Biochemical characterization of phospholipase A$_2$ (trimorphin) from the venom of the Sonoran Lyre Snake *Trimorphodon biscutatus lambda* (family Colubridae)

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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$_2$s, especially the Asp-49 enzymes. The Cys-11 residue, characteristic of the group IA PLA$_2$s, 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$_2$s 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 lyso-phospholipids (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$_2$s 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...
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$_2$S 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$_2$S from the venom (Duvernoy’s gland secretions) of the polyphylectic family Colubridae, the world’s largest snake family (Mackessy, 2002). As a result we know very little about the PLA$_2$S 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, trifuoroacetic 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% trifuoroacetic 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 (Vydac 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 PLA2 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 PLA2 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 PLA2s (*Kini, 1997*). Comparative analysis of PLA2s 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.
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\textsubscript{2}. In order to isolate and purify PLA\textsubscript{2} from the crude venom more quickly and effectively, a single-step procedure using HPLC on a reverse-phase C\textsubscript{18} column was used. The elution profile revealed nine major peaks, of which a single symmetrical peak with a retention time of \sim 29 min was found to be active PLA\textsubscript{2} (Fig. 2). Recovery of PLA\textsubscript{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\textsubscript{2} from the venom of *Bothrops jararaca*. The homogeneity of the purified PLA\textsubscript{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. 2. Reverse-phase HPLC chromatogram of crude venom of *T. biscutatus lambda*. (*) Indicates the peak containing PLA\textsubscript{2} (trimorphin), which is well-separated from other fractions.

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 \mu g protein; note lack of contaminant bands. Lane 4: Crude venom from *T. biscutatus lambda*, 35 \mu g protein.
3.3. Effect of EDTA and pH on enzyme activity

At concentrations above 50 μM, the metal ion chelator EDTA completely inhibited PLA₂ activity, demonstrating the requirement of divalent cation for activity (likely Ca²⁺, as for other PLA₂s); the IC₅₀ 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 PLA₂s (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 PLA₂ 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 PLA₂ from *L. semifasciata* (Fujimi et al., 2002), and a high degree of sequence homology with the group IA PLA₂ s, particularly the Asp-49 enzymes from several hydrophiine venoms, is apparent. The cysteine at position 11, which is characteristic of the type IA PLA₂s, is conserved in trimorphin. The amino acid residues involved in Ca²⁺ 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 Ca²⁺ binding of PLA₂, and even the conservative substitution of Asp-49 → Glu-49 resulted in a 12-fold decrease in Ca²⁺-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 PLA₂s, is conserved in trimorphin. Together with Asp-49 and Ca²⁺ ion, His-48 is believed to play a key role in the catalytic activity of PLA₂ 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...
loss of enzymatic activity in equine pancreatic PLA₂, even
though the binding of monomeric substrate and cofactor
Ca²⁺ 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
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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 selected Group I PLA₂ enzymes and cofactor
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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$_2$s 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 Słowiński et al., 1997) is largely preserved. Trimorphin is nested within a group including primarily the hydrophiine elapid PLA$_2$s (PLA$_2$s 2-44).
Acknowledgements

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Appendix A

Phospholipase A2 toxins and snake species included in cladistic analysis of PLA2 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)

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References


