ggaaagctgaggggaagaggaagacgccttgaaagagaggagggggatttggggggaagg	2240
atggcagg	2248

Figure 5 Nucleotide sequence of the *Pt-bp* gene

Exons are represented in capitals. The deduced amino acid sequences are shown in the single-letter code in upper case, and the amino acid sequence of the mature neurotoxin is bold. Intron sequences and the 5' and 3' non-coding regions of the gene are given in lower-case letters. The TIS is numbered +1 and the stop codon is indicated by asterisks. The consensus sequence for polyadenylation, AATAAA, at the 3' end is also highlighted. Consensus sites for some putative transcription factors and TATA box motifs are also shown at the 5' end of the gene.

Both the translation-initiation codon and stop codon can be located in the gene sequence, as ATG and TGA respectively. The 3' end of the neurotoxin gene contains a polyadenylation signal, AATAAA, at nts 1894–1990. The sequence TGTTTTG has also been located downstream of the poly-A signal, which is known to regulate the half-life of the mRNA [37].

TIS and regulatory elements of the neurotoxin gene

The TIS of the *Pt-bp* gene has been determined by primer-extension analysis using reverse transcriptase and the ³²P-labelled primer LPEXT, which is complementary to the 5' end of the neurotoxin mRNA sequence. The TIS has been assigned to

adenosine (A⁺) at the same position (Figure 5) as the TIS for Pt-Sntx [18]. The TIS of Pt-bp was further confirmed by a putative TATA box located 31 bp immediately upstream of it. However, no CAAT box, which is known to increase the efficiency of the promoter, was observed.

A search for the regulatory elements at the 5'-flanking region of the neurotoxin gene using TFSEARCH (TFSEARCH: Searching Transcription Factor Binding Sites, <http://www.rwcp.or.jp/papia/>) showed the presence of a GC box [38], which may be recognized by transcription factors (Figure 5) such as Sp-1, and binding sites for the transcription factors AP-2 [39] and GATA-2 [40].

The gene was confirmed as a functional gene by determining its promoter activity. The promoter was cloned upstream of the *CAT* gene in pMAMneo-CAT [41]. The promoter activity was assayed quantitatively by measuring both the *CAT* activity and the amount of *CAT* mRNA that is found in CHO cells transfected with the promoter cloned in both orientations (forward, LF-pMAMneo-CAT, and reverse, LR-pMAMneo-CAT). The promoter cloned in the opposite orientation to the *CAT* gene, LR-pMAMneo-CAT, served as a negative control. The transfected CHO cell lysates were assayed for *CAT* activity using [¹⁴C]chloramphenicol and *n*-butyryl-CoA. The counts of butyrylated chloramphenicol formed for the control (CHO cells), pMAMneo-CAT, LR-pMAMneo-CAT and LF-pMAMneo-CAT were 400 ± 50, 1200 ± 100, 500 ± 50 and 13500 ± 100 c.p.m. respectively. The *CAT* mRNA concentration was determined by a new method using Real-Time PCR [41]. The total RNA was isolated separately from the cell lines transfected with the above plasmids and used in PCR. The Real-Time PCR studies confirmed that the promoter is active with a threshold cycle (*C_T*) value of 19.0 for the LF-pMAMneo-CAT and 29.0 for the MMTV-pMAMneo-CAT [a commercial vector from Clontech carrying the mouse mammary tumour virus (MMTV) promoter] respectively. The LR-pMAMneo-CAT showed no activity for *CAT*. The *C_T* value for the non-template control was 40.0. The *C_T* values for the internal controls (rRNA) were 13.1 and 19.6 for LF-pMAMneo-CAT and MMTV-pMAMneo-CAT, respectively. From the results, the $\Delta\Delta C_T$ for the toxin promoter will be -3.5 when normalized to the rRNA *C_T* values [41]. Hence, the relative expression of the toxin gene promoter, calculated as $2^{-\Delta\Delta C_T}$, will be 11.3. This indicates that the toxin gene promoter is at least 10 times more active than the general MMTV promoter used in gene-expression studies.

