# Molecular cloning and characterization of a neurotoxic phospholipase $A_2$ from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*)

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Using gel-filtration chromatography and reverse-phase (RP) HPLC we have purified a presynaptic neurotoxin (designated as trimucrotoxin) from the crude venom of Taiwan habu (*Trimeresurus mucrosquamatus*). Its complete primary structure was solved by an automated N-terminal sequencing and cDNA sequencing method. The enzyme inhibited the twitch of the chick biventer cervicis muscle at 0.1–1 $\mu$g/ml and showed lethality in mice ($LD_{50} = 1.2$ $\mu$g/g, when given intravenously). Trimucrotoxin exists mainly as a homodimer of 14 kDa subunits as shown by a gel-filtration experiment, and dissociates into monomers during SDS/PAGE in the absence of $Ca^{2+}$. However, most of trimucrotoxin migrated as slowly as a trimer during non-denaturing SDS/PAGE in the presence of $Ca^{2+}$ or $Sr^{2+}$. Its amino acid sequence identity to crotoxin B and agkistrodotoxin is about 75%, and its cDNA sequence is 82% identical to that of crotoxin B. Rabbit antiserum against trimucrotoxin also cross-reacted with the other crotalid neurotoxic phospholipases $A_2$. Furthermore, the purified acidic subunit of crotoxin potentiated the neurotoxicity of trimucrotoxin. A comparison of the sequences of these crotalid neurotoxins revealed some common features of the possible neurotoxic sites, including residues 6, 11, 76–81 and 119–125.

## INTRODUCTION

The extracellular phospholipases $A_2$ ($PLA_2$s) constitute a large family of homologous 14 kDa proteins which are the major components of snake venoms [1–3]. The venom $PLA_2$s, in the presence of $Ca^{2+}$, catalyse the hydrolysis of the 2-acyl ester bond of 1,2-diacyl-*sn*-3-phosphoglycerol lipids. Many of the purified enzymes have been shown to possess different pharmacological actions: neurotoxic [4–6], haemolytic, myonecrotic, anticoagulant [7], convulsant, hypotensive [8,9] or an oedema-inducing action [10]. Such a functional diversity within this group of structurally similar proteins raises interest in the study of their structure–function relationships.

The neurotoxicity of $PLA_2$ appears to arise from specific binding and subsequent hydrolysis of certain phospholipids on the presynaptic membrane [5,11–14]. We previously suggested that some structure feature on the $PLA_2$ surface might be responsible for the initial hydrolysis-independent step leading to the neurotoxicity [15,16]. It was found that depression of respiration took place 2–5 min after injection of 1 mg of the venom of the Taiwan habu (*Trimeresurus mucrosquamatus*) into a rabbit (1.5 kg body weight), and this paralysis may complement the venom-induced circulatory failure which is the major cause of death by the envenomation [17]. It has also been reported that a $PLA_2$ from *T. mucrosquamatus* venom is neurotoxic [18]; we have called it 'trimucrotoxin' hereafter. This was the first neurotoxic $PLA_2$ found in the venom of this old Asian genus, *Trimeresurus*, although neurotoxic $PLA_2$s from another crotalid genus (e.g. crotoxin [5,12,14] and agkistrodotoxin [13]) are well-studied. Crotoxin consists of the non-covalent association of two subunits: a basic and weakly toxic $PLA_2$ subunit and a non-toxic, non-enzymic subunit (crotoxin A) that enhances the toxicity of the basic subunit (crotoxin B). We herein determined the cDNA sequences of trimucrotoxin to solve its primary structure and compared it with the sequences of other group-II $PLA_2$s.

## MATERIALS AND METHODS

### Materials

The crude venom and fresh glands of *T. mucrosquamatus* (Taiwan habu) were provided by Dr. M. Y. Liau (Institute of Preventive Medicine, Taiwan). The source of [$\alpha$-$^{32}$P]dATP, DNA sequencing kits, enzymes and phospholipids are the same as described previously [15].

