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Biochemical characterization of phospholipase A_2 (trimorphin) from the venom of the Sonoran Lyre Snake *Trimorphodon biscutatus lambda* (family Colubridae)

Ping Huang, Stephen P. Mackessy*

Department of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA

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Abstract

Phospholipases A_2 (PLA_2), common venom components and bioregulatory enzymes, have been isolated and sequenced from many snake venoms, but never from the venom (Duvernoy's gland secretion) of colubrid snakes. We report for the first time the purification, biochemical characterization and partial sequence of a PLA_2 (trimorphin) from the venom of a colubrid snake, *Trimorphodon biscutatus lambda* (Sonoran Lyre Snake). Specific phospholipase activity of the purified PLA_2 was confirmed by enzyme assays. The molecular weight of the enzyme has been determined by SDS-PAGE and mass spectrometry to be 13,996 kDa. The sequence of 50 amino acid residues from the N-terminal has been identified and shows a high degree of sequence homology to the type IA PLA_2s, especially the Asp-49 enzymes. The Cys-11 residue, characteristic of the group IA PLA_2s, and the Ca^{2+} binding loop residues (Tyr-28, Gly-30, Gly-32, and Asp-49) are conserved. In addition, the His-48 residue, a key component of the active site, is also conserved in trimorphin. The results of phylogenetic analysis on the basis of amino acid sequence homology demonstrate that trimorphin belongs to the type IA family, and it appears to share a close evolutionary relationship with the PLA_2s from hydrophiine elapid snakes (sea snakes and Australian venomous snakes).

Keywords: Amino acid sequence; Catalytic site residue; Calcium binding site residues; Colubrid snake; Duvernoy's gland; Elapidae; Enzyme; Evolution; Mass spectrometry; Phospholipase A_2; Phylogenetic analysis

1. Introduction

Snake venoms are complex mixtures of components with a diverse array of actions both on prey and human victims, and they are generally rich sources of water-soluble enzymes and polypeptides. Among these enzymes, the secreted phospholipases A_2 are widely distributed among various species, and those from the venoms of reptiles and the pancreatic tissues of mammals are particularly well characterized (Danse et al., 1997). Phospholipases A_2 are esterolytic enzymes which hydrolyze acyl-ester bonds at the *sn*-2 position of 1,2-diacyl-3-*sn*-phosphoglycerides and release fatty acids and the corresponding 1-acyl lysophospholipids (van Deenen et al., 1963; Kini, 1997). Especially noteworthy are various types of phospholipase A_2 (PLA_2) toxins which are neurotoxins and cardiotoxins (Lee, 1979; Dufton and Hider, 1983; Mukherjee, 1990), which have important pharmacological applications in the understanding of biochemical functions of human cells and diseases. Snake venom PLA_2s are enzymes primarily used for trophic and defense functions, and they exhibit a wide variety of pharmacological activities including neurotoxic, cardiotoxic, hemolytic, anticoagulant and myonecrotic actions, among others (Chang, 1985; Rosenberg, 1990; Hawgood and Bon, 1991; Yang, 1994; Zhang and

* Corresponding author. Present address: Atrix Laboratories, Inc., 2579 Midpoint Drive, Fort Collins, CO 80525, USA. Tel.: +1-970-351-2429; fax: +1-970-351-2335.

E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).

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Gopalakrishnakone, 1999). By comparative sequence analysis, the venom PLA_2 enzymes from various snake families are found to be closely related to mammalian pancreatic PLA_2 enzymes (Kini, 1997).

Phospholipases A_2s are among the most extensively studied and characterized proteins (Yang, 1994; Tsai, 1997; Danse, 1997). However, the efforts on the isolation and characterization of venom PLA_2 enzymes have so far been directed toward the venoms of snakes from the families Elapidae and Viperidae (e.g. Kini, 1997). Little attention has been paid to the isolation and characterization of PLA_2s from the venom (= Duvernoy's gland secretions) of the polyphyletic family Colubridae, the world's largest snake family (Mackessy, 2002). As a result we know very little about the PLA_2s from colubrid snakes. The current study focuses on the isolation, purification and biochemical characterization of a phospholipase A_2, termed trimorphin, from the venom of the colubrid snake *Trimorphodon biscutatus lambda* (Sonoran Lyre Snake). We developed a single-step HPLC procedure to purify the PLA_2 from this venom. The sequence of 50 amino acid residues from the N-terminus has also been determined, representing the first sequence data for any colubrid snake venom PLA_2.

2. Materials and methods

2.1. Materials

Ketamine (2-[2-chlorophenyl]-2-[methylamino]-cyclohexanone-HCl) was purchased from Fort Dodge Laboratories, Inc. (Ft. Dodge, IA, USA). Pilocarpine, 4-nitro-3-(octanoyloxy) benzoic acid, trifluoroacetic acid and other biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Novex Mark 12 molecular weight markers and precast tris–glycine gels were products of Invitrogen Corp. (Carlsbad, CA, USA). Protein concentration reagent and bovine γ-globulin were purchased from BioRad, Inc. (San Diego, CA, USA). All chemicals and solvents were of the highest quality commercially available.