Properties of native and recombinant neurotoxins

The pGEX vector system was used for the expression of neurotoxins. The resulting recombinant plasmids produced GST-Pt-bp and GST-Pt-b proteins upon expression in *E. coli*.

The fusion proteins were separated from *E. coli* proteins by affinity chromatography on a glutathione-agarose column. After removing all the contaminating proteins from the resin-bound fusion proteins, the recombinant neurotoxins were cleaved from GST by digesting with thrombin. The toxins eluted using 50 mM Tris/HCl (pH 7.4) were collected and further purified by RP-HPLC. The GST-fusion proteins and the recombinant neurotoxins were found to cross-react with antibodies raised against the crude venom of *P. textilis*.

Each of the recombinant neurotoxins contained three extra amino acids (GSM) at the N-terminal end following the removal of GST from the fusion protein, of which G and S were introduced by the thrombin cleavage site while M was introduced by the forward primer during subcloning. Methionine was initially introduced with a view to cleaving the recombinant toxin with

Table 1 Properties of Lntxs

For native Pt-N4, molecular mass is based on ESI-MS; for Pt-bp and Pt-b, molecular mass and pI values were calculated from their sequences. IC₅₀ values for both native and recombinant neurotoxins were obtained from binding assays carried out on *T. californica* nAChRs. N.D., not determined.

Protein	Molecular mass (Da)	pI	IC ₅₀ (M)	LD ₅₀ (μg/g of mouse)
Pt-N4 (native)	7892.91	N.D.	3.1 × 10 ⁻⁸	0.15
Pt-bp (recombinant)	8962	8.25	2.7 × 10 ⁻⁸	0.15
Pt-b (recombinant)	7715	7.91	3.3 × 10 ⁻⁸	0.15

Figure 6 Phylogenetic analysis of Lntxs and Sntxs

A cladogram, constructed using intron 2 sequences of various genes by MegAlign from DNASTAR, is shown. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events. See Figures 2 and 3 for abbreviations.

CNBr. However, we found that the extra three amino acid residues at the N-terminus did not affect the function of the toxin significantly. Hence, CNBr cleavage was not carried out. The recombinant proteins and the native Pt-N4 were tested for lethality (LD₅₀) and binding affinity to nAChRs. The results, shown in Table 1, demonstrate that recombinant Pt-bp and Pt-b are lethal Lntxs with the capacity to compete with the binding of ¹²⁵I-α-bungarotoxin on AChRs from *Torpedo*, with almost the same affinity as the native Lntx, Pt-N4.

Thus the major structural differences between Pt-bp and Pt-b or Pt-N4 could be the absence of 11 amino acid residues from the C-terminal end. The LD₅₀ and IC₅₀ values of these toxins suggest that the extra C-terminal end of Pt-bp does not play any important role for its function in lethality or binding affinities to nAChRs. However, Pt-bp showed a slightly higher lethality and binding affinity to nAChRs compared with Pt-b. This could be accounted for partly by the differences of pI caused by the different number of basic amino acid residues located at the C-terminal end. Hence, our results also provide indirect evidence to support the view that the presence of the more basic amino acids at the C-terminal end may contribute to enhanced lethality [42].

Phylogenetic analysis of Lntxs and Sntxs

The structure and organization of the genes encoding Lntxs and Sntxs are generally similar (Figure 4b), all consisting of three exons interrupted by two introns. Intron 1 always occurs at the same position, separating the codon for Gly⁻³ in the signal peptide, whereas intron 2 always occurs at a similar position with slight variation between Lntx and Sntx genes (Cys⁻³⁴ for Lntxs and Asn/Asp/Gly⁻³⁸ for Sntxs). Comparison of the nucleotide sequences of the corresponding exons and introns as well as the 5'-flanking regions of these neurotoxin genes shows high similarity between genes, suggesting that both Sntxs and Lntxs must have evolved from the same ancestral molecule. The similarity in non-coding regions has been found to be higher than that in the

coding region, except in exon 1, which remains very much conserved in both Sntxs and Lntxs, probably due to its indispensable role in the translocation and secretion of these toxins. Therefore, it seems that introns and exons may have undergone evolution in different ways.

