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Biochemical and functional studies of ColTx-I, a new myotoxic phospholipase A_2 isolated from *Crotalus oreganus lutosus* (Great Basin rattlesnake) snake venom

J.R. Almeida [a, d, e], L.M. Resende [a], A.G. Silva [c], R.I.M.A. Ribeiro [c], R.G. Stábeli [b, e], A.M. Soares [b, e], L.A. Calderon [b, e], S. Marangoni [a], S.L. Da Silva [d, e, *]

[a] Department of Biochemistry and Tissue Biology, Institute of Biology, Campinas State University (UNICAMP), Campinas, SP, Brazil
[b] Oswaldo Cruz Fundation (FIOCRUZ), CEBio, Fiocruz Rondônia and Federal University of Rondônia, Porto Velho, RO, Brazil
[c] CCO, Universidade Federal de São João del Rei, Divinópolis, MG, Brazil
[d] IKIAM – Universidad Regional Amazónica, Tena, Napo, Ecuador
[e] International Network of Ecuadorian Snakes Venoms Studies (RIEVSE), Ecuador

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ABSTRACT

Commonly, phospholipases A_2 (PLA_2s) play key roles in the pathogenesis of the local tissue damage characteristic of crotaline and viperine snake envenomations. *Crotalus oreganus lutosus* snake venom has not been extensively studied; therefore, the characterization of its components represents a valuable biotechnological tool for studying pathophysiological processes of envenoming and for gaining a deeper understanding of its biological effects. In this study, for the first time, a basic PLA_2 myotoxin, ColTx-I, was purified from *C. o. lutosus* through two chromatographic steps. ColTx-I is monomeric with calculated molecular mass weight (Mw) of 14,145 Da and a primary structure closely related to basic PLA_2s from viperid venoms. The pure enzyme has a specific activity of 15.87 ± 0.65 nmol/min/mg at optimal conditions (pH 8.0 and 37 °C). ColTx-I activity was found to be dependent on Ca^{2+}, as its substitution by other ionic species as well as the addition of chelating agents significantly reduced its phospholipase activity. *In vivo*, ColTx-I triggered dose-dependent inflammatory responses, measured using the paw edema model, with an increase in IL-6 levels, systemic and local myotoxicity, characterized by elevated plasma creatine kinase activity. ColTx-I induced a complex series of degenerative events associated with edema, inflammatory infiltrate and skeletal muscle necrosis. These biochemical and functional results suggest that ColTx-I, a myotoxic and inflammatory mediator, plays a relevant role in *C. o. lutosus* envenomation. Thus, detailed studies on its mechanism of action, such as evaluating the synergism between ColTx-I and other venom components may reveal targets for the development of more specific and effective therapies.

1. Introduction

Snake venoms are libraries of pharmacologically active substances that are naturally designed to act on the specific targets of the prey or victim (Calvete, 2009; Da Silva et al., 2011), which proteins compose more than 90% of the venom dry weight, with phospholipases A_2 (PLA_2 E.C.3.1.1.4) generally being one of the most abundant (Calderon et al., 2014; Calvete, 2009). Secreted PLA_2s are stable, ubiquitous, versatile, relatively small (~14 kDa), calcium-dependent and disulfide-rich enzymes that catalyze the membrane phospholipids' hydrolysis at *sn*-2 position, generating lysophospholipids and biologically active fatty acids (Schaloske and Dennis, 2006; Van and De Haas, 1963) being usually responsible by muscle damage and playing other important toxic and digestive roles in prey capture and immobilization (Gutierrez and Ownby, 2003; Schaloske and Dennis, 2006).

Despite sharing high identity and an apparent molecular simplicity, these enzymes exert an exciting variety of pharmacological effects, which include neurotoxicity, cytotoxicity, edema-

* Corresponding author. IKIAM – Universidad Regional Amazónica, Km 7, Via Muyuna, Tena, Napo, Ecuador.
E-mail addresses: biomol2@hotmail.com, slsilva@pq.cnpq.br (S.L. Da Silva).

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forming activity, bactericidal activity, myotoxicity, among others (Castillo et al., 2012; Gutierrez and Lomonte, 2013; Kini, 2003; Montecucco et al., 2008; Samel et al., 2013). The characterization of this functionally versatile group of toxins awakens medical-scientific interest due to the number of potential applications for understanding envenomation, making clinical diagnosis, developing therapeutic strategies and using these toxins as molecular and biotechnological tools in pathophysiological, taxonomic, ecological studies as well as parameters for hemostatic assays (Calderon et al., 2014; Carvalho et al., 2013; Cecilio et al., 2013; Murakami and Lambeau, 2013).