2.2. Venom extraction

Venom from the Duvernoy's gland was extracted repeatedly from three adult *T. biscutatus lambda* (from Cochise Co., AZ, USA) using ketamine–HCl (20 μg/g of body weight) and pilocarpine–HCl (7.5 μg/g of body weight) as described previously (Hill and Mackessy, 1997). The subjects were first anesthetized with ketamine–HCl followed by parasympathetic stimulation with pilocarpine–HCl to increase the venom yield. The venom samples were collected using a 50 μl capillary tube placed over the enlarged rear maxillary fangs to minimize contamination by saliva. The secretion volume was estimated and recorded and the venom samples were transferred to microcentrifuge tubes, immediately frozen and lyophilized, and stored frozen at −20 °C until used.

2.3. Protein assay

The protein concentration of the samples was determined by the method of Bradford (1976) as modified by BioRad Laboratories (San Diego, CA, USA). Venom samples were prepared at an apparent concentration of 4.0 μg/μl. Bovine γ-globulin protein standards were also prepared at concentrations of 5, 10, 15, 20, and 30 μg/ml.

2.4. Purification of Tb-PLA_2

Lyophilized crude venom of *Trimorphodon biscutatus* was dissolved in 0.1% trifluoroacetic acid (TFA) at a concentration of 10 mg/ml, followed by a 2-min centrifugation with a bench-top centrifuge and filtration with a 0.22 μm syringe filter to remove any colloidal or particulate material. The samples were loaded on a reverse-phase C_{18} HPLC column (Vydak column, 4.6 × 250 mm, Waters Empower HPLC System) and elution was performed with 0.1% TFA and a gradient of 15–75% buffer B (80% acetonitrile in 0.1% TFA) over 30 min at a flow rate of 0.8 ml/min. Protein fractions were collected with a Gilson FC 203B fraction collector (0.5 min) and related fractions (PLA_2) were pooled for further analysis.

2.5. Phospholipase A_2 activity assay

PLA_2 enzyme activity was determined by the method of Holzer and Mackessy (1996) using 50 μl of venom or 100 μl of fraction sample, using 4-nitro-3-(octanoyloxy) benzoic acid as substrate in the presence of Ca^{2+}. The assay buffer was 10 mM Tris–HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.1 M NaCl. The effect of the metal chelator diNa-EDTA was also evaluated using this method; enzyme (5 μg protein) and EDTA were incubated in buffer (lacking added calcium) for 30 min at RT prior to assay for activity. The pH profile of trimorphin (5 μg protein) was determined as above using the following buffers: 0.1 M sodium acetate (pH 5.0), 0.1 M MES (pH 5.5 and 6.0), 0.1 M PIPES (pH 6.5), 0.1 M HEPES (pH 7.0–8.0), 0.1 M Tris–HCl (pH 8.5), 0.1 M CHES (pH 9.0–10.0) and 0.1 M CAPS (pH 10.5–11.0). All of these buffers also contained 10 mM $CaCl_2$ and 0.1 M NaCl.

2.6. SDS-PAGE

The purity of isolated trimorphin was verified using SDS-PAGE with Novex precast gels (14% acrylamide Tris–glycine). Immediately prior to loading on the gel (2 and 5 μg protein per lane), the samples were treated with 5% 2-mercaptoethanol, heated at 100 °C for 5 min, allowed to cool to room temperature and centrifuged. Crude venoms

(35 μg protein per lane; *T. biscutatus*) were also reduced. Gels were imaged using a Kodak DC-120 digital camera.

2.7. Reduction and alkylation

Purified trimorphin (approx. 250 μg) was dissolved in 1.0 ml of 0.1 M Tris buffer, pH 7.5, containing 1% SDS and 0.1 M dithiothreitol (DTT). The mixture was boiled for 3 min and then incubated under nitrogen for 1 h at room temperature. An aliquot of 40 μl of a freshly prepared 100 mM stock solution of 4-vinylpyridine was added to the solution and followed by incubation overnight under nitrogen at room temperature. The resultant mixture was transferred into washed dialysis tubing (3.5 kDa cutoff) and dialyzed against 1.0 l of 0.1% SDS for three changes.

2.8. Amino acid sequence analysis

The N-terminal amino acid sequence (first 50 residues) of the S-pyridylated PLA_2 enzyme was determined by automated Edman degradation using an Applied BioSystems 473a pulsed liquid-phase sequencer at the Protein Structure Core Facility, University of Nebraska Medical Center.

2.9. Mass spectroscopy

Mass spectroscopic analysis of the purified PLA_2 was carried out at MacroMolecular Resources, Colorado State University (Fort Collins, CO, USA). Native protein sample was dissolved in 0.2% formic acid in 50/50 acetonitrile/water at a concentration of 1.5 mg/ml. The mass was determined by MALDI MS spectroscopy (Kratos, MALDI I equipment).

