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CoaTx-II, a new dimeric Lys49 phospholipase A_2 from *Crotalus oreganus abyssus* snake venom with bactericidal potential: Insights into its structure and biological roles

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ABSTRACT

Snake venoms are rich and intriguing sources of biologically-active molecules that act on target cells, modulating a diversity of physiological functions and presenting promising pharmacological applications. Lys49 phospholipase A_2 is one of the multifunctional proteins present in these complex secretions and, although catalytically inactive, has a variety of biological activities, including cytotoxic, antibacterial, inflammatory, antifungal activities. Herein, a Lys49 phospholipase A_2, denominated CoaTx-II from *Crotalus oreganus abyssus*, was purified and structurally and pharmacologically characterized. CoaTx-II was isolated with a high degree of purity by a combination of two chromatographic steps; molecular exclusion and reversed-phase high performance liquid chromatography. This toxin is dimeric with a mass of 13868.2 Da (monomeric form), as determined by mass spectrometry. CoaTx-II is rich in Arg and Lys residues and displays high identity with other Lys49 PLA_2 homologues, which have high isoelectric points. The structural model of dimeric CoaTx-II shows that the toxin is non-covalently stabilized. Despite its enzymatic inactivity, *in vivo* CoaTx-II caused local muscular damage, characterized by increased plasma creatine kinase and confirmed by histological alterations, in addition to an inflammatory activity, as demonstrated by mice paw edema induction and pro-inflammatory cytokine IL-6 elevation. CoaTx-II also presents antibacterial activity against gram negative (*Pseudomonas aeruginosa* 31NM, *Escherichia coli* ATCC 25922) and positive (*Staphyloccocus aureus* BEC9393 and Rib1) bacteria. Therefore, data show that this newly purified toxin plays a central role in mediating the degenerative events associated with envenomation, in addition to demonstrating antibacterial properties, with potential for use in the development of strategies for antivenom therapy and combating antibiotic-resistant bacteria.

1. Introduction

Antimicrobial resistance is one of most serious and alarming public health problems, spreading faster than the discovery and introduction of new therapeutic molecules into clinical practice. The prevalence of this bacterial resistance and the side effects of conventional antibiotics have prompted the search and development of more efficient strategies, as well as powerful and safe compounds to aid in the fight against infectious diseases (Ling et al., 2015; Sudharshan and Dhananjaya, 2015). As such, proteins and peptides from snake venoms represent valuable and attractive sources of bioactive molecules against Gram-positive and Gram-negative bacteria (Oliveira-Junior et al., 2013; Corrêa et al., 2016).

Snake venoms constitute a complex and natural library of proteins and peptides that present valuable structural and functional diversity (Calvete et al., 2007; McCleary and Kini, 2013). A deeper

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understanding of the structural features, biological effects and biotechnological/medical applications of these compounds is instrumental in interpreting symptoms of snakebite envenomation and the identification and development of novel therapeutic agents (Angulo and Lomonte, 2009; Brahma et al., 2015). Among these numerous proteins and peptides, phospholipases A_2 (PLA_2s) are one of the most studied and well described group of proteins; these proteins generally present promising antibacterial activity (Lomonte et al., 2009; Gutierrez and Lomonte, 2013).

PLA_2s are stable, conserved, multifunctional, of low molecular weight and contain disulfide-rich enzymes that are involved in lipid metabolism and membrane remodeling (Van and De Haas, 1963; Schaloske and Dennis, 2006). This group of toxins shares significant sequential and structural similarity, however it exhibits an intriguing array of biological functions, which should be explored and studied from a toxicological, biotechnological and biomedical point of view (dos Santos et al., 2011; Carvalho et al., 2013; Gutierrez and Lomonte, 2013).

There are several isoforms of PLA_2, and these can be isolated from different sources. The most important known PLA_2s isolated from snakes venoms are: 1) The catalytically-active variant (Asp49 PLA_2), presenting a conserved aspartic acid residue at position 49, located in a catalytic center (key residue for binding essential Ca^{2+}), and 2) the catalytically-inactive homologues (Lys49 PLA_2), which lose the ability to cleave phospholipids (Ward et al., 2002; Lomonte et al., 2003a). Despite their catalytic inactivity, interestingly, Lys PLA_2s are biologically active and are involved in pathophysiology envenomation by snakes. These PLA_2s have been studied as structural models for the drug design of molecules with antitumor and antibacterial activities (Lomonte and Rangel, 2012; Azevedo et al., 2016). Therefore, the identification of new Lys PLA_2s from snake venom may be important for certain areas of health, such as serum therapy, treatment of cancer and infectious diseases (Lomonte et al., 2009; McCleary and Kini, 2013).