### Purification of $PLA_2$

The crude venom of Taiwan habu (100–150 mg) was fractionated on a Sephadex G-100 column (1.5 cm × 120 cm, Pharmacia, Sweden) in 0.1 M ammonium acetate, pH 6.8, at 4 °C. The $PLA_2$ fractions were further purified by reverse-phase (RP) HPLC using a column of silica gel (ODS-H, 5 mm, 1 cm × 25 cm; Chemcosorb Scientific Co., Osaka) in 0.07% aqueous trifluoroacetic acid. They were eluted with a 25–50% linear gradient of $CH_3CN$ containing 0.07% trifluoroacetic acid. The conditions used did not have an adverse effect on the venom $PLA_2$.

### Protein quantification and assay of enzymic activity and neurotoxicity

The dye-staining method of Bradford was used for quantification of $PLA_2$ [19]. BSA (1 mg/ml, $A_{280} = 0.56$) was used to establish the standard curve.

Abbreviations used: $diC_{16}PC$, L-dipalmitoylglycerophosphocholine; $PLA_2$, phospholipase $A_2$; RP, reverse-phase.

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The sequence data reported in this paper have been deposited in the EMBL/GenBank Databases under the accession number X77645.

$PLA_2$ activity was measured by the pH-stat titration method [10]. L-Dipalmitoylglycerophosphocholine ($diC_{16}PC$; 3 mM) was mixed with 3 mM sodium deoxycholate or 6 mM Triton X-100 and 100 mM NaCl in a glass–Teflon tissue homogenizer to form micelles. $CaCl_2$ (5 mM) was added just before addition of the enzyme. The liberated fatty acid was titrated with 4 mM NaOH at 37 °C and pH 7.4 using a pH-stat apparatus (Radiometer, RTS 822, Denmark). The reaction rate was corrected for the non-enzymic spontaneous rate.

Neurotoxicity was assessed using the biventer cervicis muscle of chick, and lethal potency ($LD_{50}$) was assessed using ICR mice (18–20 g) as described previously [15].

### SDS/PAGE analysis

The molecular masses of the $PLA_2$s were analysed by SDS/PAGE according to Laemmli [20]. For SDS/PAGE under non-denaturing conditions, reducing agent was omitted from the sample buffer, and the sample was not heated. The gel was stained with Coomassie Blue and the mobilities of the protein standards were plotted against the logarithm of their molecular masses (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; cytochrome *c*, 13.5 kDa).

### Preparation of polyclonal antibodies and immunodiffusion

Antiserum against trimucrotoxin was prepared by injecting subcutaneously 0.2 mg of the purified $PLA_2$ in 0.2 ml of PBS homogenized with 0.2 ml of complete Freund's adjuvant into the back skin of a Wistar rat, and subsequent biweekly immunizations with antigen emulsified in incomplete Freund's adjuvant. Antiserum was then collected. Double immunodiffusion was performed in 1% agarose [21].

### Construction of venom gland cDNA

Total RNA was isolated by the guanidinium thiocyanate and CsCl method [22] and poly(A)-rich RNA was prepared using an oligo(dT)–cellulose column (Pharmacia). Complementary DNA (cDNA) was synthesized from 1 $\mu$g of the poly(A)-rich RNA by using a 'cDNA synthesis system plus' kit (Amersham, U.K.).

### Cloning and sequencing of trimucrotoxin

In order to amplify the $PLA_2$ cDNA, the PCR [23] was conducted by using the cDNA of the total mRNA of the venom gland as the template. A pair of mixed-base oligonucleotide primers, 21 and 18 residues [15], were designed based on the highly conserved cDNA regions for the known group-II $PLA_2$s from snake venoms.

The PCR procedures were performed with Vent DNA polymerase. A 0.45 kb fragment was specifically amplified as shown by 1% agarose gel electrophoresis. After being treated with Klenow fragment and polynucleotide kinase [22], the amplified DNA was inserted into the *Hin*cII site of the pGEM-4Z plasmid (Promega Biotec., Wisconsin, U.S.A.). Then, it was transformed into *Escherichia coli* strain JM101. White transformants were picked-up and cDNA clones were selected. Both strands of the cDNA were sequenced by the dideoxynucleotide method [22] using a sequencing kit (Sequenase version 2.0, U.S. Biochemical).

