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Divergent functional profiles of acidic and basic phospholipases A_2 in the venom of the snake *Porthidium lansbergii lansbergii*

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ABSTRACT

The Lansberg's hognose pitviper, *Porthidium lansbergii lansbergii*, inhabits northern Colombia. A recent proteomic characterization of its venom (J. Proteomics [2015] 114, 287–299) revealed the presence of phospholipases A_2 (PLA_2) accounting for 16.2% of its proteins. The two most abundant PLA_2s were biochemically and functionally characterized. Pllans-I is a basic, dimeric enzyme with a monomer mass of 14,136 Da, while Pllans-II is an acidic, monomeric enzyme of 13,901 Da. Both have Asp49 in their partial amino acid sequences and, accordingly, are catalytically active upon natural or synthetic substrates. Nevertheless, these two enzymes differ markedly in their bioactivities. Pllans-I induces myonecrosis, edema, and is lethal by intracerebro–ventricular injection in mice, as well as cytolytic and anticoagulant *in vitro*. In contrast, Pllans-II is devoid of these effects, except for the induction of a moderate edema. In spite of lacking myotoxicity, Pllans-II enhances the muscle damaging action of Pllans-I *in vivo*. Altogether, results further illustrate the divergent functional profiles of basic and acidic PLA_2s in viperid venoms, and suggest that Pllans-I plays a myotoxic role in envenomings by *P. l. lansbergii*, whereas Pllans-II, apparently devoid of toxicity, enhances muscle damage caused by Pllans-I.

1. Introduction

The Lansberg's hognose pitviper, *Porthidium lansbergii lansbergii*, locally known as 'patoco' or 'patoquilla', is found in the Caribbean region of Colombia, inhabiting arid and semi-arid lands and humid zones of the Magdalena River Valley, from low elevations up to 1400 m. Its diet appears to be generalist, including preys such as frogs, lizards and small rodents (Campbell and Lamar, 2004; Otero, 2014). Although snakebite accidents have been reported for this species (Paredes, 2012), specific clinical features of these envenomings are lacking in the medical literature. Only few studies on its venom are available, and none of its proteins, to the best of our knowledge, have been isolated and characterized.

In a previous study, the whole venom of *P. l. lansbergii* was analyzed by a combined proteomic and toxicological approach (Jiménez-Charris et al., 2015). In resemblance with two other *Porthidium* species (*Porthidium nasutum* and *Porthidium ophryomegas*) from Costa Rica (Lomonte et al., 2012), the proteome of *P. l. lansbergii* venom predominantly contains metalloproteinases, and presents phospholipases A_2 (PLA_2; EC 3.1.1.4) as the second most abundant proteins (Jiménez-Charris et al., 2015). However, *P. l. lansbergii* venom showed low proportions of additional protein types that were not found in the other two *Porthidium* species, such as phosphodiesterase, phospholipase B, and vascular endothelium growth factor components. Furthermore, its relative content of PLA_2s was slightly higher than that observed in *P. nasutum* and *P. ophryomegas*, and accordingly, *P. l. lansbergii* venom showed higher PLA_2 activity in comparison to *P. nasutum* venom (Jiménez-Charris et al., 2015). In consequence, and to gain further knowledge on the toxic components of the venom of *P. l. lansbergii*, in this study we aimed for the isolation of its two most abundant PLA_2s in order to characterize their biochemical and functional properties.

Snake venom PLA_2s are heat-stable enzymes which, in spite of their conserved architecture, show remarkable functional diversity (Arni and Ward, 1996; Kini, 2003). They catalyze the hydrolysis of

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the 2-acyl bond of phospholipids, releasing fatty acids and lysophospholipids in a calcium-dependent reaction (Scott et al., 1990). Based on their disulfide bonding patterns and other distinctive structural features, snake venom PLA_2s have been classified into group I (in elapids) or II (in viperids) (Doley et al., 2010). In turn, group II PLA_2s have been divided into two subtypes: Asp49–PLA_2s, which are catalytically active, and 'PLA_2 homologues' or 'PLA_2-like' proteins, which are catalytically inactive and most commonly possess a Lys49 substitution (Lomonte et al., 2003; Lomonte and Rangel, 2012). Among their diverse effects, snake venom PLA_2s can exert neurotoxic, myotoxic, hemolytic, inflammatory, anticoagulant, bactericidal, and platelet-inhibiting activities (Páramo et al., 1998; Kini, 2003, 2005; Gutiérrez and Lomonte, 1995; Lomonte et al., 2009; Huancahuire-Vega et al., 2011). The subtle structural features and biochemical properties that define specific bioactivities in different venom PLA_2s have been difficult to pinpoint, and many key molecular aspects of their mechanisms of action are still unknown (Gutiérrez and Lomonte, 2013). Therefore, it is of interest to explore the vast biodiversity of venomous snakes to search and characterize novel variants of PLA_2s which could shed light on understanding their diversification, biological roles, and complex structure-function relationships.

2. Materials and methods

2.1. Snake venom

Venom of *P. l. lansbergii* was obtained from three adult specimens found in Atlantic Department, Colombia. Snakes were released after collection of their venoms, which were pooled, centrifuged to remove debris, dried *in vacuo*, and stored at −20 °C.