The *Crotalus oreganus abyssus* (Grand Canyon rattlesnake) is a venomous pitviper belonging to the Viperidae family and, like several other pitviper subspecies, biological and structural characterization of its venom composition is incomplete (Da Silva et al., 2011; Martins et al., 2014; Almeida et al., 2016). This pitviper subspecies also belongs to a group of snakes, commonly referred to as the Western Rattlesnake complex, and is considered a powerful tool for understanding venom profiles, variation and evolution, as described by Mackessy (2010). Given the molecular proprieties of Lys49 PLA_2s and the incomplete characterization of compounds from *C. o. abyssus* snake venom, we herein isolated and characterized a catalytically-inactive Lys49 PLA_2 (CoaTx-II) with antibacterial activity, in order to obtain information regarding its structure, biological roles and pharmacological potential.

2. Materials and methods

2.1. Venom and reagents

Crotalus oreganus abyssus snake venom was obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University - Kingsville (Kingsville, TX, USA) and stored at −20 °C. All reagents were of analytical or sequencing grade.

2.2. Animals

Male Swiss mice (18–20 g; 7–8 weeks) used in assays were kept under specific pathogen-free conditions, a 12:12 h light–dark cycle and received water and food *ad libitum*. Mice were housed in laminar-flow cages maintained at a temperature of 22 ± 2 °C and a relative humidity of 50–60%. The animal experiments were carried out according to the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA) and approved by the University's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP).

2.3. Isolation of CoaTx-II from Crotalus oreganus abyssus snake venom

Lys49 PLA_2 was purified from the venom of *C. o. abyssus* using two chromatographic steps. The snake venom (100 mg) was fractioned by chromatography on a G75-Sephadex column, previously equilibrated with 0.20 M ammonium bicarbonate buffer (AMBIC - pH 7.8). Initially, the venom was dissolved in AMBIC 1M, homogenized and centrifuged at 9000*g* for 3 min. The supernatant obtained was loaded on a Sephadex G75 column (1.5 cm × 90 cm, Amersham Pharmacia Biotech), previously equilibrated with 80 mM ammonium bicarbonate buffer, pH 7.8, under a flow rate of 21 ml/h. Three peaks (Coa-I, Coa-II and Coa-III – Fig. 1A) were obtained and these were lyophilized and stored frozen at −20 °C. Fraction II (Coa-II peak – Fig 1A) was selected for the next purification step due to the presence of proteins with a molecular mass compatible with that of the protein of interest (Lys49 PLA_2).

For the HPLC separation, the Coa-II peak (pool of protein with molecular mass similar to Lys49 PLA_2s) was dissolved in 120 μl 0.1% (v/v) trifluoroacetic acid (solvent A) and 80 μl 1 M ammonium bicarbonate. The resulting solution was centrifuged at 9000*g* for 3 min and the supernatant was further submitted to a reverse-phase HPLC (model 2010, Shimadzu, Japan) using an analytical C18 column (μ-Bondapak, 0.78 × 30 cm). The C18 column was equilibrated in solvent A and the proteins eluted with a linear gradient from 0 to 100% of solvent B (66% acetonitrile, 0.1% TFA), at a flow rate of 1 ml/min, for 60 min. The elution profile of both chromatographic steps was monitored at 280 nm, and the collected fractions of 2 ml were lyophilized and conserved at −20 °C. All groups of peaks obtained were assayed for PLA_2 activity, and those without enzymatic activity were selected and tested for myotoxicity activity. The peak with the highest myotoxic activity, shown in Fig. 1B, was named CoaTx-II and chosen for this study. This peak was re-chromatographed to evaluate its purity, under the same conditions as described above (reverse-phase chromatography) and further functionally and structurally characterized.

2.4. N-terminal sequencing

The N-terminal sequence of 50 μg CoaTx-I provided by Martins et al. (2014) and CoaTx-II (purified in this work) were obtained directly by automated Edman sequencing on a PPSQ-33A (Shimadzu) automatic sequencer (Edman, 1950).

2.5. Phospholipase A_2 activity

Two methods using different substrates, a non-micellar (4-nitro-3-octanoyloxy benzoic acid - NOBA) and a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol - HPGP), were used to determine enzymatic activity.

2.5.1. Determination of PLA_2 activity using a non-micellar substrate (NOBA)

PLA_2 activity was determined using the assay method described by Holzer and Mackessy (1996), adapted for 96-well microplates according to Calgarotto et al. (2008). Enzymatic activity was expressed as the initial velocity of the reaction (Vo) and calculated based on absorbance after 20 min at 425 nm using a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA). PLA_2