2.10. Protein sequence homology

Type classification of trimorphin was accomplished by comparison of key amino acid residues with characteristic residues of other venom PLA_2s (Kini, 1997). Comparative analysis of PLA_2 s was performed using MacClade 4 and PAUP (Phylogenetic Analysis Using Parsimony) 4.0 software with previously published protein sequence data (largely summarized in Kini, 1997) which is also available via the Internet at the National Center for Biotechnology Information's NR Protein Database (FASTA) (Pearson and Lipman, 1988). Species and toxin names are given in Appendix A. The cladogram tree was generated using MacClade 4.

3. Results and discussion

3.1. Venom production in trimorphodon

Due to low yields (relative to front-fanged snakes), venom samples were extracted repeatedly from adult snakes in captivity over a period of time. Larger snakes produced greater yields (Fig. 1A), and an exponential relationship exists between snake length and venom mass. This type of relationship has been observed both for other colubrids (*Boiga irregularis*: Mackessy, 2002) and for rattlesnakes (Mackessy, 1988; Mackessy et al., 2003). A strong linear relationship exists between venom volume and mass (Fig. 1B), and as has been observed previously (Hill and Mackessy, 1997, 2000), pilocarpine-induced venom is of low protein concentration (~48 mg solids/ml venom; 80–90% protein) relative to front-fanged snake venoms (e.g. rattlesnakes: 225–280 mg/ml, 90–92% protein; unpubl. data). However, the largest single yield, 20 mg, is comparable to yields of many species of smaller front-fanged snakes (pers. obs.).

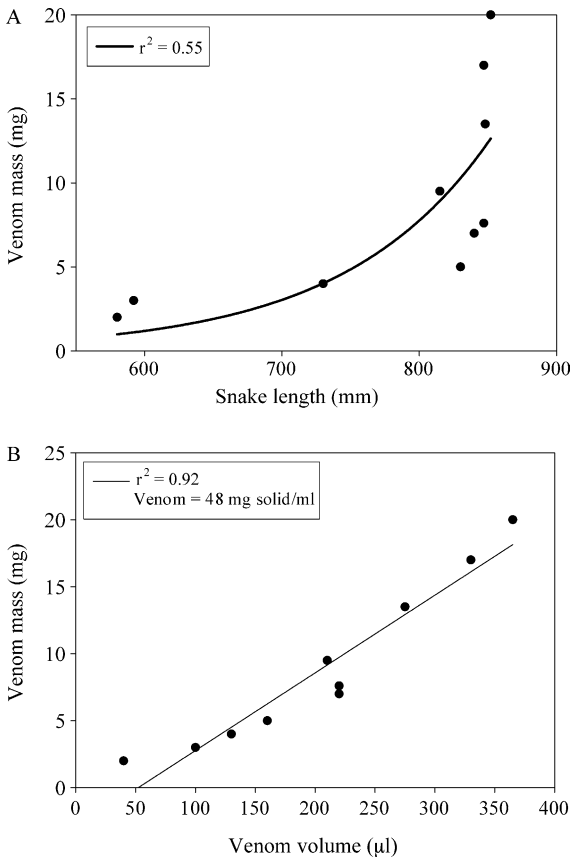

Fig. 1. Venom yields for *T. biscutatus lambda* increase exponentially with snake length (A), and venom mass shows a close linear relationship with venom volume (B). Mass of solids (primarily protein) in the venom averages 48 mg/ml.