## RESULTS

### Purification of trimucrotoxin

The *T. mucrosquamatus* venom was separated by Sephadex G-100 gel-permeation chromatography into four major protein fractions (Figure 1). The $PLA_2$ activity was found mainly in fraction III, which was further fractionated by RP-HPLC (Figure 2). The basic Lys-49 $PLA_2$ homologue [10] and trimucrotoxin were found to be present in peaks 4 and 5 respectively. The acidic $PLA_2$s were eluted immediately after them.

Based on the results of SDS/PAGE, the doubly HPLC-purified trimucrotoxin was found to be very pure (Figure 2, inset). The purity was further confirmed by amino acid sequence

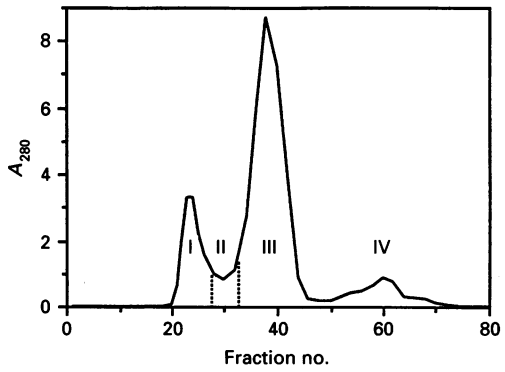


**Figure 1 Gel-permeation chromatography of *T. mucrosquamatus* venom on Sephadex G-100**

The column was equilibrated and eluted with 0.1 M ammonium acetate (pH 6.8), 2.5 ml fractions were collected and the flow rate was adjusted to 18 ml/h. Fraction III had $PLA_2$ activity.

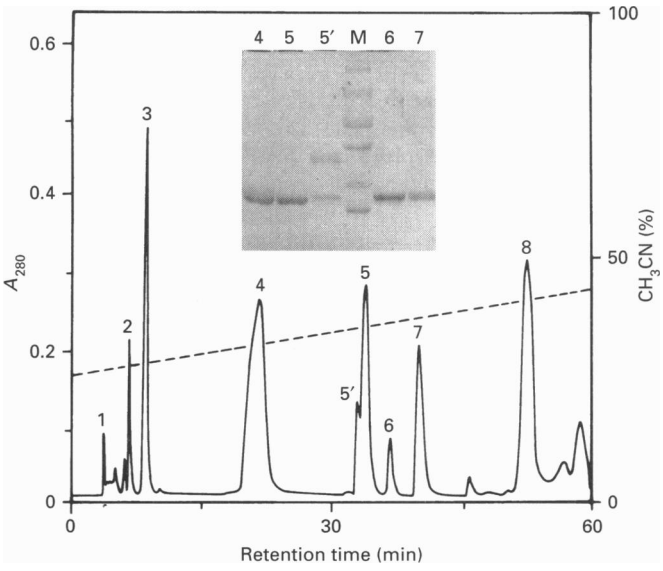


**Figure 2 RP-HPLC purification of $PLA_2$s after gel-filtration**

A Chemcosorb RP-column (ODS-H, 5 mm, 1 cm × 25 cm) was used to isolate $PLA_2$s from fraction III (Figure 1). Solvent A was 0.07% trifluoroacetic acid in water, solvent B was 0.07% trifluoroacetic acid in $CH_3CN$, and the elution was by a linear gradient (21–46%) over 60 min at a flow rate of 2 ml/min. Fractions 4–7 except 5′ had $PLA_2$ activities. Inset: SDS/PAGE (12.5%) showing the purity and molecular masses of peaks 4–7. Lane 'M' contains the molecular-mass markers, from top to bottom: 94, 67, 43, 30, 20.1 and 13.5 kDa.

**Table 1 Neuromuscular blocking actions of trimucrotoxin, crotoxin, crotoxin A subunit and their combination**

The time to inhibit 90% of the contraction of chick neck muscle was measured and shown as mean ± S.E.M. The number of experiments was given in parentheses.

