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Research paper

Primary structures and partial toxicological characterization of two phospholipases A_2 from *Micrurus mipartitus* and *Micrurus dumerilii* coral snake venoms

Paola Rey-Suárez [a, *], Vitelbina Núñez [a, b], Mónica Saldarriaga-Córdoba [c, d], Bruno Lomonte [e]

[a] Programa de Ofidismo y Escorpionismo, Universidad de Antioquia, Medellín, Colombia
[b] Escuela de Microbiología, Universidad de Antioquia, Medellín, Colombia
[c] Departamento de Ciencias, Universidad Iberoamericana de Ciencias y Tecnología, Chile
[d] Centro de Investigación en Recursos Naturales y Sustentabilidad, Universidad Bernardo O'Higgins, Fábrica 1990, Segundo Piso, Santiago, Chile
[e] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

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ABSTRACT

Snake venom phospholipases A_2 (PLA_2) share high sequence identities and a conserved structural scaffold, but show important functional differences. Only a few PLA_2s have been purified and characterized from coral snake (*Micrurus* spp.) venoms, and their role in envenomation remains largely unknown. In this report, we describe the isolation, sequencing and partial functional characterization of two *Micrurus* PLA_2s: $MmipPLA_2$ from *Micrurus mipartitus* and $MdumPLA_2$ from *Micrurus dumerilii*, two species of clinical importance in Colombia. $MmipPLA_2$ consisted of 119 amino acid residues with a predicted pI of 8.4, whereas $MdumPLA_2$ consisted of 117 residues with a pI of 5.6. Both PLA_2s showed the conserved 'group I' cysteine pattern and were enzymatically active, although $MdumPLA_2$ had higher activity. The two enzymes differed notably in their toxicity, with $MmipPLA_2$ being highly lethal to mice and mildly myotoxic, whereas $MdumPLA_2$ was not lethal (up to 3 μg/g body weight) but strongly myotoxic. $MdumPLA_2$ displayed higher anticoagulant activity than $MmipPLA_2$ *in vitro* and caused more sustained edema in the mouse footpad assay. Neither of these enzymes was cytolytic to cultured skeletal muscle C2C12 myotubes. Based on their structural differences, the two enzymes were placed in separate lineages in a partial phylogeny of *Micrurus* venom PLA_2s and this classification agreed with their divergent biological activities. Overall, these findings highlight the structural and functional diversity of *Micrurus* venom PLA_2s.

1. Introduction

Phospholipases A_2 (PLA_2) are esterases that hydrolyze glycerophospholipids at the *sn*-2 ester bond of the glycerol backbone releasing free fatty acids and lysophospholipids [1]. These enzymes are widespread in nature, but have been especially well characterized in mammalian tissues and in arthropod and snake venoms. In the latter, PLA_2s often play key roles in the immobilization, killing, and digestion of the prey [2]. These roles result from their bioactivities, which include neurotoxicity (pre- or post-synaptic), myotoxicity (local or systemic), cardiotoxicity, anticoagulant activity, inhibition or stimulation of platelet aggregation, hypotension, and edema-formation, among others [3,4]. Amino acid sequences of many snake venom PLA_2s and crystal structures have been reported for some, showing that they share high sequence identities and a conserved structural scaffold [1], but have important differences in their functional properties [4]. On the basis of their primary structures and disulfide bond patterns, PLA_2s from snake venoms are classified into group IA, found in the Family Elapidae, and group IIA found in the Family Viperidae [5].

In the Americas, elapids are represented by coral snakes (genera *Micrurus, Micruroides* and *Leptomicrurus*). The genus *Micrurus*, with 85 species currently accepted by The Reptile Database (http://

* Corresponding author.
E-mail address: ofidpa@gmail.com (P. Rey-Suárez).

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www.reptile-database.org), is by far the most diverse and abundant [6]. Proteomic and transcriptomic studies of several *Micrurus* venoms have shown that three-finger toxins (3FTxs) and PLA_2s constitute the most abundant components [7]. However, due to the very small amounts of venom produced by these snakes, very few of their components have been isolated and characterized. PLA_2s have been isolated from the venoms of only six species: *M. fulvius* [8,9], *M. dumerilii carinicauda* [10,11], *M. nigrocinctus* [12–14], *M. frontalis frontalis* [15], *M. spixi* [16], and *M. lemniscatus carvalhoi* [17–19]. An additional PLA_2 was cloned from *M. corallinus* venom gland and characterized as a recombinant protein [20]. Thus, PLA_2s present in the venoms of *Micrurus* spp. are still largely uncharacterized.

Recent proteomic studies on *Micrurus* venoms have revealed two divergent compositional patterns [7,21]. A 'PLA_2-rich' phenotype has been observed mainly in species that are phylogenetically more derived, whereas a '3FTx-rich' phenotype occurs in species that are phylogenetically more basal, and that probably represents the ancestral venom condition. Moreover, it has been observed that 'PLA_2-rich' venoms generally present more complex protein composition than their '3FTx-rich' counterparts. This complexity is mainly determined by an expansion in the number of PLA_2 isoforms, especially those associated with myotoxicity [7]. As a contribution to knowledge on the diversity of coral snake PLA_2s, in this report we describe the isolation, sequencing, and partial functional characterization of two these enzymes: $MmipPLA_2$ from *Micrurus mipartitus*, and $MdumPLA_2$ from *Micrurus dumerilii*, respectively. These two species are responsible for the majority of coral snake bites in Colombia, and envenomings inflicted by them induce mainly neurotoxic effects leading to progressive paralysis of skeletal muscle, including respiratory muscles [22]. *M. mipartitus* and *M. dumerilii* produce venoms that are '3FTx-rich' and 'PLA_2-rich', respectively: in the venom of *M. mipartitus*, 3FTxs (60% of the total protein content) predominate over PLA_2s (30%), whereas in the venom of *M. dumerilii* PLA_2s (52%) predominate over 3FTxs (28%) [23,24]. As the most abundant PLA_2s in their respective venoms, $MmipPLA_2$ and $MdumPLA_2$ exemplify the great diversification of structural and functional characteristics of toxic PLA_2s within the genus *Micrurus*.