Myotoxic PLA_2s are involved in local and systemic skeletal muscle degeneration, a common pathophysiological event in viperid snakebite envenomation (Gutierrez and Ownby, 2003; Hernandez et al., 2011). These events are accompanied by an acute inflammatory reaction associated with swelling, pain and the recruitment of macrophages and polymorphonuclear leukocytes (Chacur et al., 2003; Gutierrez et al., 2009; Mamede et al., 2013). Activated leukocytes secrete a wide range of chemical mediators such as IL-1, IL-6 and IL-8, which along with the biological effects induced by the toxin, may contribute to the evolution of local myotoxicity and regeneration (Oliveira et al., 2009; Voronov et al., 1999). Local myonecrosis is a difficult medical challenge owing to its rapid development and poor tissue regeneration characterized by dysfunction and tissue loss, which have negative social and psychological consequences (Hernandez et al., 2011; Tonello et al., 2012). The effects triggered by PLA_2s occur upon interaction with lipids or proteins, altering the plasma membrane integrity by catalytically dependent or independent mechanisms that consequently mediate a number of associated events, such as: calcium influx, depolarization, loss of ionic gradients and efflux of cytosolic molecules (Kini, 2003; Montecucco et al., 2008).

The biochemical and functional characteristics of several crotalic and bothropic phospholipases have been described (Damico et al., 2008; Gutierrez and Lomonte, 2013; Higuchi et al., 2007a); however, no information is available regarding the toxic activity of purified proteins from *Crotalus oreganus lutosus,* (Great Basin rattlesnake), a venomous pitviper subspecies belonging to the Viperidae family and Western Rattlesnake complex, that is found in the Great Basin region of the United States. The Western Rattlesnakes occur across a broad geographical area and disjunct populations occur in much of the mountainous west, resulting in potential disruption of gene flow and local variation (Ashton and De Queiroz, 2001; Mackessy, 2010). Due to this, these venomous pitviper subspecies are considered an ideal species group to understand questions of what differences in venom composition occur, why these differences evolve and how composition affects the biological role of venom, as described by Mackessy (2010). In the present study, a myotoxic PLA_2 (ColTx-I) from *C. o. lutosus* was isolated and characterized in order to obtain insights into its primary structure, biological effects, induction of morphological changes of skeletal muscle and its relevance to the pathophysiology of envenomations produced by this species.

2. Material and methods

2.1. Venom and reagents

C. o. lutosus snake venom was obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University – Kingsville (Kingsville, TX, USA). All reagents were of analytical or sequencing grade.

2.2. Animals

The male Swiss mice (18–20 g) used in this study were maintained under specific pathogen-free conditions and had free access to food and water. The animals were housed in laminar-flow cages maintained at a temperature of 22 ± 2 °C and a relative humidity of 50–60%, with a 12:12 h light–dark cycle. Animal procedures were performed in accordance with 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), number 3245-1.

2.3. Isolation of ColTx-I from C. o. lutosus snake venom

One hundred mg of *C. o. lutosus* snake venom were dissolved in 1 ml of ammonium bicarbonate buffer (0.2 M, pH 7.8), homogenized until reaching complete dissolution and centrifuged at 5000 × g (5 min). The supernatant obtained was loaded onto a Sephadex G75 column (1.5 cm × 90 cm, Amersham Pharmacia Biotech), previously equilibrated with ammonium bicarbonate buffer (50 mM, pH 7.8) and run at a flow rate of 0.2 ml/min. Three peaks were obtained (Col-I, Col-II and Col-III – Fig. 1A), which were lyophilized and stored at − 20 °C. Subsequently, 5 mg from the lyophilized fraction of Col-II, which showed PLA_2 activity (Fig. 1A), were dissolved in 120 μl of 0.1% (v/v) trifluoroacetic acid (solvent A) and 80 μl of 1 M ammonium bicarbonate. The resulting solution was centrifuged at 5000 × g (2 min) and the supernatant was submitted to reverse-phase chromatography (Waters 991-PDA system) using an analytical C18 column (Supelco, 250 mm × 4.6 mm). The C18 column was equilibrated in solvent A and the elution proceeded with a concentration gradient from 0 to 100% of solvent B (66% acetonitrile, 0.1% TFA), at a flow rate of 1 ml/min (60 min). The elution profile of both chromatography analyses was monitored at 280 nm, and the collected fractions of 3 ml were lyophilized and stored at −20 °C. All peaks (Fig. 1B) were tested for PLA_2 activity and the active peak was named ColTx-I (Fig. 1B). This peak was re-chromatographed (Fig. 1C) to evaluate its purity, under the same conditions as described above (RP-HPLC) and was further biochemically and functionally characterized.

2.4. Electrophoresis

The relative molecular mass of ColTx-I and homogeneity was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described by Laemmli (1970). Samples were heated at 100 °C (5 min) and then run under reducing and non-reducing conditions (Vargas et al., 2012; Vieira et al., 2013).

2.5. Phospholipase A_2 activity

PLA_2 activity was measured using a non-micelar substrate, 4-nitro-3-octanoyloxy benzoic acid (NOBA), according to the assay method described by Cho and Kezdy (1991) and Holzer and Mackessy (1996) adapted for 96-well plates (Calgarotto et al., 2008; Damico et al., 2005). The PLA_2 activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase in absorbance at 425 nm after 20 min. The effects of pH, temperature, substrate concentration and divalent ions on PLA_2 activity were also investigated.

2.6. MALDI-TOF mass spectrometry analysis of ColTx-I

The molecular mass of ColTx-I was determined by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)