| Toxins | Concentration ($\mu$g/ml) | Time to 90% block (min) |
|---|---|---|
| Trimucrotoxin | 0.10 | 134 ± 18 (2) |
| | 0.25 | 115 ± 15 (3) |
| | 0.30 | 86 ± 7 (2) |
| | 0.50 | 72 ± 5 (5) |
| Crotoxin | 0.05 | 95 ± 7 (4) |
| | 0.10 | 68 ± 10 (3) |
| Crotoxin A | 0.60 | > 300 (2) |
| Trimucrotoxin | 0.25 | |
| + Crotoxin | 0.05 | 76 ± 12 (4) |
| Trimucrotoxin | 0.30 | |
| + Crotoxin A | 0.30 | 70 ± 10 (2) |
| Trimucrotoxin | 0.30 | |
| + Crotoxin A | 0.60 | 46 ± 4 (2) |
| Trimucrotoxin | 0.10 | |
| + Crotoxin A | 0.20 | 62 (1) |

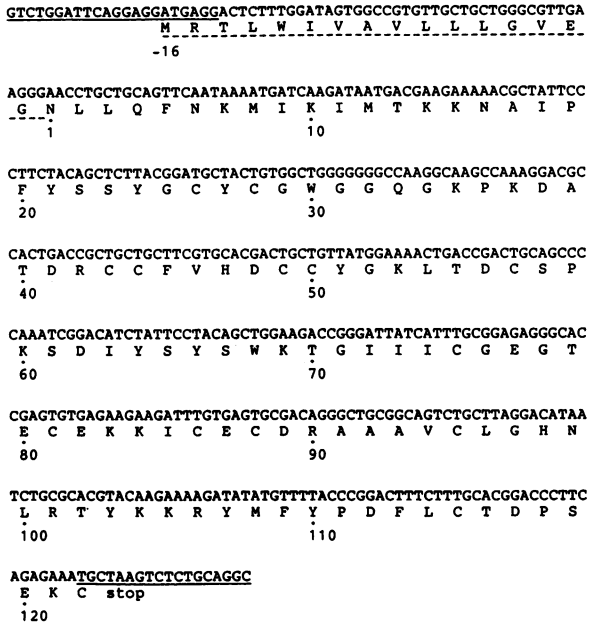


**Figure 3 The cDNA nucleotide sequence and the predicted protein sequence for trimucrotoxin**

Underlining indicates the primers used in the PCR-amplification of the cDNA. Broken lines mark the signal peptides.

analysis up to the 47th residue using an automated protein sequencer [15]. We found that the toxic $PLA_2$ described in the previous publication [18] was a mixture of trimucrotoxin and the Lys-49 homologue [10].

### Enzymic and pharmacological activities

The rate of hydrolysis of $diC_{16}PC$-deoxycholate by trimucrotoxin in the presence of 5 mM $Ca^{2+}$ was 210 $\mu$mol/mg per min at pH 7.4 and 37 °C. The $Ca^{2+}$ affinity of trimucrotoxin was estimated kinetically to be about 0.1 mM. Trimucrotoxin is a presynaptic neurotoxin, although at high doses it also show other effects [18]. The ability of trimucrotoxin to block neuromuscular transmission in chick neck muscle is shown in Table 1. Its neurotoxicity is severalfold lower than that of crotoxin. The effect of the combination of both toxins is additive, not synergistic, suggesting that both toxins probably act at the same presynaptic site [4,12]. The acidic $PLA_2$s from the same venom source could not potentiate the neurotoxicity of trimucrotoxin. However, at the same or twice the molarity of trimucrotoxin, the acidic subunit of crotoxin (crotoxin A) did potentiate the neurotoxicity of trimucrotoxin (Table 1). Thus, trimucrotoxin is similar to crotoxin B and agkistrodotoxin in being able to be chaperoned by crotoxin A to its specific binding proteins [11]. Crotoxin A dose-dependently inhibited the enzymic activity of trimucrotoxin by 20–40% *in vitro* (results not shown).

### cDNA cloning and sequencing

At least two cDNA clones of trimucrotoxin from two independent PCR experiments were sequenced and consistent results were obtained (Figure 3). The cDNA of trimucrotoxin encodes a conserved signal peptide of 16 amino acids followed by the $PLA_2$ polypeptide chain of 122 residues. The deduced protein sequences after the signal peptide match exactly those obtained by the automated protein sequencer. The amino acid sequence is 75% and 74% identical to those of crotoxin B and agkistrodotoxin respectively, and also similar to other basic crotalid $PLA_2$s (Figure 4). The cDNA sequence is 82% identical to that of crotoxin B.