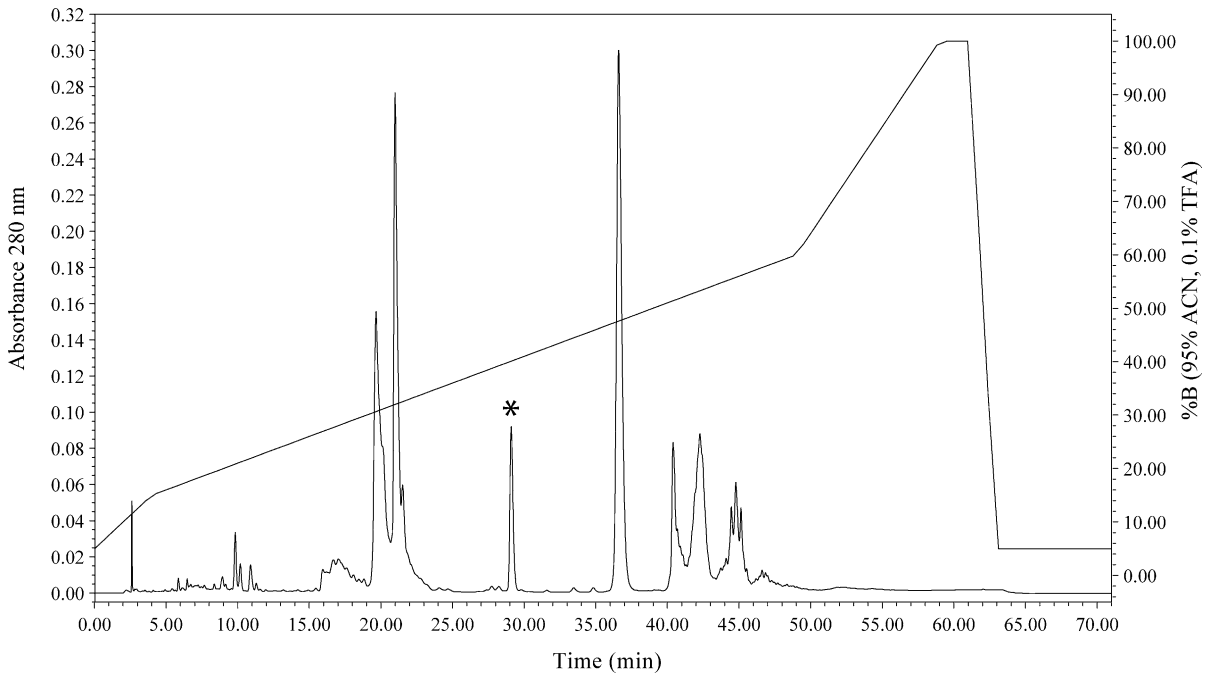

Fig. 2. Reverse-phase HPLC chromatogram of crude venom of *T. biscutatus lambda*. (*) Indicates the peak containing PLA_2 (trimorphin), which is well-separated from other fractions.

3.2. Purification of trimorphin

Like the venoms of most other snakes, the venom of *T. biscutatus* is a mixture of pharmacologically active proteins and polypeptides, including metalloproteases and phospholipase A_2. In order to isolate and purify PLA_2 from the crude venom more quickly and effectively, a single-step procedure using HPLC on a reverse-phase C_{18} column was used. The elution profile revealed nine major peaks, of which a single symmetrical peak with a retention time of ~29 min was found to be active PLA_2 (Fig. 2). Recovery of PLA_2 activity that was present in crude venom is 3.5% of total proteins, which is comparable to a 3.8% recovery rate achieved with a three-step isolation procedure (ammonium sulfate precipitation, DEAE-Sephacel, and reverse-phase HPLC) by Serrano et al. (1999) for PLA_2 from the venom of *Bothrops jararaca*. The homogeneity of the purified PLA_2, trimorphin, was established by SDS-PAGE and mass spectroscopy. After reduction by 2-mercaptoethanol, trimorphin appeared as a single band of 14 kDa (SDS-PAGE using Novex Mark 12 as protein standards; Fig. 3). To confirm the molecular weight estimate of native trimorphin, we carried out mass spectroscopic analysis, which revealed a single peak with a molecular mass of 13,996 Da (Fig. 4), closely agreeing with the result of SDS-PAGE. MALDI-TOF mass spectrometry has also been used to characterize complexity of colubrid venoms (Mackessy, 2002) and it provides a rapid tool for screening for smaller toxins as well.

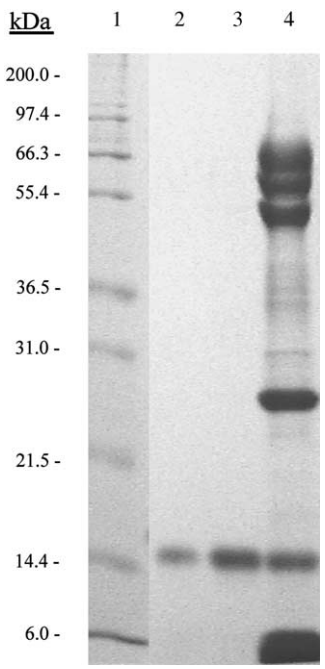

Fig. 3. SDS-PAGE of trimorphin under reducing conditions. Lane 1: Novex Mark 12 protein standards. Lanes 2 and 3: RP-HPLC purified trimorphin, approximately 2 and 5 μg protein; note lack of contaminant bands. Lane 4: Crude venom from *T. biscutatus lambda*, 35 μg protein.

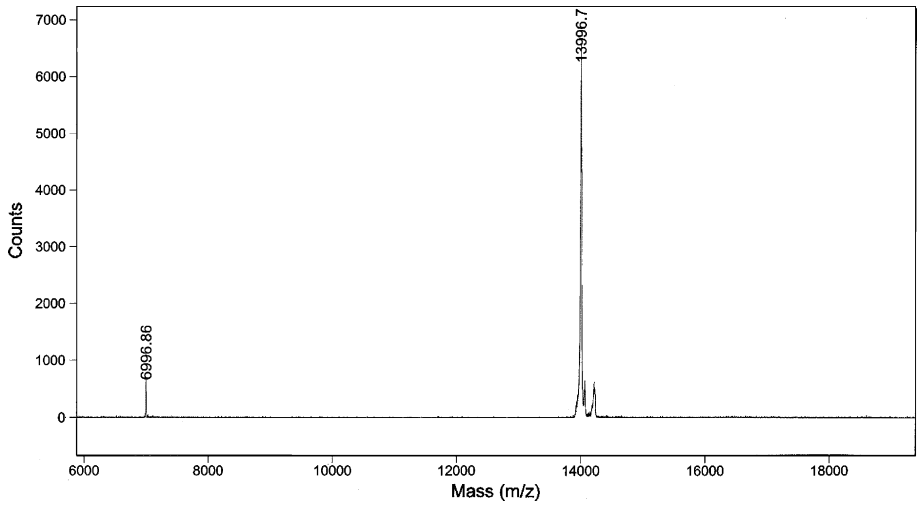

Fig. 4. Mass spectrum of trimorphin. A single peak with a molecular mass of 13,996 Da is seen; the small shoulder may represent minor isoforms, and the 6.99 kDa peak is the doubly charged ion of trimorphin. This data also shows that the single step isolation method produces a highly purified product.