2.2. Isolation of Pllans-I and Pllans-II

The reverse-phase HPLC conditions previously described for the proteomic characterization of *P. l. lansbergii* venom (Jiménez-Charris et al., 2015) were followed in order to isolate two PLA_2 enzymes, named Pllans-I and Pllans-II. In brief, aliquots of venom (2 mg) were separated in a C_{18} column (250 × 4.6 mm, 5 μm particle diameter; Teknokroma) eluted at 1 ml/min with a gradient from water/0.1% trifluoroacetic acid (TFA) to acetonitrile/0.1% TFA, as follows: 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min. The eluent was monitored at 215 nm in an Agilent 1200 chromatograph using ChemStation® software, and the targeted PLA_2 fractions were collected manually, dried by vacuum centrifugation, and stored at −20 °C.

2.3. SDS–PAGE and mass spectrometry (MS)

Protein concentration of Pllans-I and Pllans-II was estimated by measuring their absorbance at 280 nm in a NanoDrop® 2000c instrument (Thermo Scientific). Their homogeneity was then evaluated by SDS–PAGE under non-reducing or reducing (5% 2-mercaptoethanol at 100 °C for 5 min) conditions, using 12% gels, stained with Coomassie blue R–250. In addition, homogeneity and isotope-averaged molecular mass were determined by ESI–MS on a QTrap3200® mass spectrometer (Applied Biosystems). The proteins were diluted in 50% acetonitrile containing 0.1% formic acid, loaded into metal-coated disposable glass capillaries, and directly infused into a nanospray source for ionization at 1200 V. MS analysis was performed in positive enhanced multicharge mode in the range 600–1700 m/z, aided by the Analyst® v.1.5 software (ABsciex) for deconvolution.

2.4. Partial amino acid sequencing by tandem mass spectrometry

Pllans-I and Pllans-II were digested with sequencing grade trypsin (Sigma) or chymotrypsin (G–biosciences) after their reduction with dithiothreitol (10 mM) and alkylation with iodacetamide (50 mM) as described (Van der Laat et al., 2013). Peptide products were desalted using C_{18} ZipTips® (Millipore), diluted with an equal volume of a saturated α-cyano4-hydroxycinnamic acid solution in 50% acetonitrile/0.1% TFA, and spotted onto an Opti-TOF384 plate for MALDI–TOF/TOF analysis on a Proteomics Analyzer 4800–Plus (Applied Biosystems) as described (Lomonte et al., 2014). Spectra were acquired in positive reflector mode at 2 kV in the 900–4000 m/z range, using 1500 shots and a laser intensity of 3000. External calibration was performed using CalMix® standards (ABsciex) spotted on the same plate. In addition, some peptides were analyzed by nESI–MS/MS on a QTrap3200 instrument by direct infusion. Doubly- or triply-charged ions selected from scans in positive enhanced resolution mode (250 amu/s) were subjected to fragmentation using the enhanced product ion tool with Q_0 trapping. Settings were: Q_1, unit resolution; collision energy, 25–40 eV; linear ion trap Q_3 fill time, 250 ms; and Q_3 scan rate, 1000 amu/s (Calvete et al., 2007). All fragmentation spectra obtained by MALDI– or ESI–mass spectrometry were interpreted manually to derive *de novo* amino acid sequences.

2.5. Sequence alignment of Pllans-I and Pllans-II

Four available sequences corresponding to basic and acidic Asp49-PLAs characterized from Viperidae family, with amino acid sequence identity values of at least 59% and 45% in comparison to Pllans-I and Pllans-II, were retrieved after a BLAST search (http://blast.ncbi.nlm.gov). These proteins, together with the manually added sequence of PhTX–II isolated from *Porthidium hyoprora* venom (Huancahuire-Vega et al., 2014), were aligned with the program MUSCLE (Edgar, 2004) using the MEGA 6.06 software (Tamura et al., 2013) and the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992).

2.6. Immunochemical cross-recognition screening

The purified Pllans-I and Pllans-II enzymes were tested by gel immunodiffusion (Ouchterlony and Nilsson, 1978) to determine their possible cross-recognition by rabbit polyclonal antibodies raised against myotoxin II, a group II PLA_2 present in the venom of *Bothrops asper* (Lomonte and Gutiérrez, 1989). The central well of a 1% agarose gel, dissolved in 0.12 M NaCl, 0.04 M sodium phosphate (PBS; pH 7.2) was filled with 30 μL of rabbit antiserum to myotoxin II, while 30 μL of two dilutions of Pllans-I and Pllans-II (0.25 and 0.5 μg/μL) were dispensed in the peripheral wells. Purified myotoxin II was utilized as a positive control for immunoprecipitation. After 24 h of diffusion at room temperature, precipitation lines were recorded using a ChemiDoc® imager with ImageLab® software (Bio–Rad).

2.7. Toxicological profiling

Animal experiments were performed in CD–1 mice of either sex, following protocols approved by the Institutional Committee for the Use and Care of Animals (CICUA), University of Costa Rica.

2.7.1. Intracerebro-ventricular lethal activity

Groups of three mice (16–18 g body weight) were injected with 5 μg of Pllans-I or Pllans-II, respectively, dissolved in 5 μL of PBS, by intracerebro-ventricular route, using a Hamilton microsyringe. This dose was selected on the basis of previous studies with related