### Immunochemical analysis

Rabbit antiserum against purified trimucrotoxin formed precipitation bands with purified agkistrodotoxin and crotoxin upon immunodiffusion test (Figure 5a). This antiserum also showed cross-reactivities with the crude venoms of *Trimeresurus flavoviridis*, *Trimeresurus okinavensis* and *Trimeresurus gramineus* (Figure 5b). However, the antiserum did not react with the Lys-49 protein [10] and the acidic $PLA_2$s [24] from the *T. mucrosquamatus* venoms. Nor did it react with crude venoms of the following Crotalidae: *Trimeresurus wagleri*, *Trimeresurus tokarensis*, *Trimeresurus popeorum*, *Trimeresurus albolabris*, *Calloselasma rhodostoma* and *Bothrops alternatus*, nor the venom of *Vipera r. russellii*.

### Molecular mass analysis

The native molecular mass of trimucrotoxin was analysed by gel filtration using an HPLC TSK G2000SW column calibrated with standards as previously described [15]. The results showed that trimucrotoxin is a 28 kDa homodimer in buffer of pH 6.8 with or without $Ca^{2+}$. This is different from the situation with many acidic group-II $PLA_2$s which dissociate into monomers (14 kDa) upon the removal of $Ca^{2+}$ by EDTA [24]. However, if 0.1% SDS is added in the gel-filtration buffer, trimucrotoxin partially dissociates into monomers.

## DISCUSSION

In this study, both automated protein sequencing and cDNA sequencing have been used to solve the primary structure of trimucrotoxin. This strategy is especially convenient since these $PLA_2$s are specifically and abundantly expressed in the venom gland. The primers used in our PCR amplification of the $PLA_2$ cDNA were designed on the basis of sequences from the 5′ and 3′-non-translated regions, and the beginning of the signal peptides

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                          1         10        20        30        40        50
                          +         +         +         +         +         +
Trimucrotoxin             NLLQFNKMIKIMTK-KNAIPFYSSYGCYCGWGGQGKPKDATDRCCFVHDCCYGKLT---
Agkistrodotoxin           NLLQFNKMIKEETG-KNAIPFYAFYGCYCGWGGQGKPKDGTDRCCFVHDCCYGRLV---
Crotoxin B                HLLQFNKMIKFETR-KNAIPFYAFYGCYCGWGGRGRPKDATDRCCFVHDCCYGKLA---
TFV-PLX                   HLLQFRKMIKKMTG-KEPIVSYAFYGCYCGKGGRGKPKDATDRCCFVHDCCYGKVT---
A. h. b., basic           HLLQFRKMIKKMTG-KEPVISYAFYGCYCGSGGRGKPKDATDRCCFVHDCCYEKVT---
B. asper, PLA2 III        SLIEFAKMILEETK-RLPFPYYTTYGCYCGWGGQGQPKDATDRCCFVHDCCYGKLS---

60        70        80        90        100       110       120       130       %
+         +         +         +         +         +         +         +         identity
DC-----SPKSDIYSYSWKTGIIICGE-GTECEKKICECDRAAAVCLGHNLRTYKKR-YMFYPDFLC-TDPSEKC    100
NC-----NTKSDTYSYSLKEGYITCGK-GTNCEEQICECDRVAAECFRRNLDTYNNG-YMFYRDSKC-TETSEEC    74
KC-----NTKWDIYPYSLKSGYITCGK-GTWCEEQICECDRVAAECLRRSLSTYKYG-YMFYPDSRC-RGPSETC    75
GC-----DPKWSYYTYSLENGDIVCGG-DPYCTKVKCECDKKAAICFRDNLKTYKNR-YMTFPDIFC-TDPTEGC    72
GC-----KPKWDDYTYSWKNGDIVCGG-DDPCKKEICECDRAAAICFRDNLKTYKKR-YMAYPDILC-SSKSEKC    66
NC-----KPKTDRYSYSRKSGVIICGE-GTPCEKQICECDKAAAVCFRENLRTYKKR-YMAYPDLLC-KKPAEKC    71
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**Figure 4 Comparison of the amino acid sequences of basic $PLA_2$s from crotalid venoms**

Numbering of residues follows that in [15]. Deletions are denoted by dashes. References for the sequences are: agkistrodotoxin and crotoxin B [32], *T. flavoviridis* $PLA_2$-X [28], basic $PLA_2$ from *A. h. blomhoffi* [29], and myotoxic *B. asper* $PLA_2$ III [30].