3.3. Effect of EDTA and pH on enzyme activity

At concentrations above 50 μM, the metal ion chelator EDTA completely inhibited PLA_2 activity, demonstrating the requirement of divalent cation for activity (likely Ca^{2+}, as for other PLA_2s); the IC_{50} is approximately 15 μM. Fig. 5 presents the pH-activity profile of trimorphin. The enzyme shows a broad pH optimum (7.0–9.0) with an apparent peak of activity at pH 7.5. No enzymatic activity was detected at pH values below 5.5 or above 10.5. This profile is in general agreement with the values for other snake venom PLA_2s (e.g. Tu et al., 1970; Vidal et al., 1972; Joubert and van der Walt, 1975). The broadness of the pH optimum suggests that the microenvironment of active center residue His-48 is well protected from the intrusion of solvent. Specific activity of trimorphin at pH 7.5 (toward 4-nitro-3-(octanoyloxy) benzoic acid) is 27.7 nmol product formed/min/mg protein.

3.4. N-Terminal amino acid sequence

Trimorphin was reduced and pyridylethylated prior to sequence analysis. The N-terminal 50 amino acid sequence of trimorphin was determined and is presented (Table 1) in alignment with several selected type IA PLA_2s from *Laticauda semifasciata* (Chinese sea krait) pancreas, *Pseudonaja textilis* (eastern brown snake) venom, *Naja nigricollis* (African black-necked spitting cobra) venom, *Notechis s. scutatus* (Australian tiger snake) venom and bovine pancreas. The sequence comparison shows that trimorphin shares greatest sequence identity (40/50 residues, 80%) with a pancreatic PLA_2 from *L. semifasciata* (Fujimi et al., 2002), and a high degree of sequence homology with the group IA PLA_2 s, particularly the Asp-49 enzymes from several hydrophiine venoms, is apparent. The cysteine at position 11, which is characteristic of the type IA PLA_2s, is conserved in trimorphin. The amino acid residues involved in Ca^{2+} binding (Tyr-28, Gly-30, Gly-32, and Asp-49) (Scott et al., 1990a,b) are also conserved in trimorphin. Asp-49 is essential for Ca^{2+} binding of PLA_2, and even the conservative substitution of Asp-49 → Glu-49 resulted in a 12-fold decrease in Ca^{2+}-binding affinity of the enzyme with a concomitant loss of catalytic activity (Li et al., 1994). The residue His-48, another highly conserved key residue in mammalian pancreatic and snake venom PLA_2s, is conserved in trimorphin. Together with Asp-49 and Ca^{2+} ion, His-48 is believed to play a key role in the catalytic activity of PLA_2 by serving as a proton acceptor and donor (Verheij et al., 1980). Introduction of a methyl group on the N-1 position of His-48 has resulted in a total

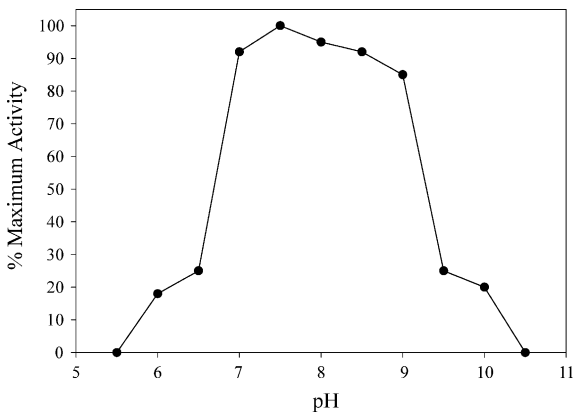

Fig. 5. pH profile of trimorphin PLA_2 enzyme activity toward synthetic substrate (4-nitro-3-(octanoyloxy) benzoic acid). Note the broad pH optimum between pH 7 and 9.