2. Materials and methods

2.1. Venoms and isolation of toxins

Venoms of *M. mipartitus* and *M. dumerilii* were donated by the serpentarium of the University of Antioquia, and were pools obtained from 15 adult specimens of both sexes of each species, from the region of Antioquia, Colombia. PLA_2s were isolated by reverse-phase-HPLC as previously described [23,24]. In brief, 2 mg venom aliquots were dissolved in 200 μL of 0.1% trifluoroacetic acid (TFA; solution A) and separated on a C_{18} column (Pinnacle, 5 μm particle diameter; 250 × 4.6 mm), using a Shimadzu Prominence-20A chromatograph monitored at 215 nm. Elution was performed at a flow rate of 1 mL/min by applying the following gradient of solution B (acetonitrile, containing 0.1% TFA): 5% B for 5 min, 5–15% B over 10 min, 15–45% B over 60 min, and 45–70% B over 12 min. Major fractions previously identified as PLA_2s in proteomic analyses of *M. mipartitus* and *M. dumerilii* venoms were manually collected, dried by vacuum centrifugation, and stored at −20 °C. These two PLA_2s correspond to peaks 20 ($MmipPLA_2$) and 26 ($MdumPLA_2$), respectively, in the numbering used in proteomic studies [23,24]. Electrophoretic homogeneity of $MmipPLA_2$ and $MdumPLA_2$ was evaluated by SDS-PAGE. After reduction with 5% 2-mercaptoethanol at 100 °C for 5 min, 20 μg of each protein was loaded onto a 15% gel, and run in a Mini-Protean Tetra® electrophoresis system (Bio-Rad) at 150 v. Proteins were visualized by Coomassie blue R-250 staining.

2.2. ESI mass spectrometry (MS)

To determine the isotope-averaged mass of $MmipPLA_2$ and $MdumPLA_2$, each protein was dissolved in 10 μL of 0.1% formic acid in 50% acetonitrile, loaded into a metal-coated capillary tip (Proxeon), and directly infused into a nano-ESI source coupled to a Q-Trap® 3200 (Applied Biosystems) mass spectrometer. Ionization was performed at 1300 V and spectra were acquired in positive Enhanced Multi-Charge mode, in the m/z range 500–1700. Charge-state and deconvolution of the ion series were analyzed with the aid of the Bayesian protein reconstruction tool of BioAnalyst® v.1.5 software (ABSciex), and confirmed by manual calculation.

2.3. Amino acid sequencing

Each toxin was dissolved in 50 mM ammonium bicarbonate and subjected to reduction with dithiothreitol (10 mM) and alkylation with iodoacetamide (50 mM). An aliquot of each reduced-alkylated protein was subjected to N-terminal sequencing on a PPSQ-33A Protein Sequencer (Shimadzu). The rest of this material was digested with sequencing grade bovine trypsin (overnight) or chymotrypsin (4 h) at 37 °C, using a protein: enzyme ratio of 100:1 [25]. The resulting peptides were separated by RP-HPLC on a C_{18} column (2.1 × 150 mm; Phenomenex), eluted at 0.3 ml/min with a 0–70% acetonitrile gradient over 40 min, and manually collected. Each peak was subjected to MS/MS fragmentation for amino acid sequencing. For MALDI-TOF-TOF analyses, fragmentation spectra were acquired on a Proteomics Analyzer 4800-Plus instrument (Applied Biosystems) using α-cyano-hydroxycinnamic acid as matrix, at 2 kV in positive reflectron mode, 500 shots/spectrum, and a laser intensity of 3000. Spectra were initially searched using the Paragon® algorithm of ProteinPilot 4.0 (ABSciex) against the UniProt/SwissProt database, and further interpreted manually. Peptides that did not produce well-resolved fragmentation spectra using MALDI were further subjected to nESI-MS/MS by direct infusion in a QTrap® 3200. Selected doubly- or triply-charged peptide ions were analyzed in Enhanced Resolution mode (250 amu/s), and fragmented using the Enhanced Product Ion tool, with Q_0 trapping. Settings were: Q1, unit resolution; collision energy, 25–45 eV; linear ion trap Q3 fill time, 250 ms; and Q3 scan rate, 1000 amu/s [26]. All resulting spectra were manually interpreted with the aid of the BioAnalyst® 1.5 Manual Sequencing tool.

2.4. Phylogenetic relationships

The search for homologous proteins to $MmipPLA_2$ and $MdumPLA_2$ was done using BLAST [27] (BLASTp: search protein databases using a protein query, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and UniProt (http://www.uniprot.org/blast/). For each query sequence, the sequences with the lowest E-value (value close to 0) which presented the highest percentage of identity were selected. A total of eight related amino acid sequences (some of them predicted from transcriptomes) of venom proteins from *Micrurus* (*M. altirostris, M. corallinus, M. dumerilii, M. fulvius, M. nigrocinctus* and *M. tener*) were selected. A *Naja naja* sequence (CAA45372-P15445) was used as an out-group. Amino acid sequences were aligned in BioEdit version 7.0 [28] using default parameters. After including gaps to maximize alignments, the final number of amino acid positions was 122.

Phylogenetic relationships among the new PLA_2s and related enzymes were analyzed using the Bayesian inference implemented in MrBayes v3.0B4 [29], which is well known for its ability to deal