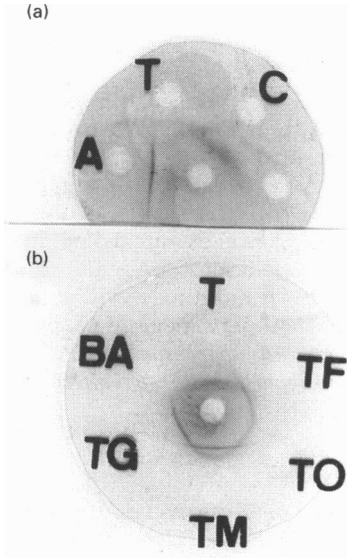


**Figure 5 Double-immunodiffusion analysis of venoms or venom $PLA_2$s**

The proteins in 10 $\mu$l of PBS buffer (pH 7.4) were added to the wells 4 h (**a**), or 24 h (**b**) after the addition of rabbit anti-trimucrotoxin serum to the central wells. (**a**) Wells A, T and C contained 3 $\mu$g each of agkistrodotoxin, trimucrotoxin and crotoxin respectively. (**b**) Outer wells contained 3 $\mu$g of trimucrotoxin (T) and 5 $\mu$g each of the crude venoms of *T. flavoviridis* (TF), *T. okinavensis* (TO), *T. mucrosquamatus* (TM), *T. gramineus* (TG) and *Bothrops alternatus* (BA). The gels were stained with Coomassie Brilliant Blue.

of the homologous $PLA_2$s. Our results confirm that the nucleotide sequences of the 5′- and 3′-flanking regions of the open-reading frames of the Crotalidae and Viperidae $PLA_2$s are highly conserved, as pointed out previously [15,25].

Examination of the primary structure of trimucrotoxin confirmed that it is a group-II $PLA_2$ (Figures 3 and 4). It is structually, immunochemically (Figure 5) and pharmacologically similar to crotoxin B and agkistrodotoxin. This conclusion is also supported by the ability of crotoxin A to complex with trimucrotoxin, thus preventing trimucrotoxin's non-specific binding. It has been reported that the antibodies against crotoxin or agkistrodotoxin are highly specific for both of the toxins [26]. Trimucrotoxin appears to share some specific epitopes with both the toxins (Figure 5), while it is apparently also related to the other basic $PLA_2$s from some crotalid venoms. This also recalls the notion that the crotalid monomeric and heterdimeric $\beta$-neurotoxins might represent different evolutionary stages or biogeographics [11]; it was suggested that *Trimeresurus* and *Agkistrodon* are prototypes of the New World Crotalinae (e.g. *Crotalus* and *Bothrops*) [27].

Caution should be taken in determining the molecular mass of $PLA_2$ by SDS/PAGE under non-denaturing conditions. Some of the basic $PLA_2$s may aggregate or migrate abnormally slowly. The trimerization of trimucrotoxin during the electrophoresis apparently depends on the occupancy of the $Ca^{2+}$ site in its catalytic centre since only in the presence of 0.1–1 mM $Ca^{2+}$, $Sr^{2+}$ and $Ba^{2+}$ was the effect observed and not with 1–5 mM $Zn^{2+}$, $Mg^{2+}$ or $Mn^{2+}$. The significance of the $Ca^{2+}$-induced trimerization is not clear. This is possibly an artifact since it did not occur in the gel-filtration analysis. Other trimeric forms of group-II $PLA_2$ have not been reported before, although a trimeric association was found in the crystal structure of the group-I $PLA_2$ from Indian cobra venom [31].