Table 1
Alignment of N-terminal amino acid sequence of trimorphin with selected Group I PLA_2 enzymes

Enzyme	10	20	30
Trimorphin	NLYQFSNMIQ	**C**TIPGSDPLS	DYGNYGC**Y**C**G**
Laticauda semi GL16-1	NLVQFSNMIK	**C**TIPGSRPLL	DYADYGC**Y**C**G**
Textilotoxin C	NLIQFSNMIK	**C**TIPGSQPLL	DYANYGC**Y**C**G**
Notexin np	NLVQFSYLIQ	**C**ANHGKRPTW	HYMDYGC**Y**C**G**
Nn-PLA_2 (basic)	NLYQFKNMIH	**C**TVP–SRPWW	HFADYGC**Y**C**G**
Bovine pancreas PLA_2	ALWQFNGMIK	**C**KIPSSEPLL	DFNNYGC**Y**C**G**
	40	50	Reference
Trimorphin	Y**G**GSGTPVDE	LLRCCQV**HD**D…	Current study
Laticauda semi GL16-1	A**G**GSGTPVDE	LDRCCQT**HD**N…	Fujimi et al. (2002)
Textilotoxin C	P**G**NNGTPVDD	VDRCCQA**HD**E…	Pearson et al. (1993)
Notexin np	A**G**GSGTPVDE	LDRCCKI**HD**D…	Halpert and Eaker (1976)
Nn-PLA_2 (basic)	R**G**GKGTPVDD	LDRCCQV**HD**N…	Yang and King (1980)
Bovine pancreas PLA_2	L**G**GSGTPVDD	LDRCCQT**HD**N…	Fleer et al. (1978)

Conserved functional residues are given in bold. Tyr-28, Gly-30, Gly-32 and Asp-49 are residues known to be involved in Ca^{2+} binding; His-48 is one of the key residues involved in catalytic activity of PLA_2; Cys-11 is characteristic of most Group I PLA_2. Laticauda semi. GL16-1, pancreatic precursor from *Laticauda semifasciata*; Textilotoxin C, from *Pseudonaja textilis* venom; Notexin np, from *Notechis s. scutatus* venom; Nn-PLA2 (basic), from *Naja nigricollis* venom.

loss of enzymatic activity in equine pancreatic PLA_2, even though the binding of monomeric substrate and cofactor Ca^{2+} to the active site remains unaffected (Verheij et al., 1980). Furthermore, a majority of residues involved in the formation of a hydrophobic channel (Leu-2, Phe-5, and Ile-9) (Scott et al., 1990b) are also conserved in trimorphin with the exception of Trp-19, which has been substituted (somewhat conservatively) by Leu-19.

3.5. Evolutionary relationships

An analysis of sequence relatedness was conducted by comparing the N-terminal amino acid sequence of trimorphin with the first 50 residues of sequence of 86 snake venom PLA_2s. The resultant cladogram (Fig. 6) strongly indicates that trimorphin is a member of the group IA PLA_2 family. Structural analysis reveals that residues which are highly conserved in elapid group IA PLA_2, are also conserved in trimorphin. Trimorphin appears to be more closely related to the PLA_2s from sea snakes and Australian elapid snake venoms (subfamily Hydrophiinae) than to the other terrestrial elapids or to viperid venoms. Phylogenetic analysis of phospholipases has been used extensively to examine evolutionary relationship among PLA_2s from various animal species (Dufton and Hider, 1983; Tamiya and Yagi, 1985; Hawgood and Bon, 1991; Kostetsky et al., 1991; Slowinski et al., 1997; Tsai, 1997). Slowinski et al. (1997) have compared the amino acid sequences of PLA_2 from 25 species of elapids in 14 genera, and their results support a division of the elapids examined into sister groups of the Australian and marine species, and African and Asian species, a conclusion also supported by DNA sequence data (Keogh, 1998). Based on cladistic analyses, trimorphin is nested within the elapid PLA_2s, with a closer homology to the marine and Australian elapids. Recently, based on mitochondrial and nuclear DNA sequence data, elapids have been shown to be nested within the 'Colubridae' subfamilies (Vidal and Hedges, 2002) or as the sister taxon to the 'Colubridae' (including several newly defined families; Vidal and David, 2004), indicating that our data (based on protein sequence) may also reflect this close relationship to the Elapidae. However, at the current stage of analysis of trimorphin, this phylogenetic comparison is for the purpose of classifying the enzyme. A more detailed relationship between the trimorphin and other snake venom PLA_2s will be obtained when the complete sequence becomes available, but we predict that the closer affinity with elapid group I enzymes (and species) than with viperid enzymes will be borne out.

Colubrid snake venoms represent a largely unexplored source of phospholipases and other enzymes and toxins (Hill and Mackessy, 2000; Mackessy, 2002), and PLA_2s will likely be isolated from venoms of numerous other colubrid species. Because many of these venoms lack the complexity of viperid and elapid venoms, the single step isolation method presented here will allow rapid isolation of colubrid PLA_2s. It is clear that colubrid PLA_2s are homologous with those found in other venoms, and as sequences become available, they undoubtedly will have great utility in helping to untangle the complex evolutionary history of this largest family of snakes.