Among intron 1 and intron 2, the latter seems to be more conserved in terms of size and nucleotide sequence similarity. Hence this sequence was used to construct a phylogenetic tree (Figure 6). From the results, it is clear that Lntx genes diverged earlier than the Sntx genes.

DISCUSSION

Features of Pt-bp and its corresponding gene

The amino acid sequence of a precursor α -neurotoxin Pt-bp from *P. textilis* has been determined by molecular cloning of its mRNA (as cDNA) and the gene from *P. textilis*. Besides determining the structure and organization of the *Pt-bp* gene, we have also shown it to be a functional gene. The Pt-bp contains a signal peptide of 21 amino acids, which is rich in hydrophobic residues, and a putative mature protein of 82 amino acids. The calculated molecular mass of the mature protein is 8962 Da. The toxin shares high similarity with other Lntxs and possesses all the characteristic features of Lntxs with respect to the amino acid sequence. In particular, it possesses ten cysteine residues, which are essential for the formation of the five disulphide bonds that are indispensable for its function, and all the amino acid residues important for the binding of Lntxs with muscular and neuronal nAChRs [32,33]. Among them, the residues responsible for the binding of α -cobrotoxin to both types of receptor are present in loop 2 of the toxin and at the C-terminal end. The residues important solely for the binding to $\alpha 7$ nAChRs have been found in loop 2, whereas those with selective binding to *Torpedo* nAChRs have been found in both loop 2 and loop 3.

The major difference in Pt-bp from other Lntxs is seen only at the C-terminal end. This is similar to the situation in Aa e from *A. antarticus* [11]. The other variations in Pt-bp have been found to be present at amino acid positions located in loop 1 and in the region between loop 1 and loop 2. However, the amino acids in loop 2 and 3 seemed to be conserved, thus confirming their important role in the binding of Lntxs to nAChRs [43].

Consistent with its structural properties, recombinant Pt-bp exhibited the functional features of Lntxs. It showed lethality and high binding affinity to nAChRs in a manner similar to the Pt-b and native Pt-N4. We could not identify the protein Pt-bp in the venom of *P. textilis* by HPLC or LC-MS. Pt-N4 appears to resemble the previously reported Pt-b in its binding affinity, molecular mass and about 95% of the N-terminal amino acids that have been sequenced. Our gene-expression studies also show that Pt-b expressed in *E. coli* behaves like the native Pt-b. Hence, the toxin Pt-bp could be considered as a precursor of Pt-b or Pt-N4. Based on LC-MS data and N-terminal amino acid sequences, Pt-N3, -N5 and -N6 could vary from Pt-N4 at the C-terminal ends. From these observations, the existence of Pt-N3–Pt-N6 in the venom, and the presence of only one gene and a single mRNA encoding Pt-bp, can only be explained by considering Pt-bp as the precursor toxin that produces Pt-b and other types of Lntx by post-translational modification leading to C-terminal cleavage. Further studies including C-terminal sequencing are needed to confirm this interpretation.

Phylogeny of Lntxs and Sntxs

Based on the comparative analysis of the nucleotide sequences of Lntxs and Sntxs, they seem to have originated from the same

ancestor by gene duplication, and evolved separately by accelerated evolution. Similar speculation has been put forward by Housset and Fontecilla-Camps [44]. The phylogenetic tree in Figure 6 shows that Lntx genes appeared earlier in evolution, suggesting that they could have served as the ancestral molecules for Sntxs. Comparison of the cDNAs encoding both Sntxs and Lntxs (Figure 2) provides additional evidence for this interpretation. At the 3' ends of the cDNAs a consistent pattern on the position of stop codons can be observed. Point mutations can be identified to cause an upstream shift in the stop codons, resulting in earlier termination of the polypeptide chain. For example, the stop codon (TGA) in Pt-bp, when shifted to the same position as the stop codon (TGA) in *N. naja sputatrix* Lntx, can result in the shortening of the polypeptide by 11 amino acid residues. Similar observations can be made for other neurotoxins including the Sntxs. It is noteworthy that Pt-bp cDNA had a much longer coding sequence, with its stop codon being placed furthest from other known Lntx mRNAs.

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