We compared the sequences of various representative group-II $PLA_2$s to find regions which apparently correlate with neurotoxicity (Table 2). The amino acid sequences of trimucrotoxin at positions 1–10, 76–81, and 119–124 are notably similar to those in crotoxin B and agkistrodotoxin. All the group-II neurotoxins have the following structural features: (1) a neutral residue at the sixth position (note that all the crotalid $PLA_2$-toxins have Asn-6; (2) none of them has a Lys residue at the eleventh position,

**Table 2. Possible toxicity-related regions of group II $PLA_2$s from snake venoms.**

The numbering of amino acid residue is according to [15]. The sequences and $LD_{50}$ other than those of trimucrotoxin are from the literature: agkistrodotoxin [32], RV-4 [15], *T. gramineus* [33], *V. a. ammodytes* $I_2$ [25] and all the rest [3]. A dash indicates a deletion and asterisks denote conserved residues.

| Toxins or $PLA_2$s | Regional amino acid sequences 6 | 11 | 76 | 81 | 119 | 125 | Charge score 76–81 | 119 & 125 | $LD_{50}$ ($\mu$g/g) |
|---|---|---|---|---|---|---|---|---|---|
| Neurotoxins | . | . | . | . | . | . | | | |
| Trimucrotoxin | FNKMIKIM | | YSWKTGII | | YMFYPDFLC | | +1 | 0 | 1.2 |
| Agkistrodotoxin | FNKMIKEE | | YSLKEGYI | | YMFYRDSKC | | 0 | +1 | 0.1 |
| Crotoxin B | FNKMIKFE | | YSLKSGYI | | YMFYPDSRC | | +1 | +1 | 0.56 |
| Caudoxin | FGNMISAM | | YKFNNGNI | | WR-YPSSKC | | +1 | +2 | 0.18 |
| Ammodytoxin A | FGMMILGE | | YHRENGAI | | YRNYPDFLC | | 0 | +1 | 0.02 |
| Ammodytoxin B | FGMMILGE | | YHRENGAI | | YMYYPDFLC | | 0 | 0 | 0.58 |
| Ammodytoxin C | FGMMILGE | | YHRENGAI | | YRNYPDILC | | 0 | +1 | 0.36 |
| Vipoxin | FAKMINGK | | YSFKKGNI | | YKFLSSSRC | | +2 | +2 | 0.1 |
| RV-4 | FARMINGK | | YSFQRGNI | | YKFLSSSKC | | +1 | +2 | 0.2 |
| | * | ** | * | * * | * | * * | | | |
| Non-neurotoxic $PLA_2$ | | | | | | | | | |
| *T. flavoviridis* PLX | FRKMIKKM | | YSLGNGDI | | YMTFPDIFC | | –1 | 0 | |
| *T. okinavensis* | FETLIMKI | | YTEEEGAI | | YWMFPAKNC | | –3 | 0 | |
| *T. gramineus* | FETLIMKI | | YSEENGAI | | YWFFPAKNC | | –2 | 0 | |
| *A. h. pallas* | FETLIMKV | | YSEENGDI | | YWAFGAKNC | | –3 | 0 | |
| *A. h. blomhoffii* | FETLIMKV | | YTEEDGAI | | YWWFPFKNC | | –3 | 0 | |
| *A. p. piscivorus* | FEKLIKKM | | YSVENGNI | | YWKYPKKNC | | –1 | 0 | |
| *C. atrox* | FETLIMKI | | YSEENGEI | | YWLFPPKDC | | –3 | –1 | |
| *B. nasicornis* | FGNMINKM | | YEFQDGDI | | YFGYSSSKC | | –3 | +1 | |
| *V. a. ammodytes* $I_2$ | FGNMIFKM | | YSFENGDI | | YKNYPSSHC | | –2 | +1 | |
| | * | ** ** | * | * * | * | * * | | | |

which is highly conserved in non-neurotoxic group-II $PLA_2$s; (3) their residues 76–81, 119 and 125 are usually more basic (Table 2). Results of chemical modification studies on $PLA_2$ toxins have also related their neurotoxicity with amino acids at positions 6–8 [16,34], 76–81 [15,34,35] and 119–125 [25,36]. Moreover, it was shown that monoclonal antibodies specific for the C-terminal regions of crotoxin B and ammodytoxin neutralized their neurotoxicity [37,38]. These structural features in the $PLA_2$ toxins presumably reduce their non-specific adsorption, and possibly potentiate their binding to the presynaptic receptor [15]. Other locations (e.g. highly conserved Tyr-21 [39], and Lys-128 [40]) have also been suspected of being involved in the neurotoxicity of $PLA_2$s. Mutagenesis at these sites and subsequent functional analysis of the mutants would help to clarify the role of these residues in the neurotoxicity of venom $PLA_2$s.

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