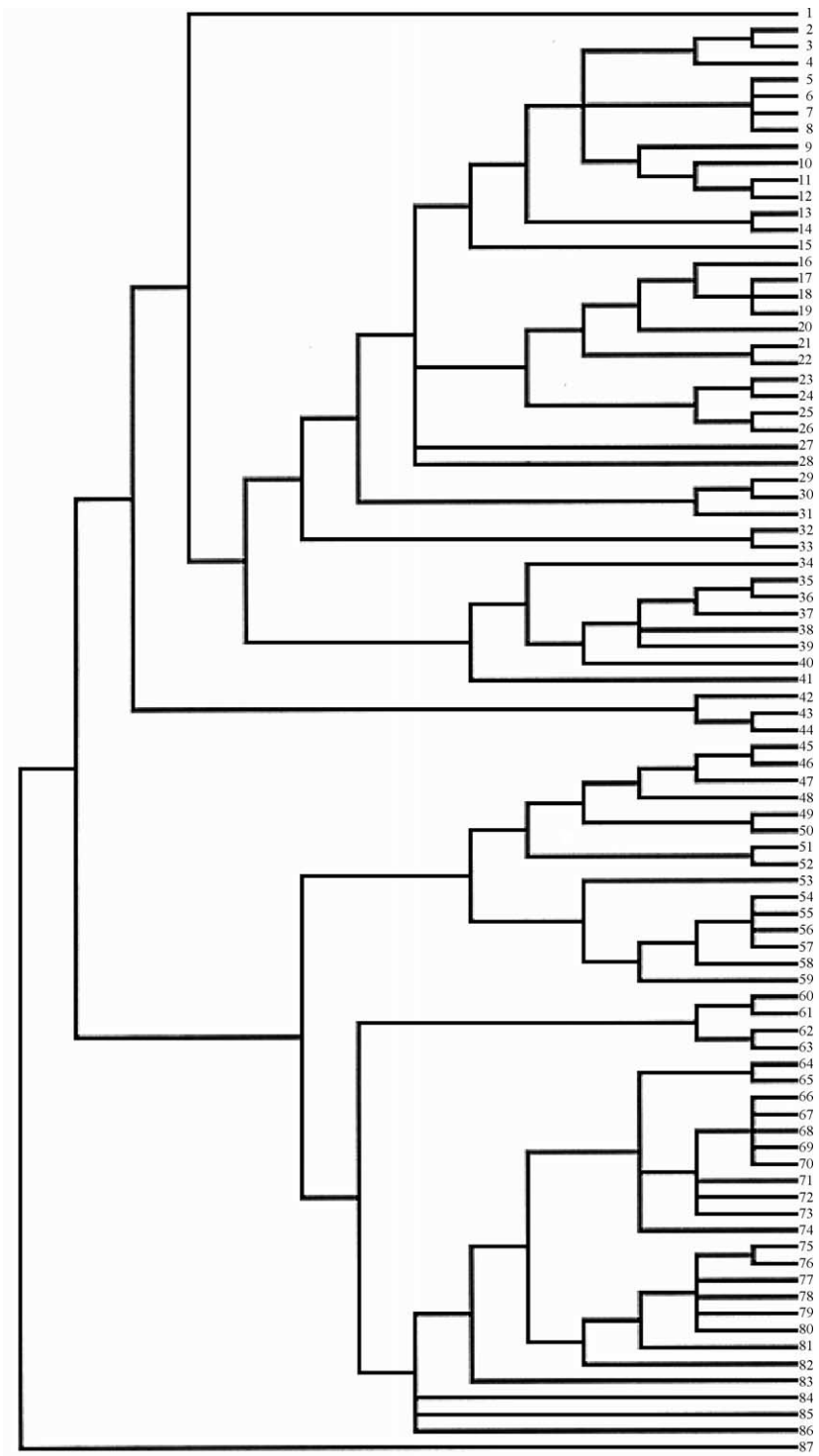

Fig. 6. Cladogram of relationship between *T. biscutatus* PLA_2 (trimorphin-1) and other snake venom group IA PLA_2 enzymes based on the first 50 amino acid residues; identity of numbered PLA_2 is given in Appendix A. The last PLA_2 (87) is Mojave toxin basic subunit PLA_2 from the viperid snake *Crotalus scutulatus* and serves as a group IIa PLA_2 outgroup representative. Note that even though the sequences represented here are truncated at residue 50, the relationship of the hydrophiine and elapine clades (as observed by Slowinski et al., 1997) is largely preserved. Trimorphin is nested within a group including primarily the hydrophiine elapid PLA_2s (PLA2s 2-44).

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Appendix A

Phospholipase A_2 toxins and snake species included in cladistic analysis of PLA_2 relationships (Fig. 6). Sequences are available in Danse et al. (1997) and via the National Center for Biotechnology Information's NR Protein Database (FASTA programs: Pearson and Lipman, 1988)

Number	Snake species	Toxin name
1	*Trimorphodon biscutatus*	Trimorphin
2	*Enhydrina schistosa*	Myotoxin
3	*Enhydrina schistosa*	Myotoxin homolog
4	*Hydrophis lapemoides*	PLA_2
5	*Notechis scutatus scutatus*	Notechis II-5
6	*Notechis scutatus scutatus*	Notexin Np
7	*Notechis scutatus scutatus*	Notexin isoform Ns
8	*Notechis scutatus scutatus*	Scutoxin
9	*Pseudonaja textilis*	Textilotoxin A subunit
10	*Laticauda semifasciata*	Ls PLA I
11	*Laticauda semifasciata*	Ls PLA III
12	*Laticauda semifasciata*	Ls PLA IV
13	*Notechis scutatus scutatus*	PLA_2 11′2
14	*Notechis scutatus scutatus*	Notechis II-1
15	*Australaps superba*	Platelet aggregation inhibitor
16	*Aipysurus laevis*	PLA_2-like
17	*Pseudechis australis*	Pa-13
18	*Pseudechis australis*	Pa-15a
19	*Pseudechis australis*	Pa-15b
20	*Laticauda colubrina*	Lc-PLA-II
21	*Laticauda laticauda*	PLA_2-like
22	*Laticauda colubrina*	Lc-PLA-I
23	*Pseudechis australis*	Pa-1Ga
24	*Pseudechis australis*	Pa-1Gb
25	*Pseudechis australis*	Pa-3a
26	*Pseudechis australis*	Pa-3b
27	*Pseudechis papuanus*	PPV PLA_2, neutral
28	*Pseudechis australis*	Pa-10a
29	*Pseudechis australis*	Pa-11
30	*Pseudechis australis*	Pa-12a
31	*Pseudechis australis*	Pa-12c
32	*Pseudechis australis*	Pa-5a
33	*Pseudechis australis*	Pa-5b
34	*Pseudechis porphyriacus*	Pseudexin A
35	*Bungarus fasciatus*	Toxin Va cardiotoxin
36	*Bungarus fasciatus*	Toxin Vb-2 cardiotoxin
37	*Bungarus fasciatus*	Toxin V-I cardiotoxin
38	*Bungarus fasciatus*	Toxin X-I basic
39	*Bungarus fasciatus*	Toxin II-2 basic
40	*Bungarus fasciatus*	Toxin III neutral
41	*Bungarus fasciatus*	Nonenzymatic acidic mutant PLA2
42	*Pseudonaja textilis*	Textilotoxin C subunit
43	*Pseudechis porphyriacus*	Pseudexin B
44	*Pseudechis porphyriacus*	Pseudexin C
45	*Oxyuranus scutellatus scutellatus*	Taipoxin α chain
46	*Pseudonaja textilis*	Textilotoxin B subunit
47	*Oxyuranus scutellatus scutellatus*	Taipoxin β1 chain
48	*Oxyuranus scutellatus scutellatus*	Taicatoxin PLA_2 1.6.4.2
49	*Oxyuranus scutellatus scutellatus*	Taicatoxin PLA_2 1.6.4.3
50	*Oxyuranus scutellatus scutellatus*	OS_2
51	*Notechis scutatus scutatus*	PLA_2 24′2
52	*Pseudechis australis*	Pa-9c
53	*Bungarus multicinctus*	Phospholipase A
54	*Bungarus multicinctus*	β-bungarotoxin, A1 chain
55	*Bungarus multicinctus*	β-bungarotoxin, A2 chain
56	*Bungarus multicinctus*	β-bungarotoxin, A2 chain variant
57	*Bungarus multicinctus*	β-bungarotoxin, A3 chain
58	*Bungarus multicinctus*	P11 PLA_2 isoform
59	*Bungarus multicinctus*	B. multicinctus A4 chain
60	*Maticora bivirgata*	PLA_2 I
61	*Maticora bivirgata*	PLA_2 II
62	*Micrurus nigrocinctus*	PLA 2.5
63	*Micrurus nigrocinctus*	PLA 3.6
64	*Micrurus nigrocinctus*	PLA 1.3
65	*Aspidelaps scutatus*	CM-II
66	*Micrurus corallinus*	PLA_2-V2
67	*Naja naja atra*	Acidic PLA
68	*Naja naja atra*	Acidic PLA, isoform

Number	Snake species	Toxin name
69	*Naja naja kaouthia*	CM-II
70	*Naja naja sputatrix*	PLA_2 clone 1
71	*Naja naja kaouthia*	CM-III
72	*Naja naja sputatrix*	PLA_2 clone 2
73	*Naja naja sputatrix*	PLA_2 clone 3
74	*Naja melanoleuca*	DE-II
75	*Naja mossambica mossambica*	CM-I
76	*Naja mossambica mossambica*	CM-II
77	*Naja mossambica mossambica*	CM-III
78	*Naja mossambica pallida*	III
79	*Naja nigricollis*	Basic PLA
80	*Naja nigricollis*	Nigexin, cytotoxin
81	*Naja melanoleuca*	DE-I
82	*Naja melanoleuca*	DE-III
83	*Hemachatus hemachatus*	DE-I
84	*Naja naja naja*	Acidic
85	*Naja naja naja*	Acidic PLA2
86	*Naja naja oxiana*	Phospholipase A E3
87	*Crotalus scutulatus scutulatus*	Mojave toxin-b, basic subunit

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