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Biochemical and biological characterization of a PLA_2 from crotoxin complex of *Crotalus durissus cumanensis*

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ABSTRACT

A new PLA_2 (Cdcum6) from crotoxin complex of Colombian *Crotalus durissus cumanensis* rattlesnake was purified using molecular exclusion chromatography and RP-HPLC. The molecular mass of Cdcum6 was determined by SDS-PAGE ~14 KDa and confirmed by MALDI-TOF (14321.98 Da). The enzyme showed K_m 6.0 mM, V_{max} 3.44 nmol/min, optimum pH was 8.0 and temperature was between 30 and 45 °C, and it had a strict requirement of Ca^{2+} for its activity. The N-terminal sequence of PLA_2 was SLVQF EKMIK EVAGK NGVPWY. Comparison of amino acid sequence data with other PLA_2 from South American *Crotalus durissus* rattlesnakes showed that Cdcum6 shares the highest sequence identity with Cdr13 an isoform PLA_2 from *Crotalus durissus ruruima*, nevertheless, Cdcum6 showed high content of basic and hydrophobic amino acids. In mice, Cdcum6 presented higher LD_{50} than crotoxin complex from *C. d. cumanensis*. Additionally, Cdcum6 induced a conspicuous local myotoxic effect and moderate footpad edema; in vitro, it was antigoagulant in doses as low as 0.5 μg/ml, and it was not cytotoxic on myoblast but Cdcum6 was able to lyse myotubes.

1. Introduction

Phospholipases A_2 (PLA_2; EC 3.1.1.4) are calcium-dependent enzymes that hydrolyze glycerophospholipids at the *sn*-2 position of the glycerol backbone, releasing lysophospholipids and fatty acids These enzymes are found in a wide variety of biological fluids and cells as well as in arthropod and snakes venom (Six and Dennis, 2000). PLA_2s from snake venoms display a variety of biological effects, such as neurotoxicity, myotoxicity, cardiotoxicity, platelet aggregation induction or inhibition, edema, hemolysis, anti-coagulation and hypotension (Kini, 2003). PLA_2 receptors classified as types M and N (Lambeau and Lazdunski, 1999), which have been identified in various types of cells, including vascular smooth muscle cells, platelets, neutrophils, chondrocytes, fibroblasts, hepatocytes and mesangial cells, as well as in brain, lung and skeletal muscle (Kudo and Murakami, 2002) could explain many of the effects caused by these toxins, although this remains to be conclusively demonstrated (Gutiérrez and Ownby, 2003).

In Colombia are reported about 3000 snakebites every year, one percent of which are inflicted by *Crotalus durissus cumanensis* rattlesnake. The envenoming caused by this snake is characterized by neurotoxicity, systemic myotoxicity, mild edema and acute renal failure (Otero et al., 2001). These activities are attributed primarily to crotoxin (CTX), the principal toxin of snakes belong to genus *Crotalus durissus* from South America.

Crotoxin is a heterodimeric complex that consists of a basic PLA_2 known as CB and an acidic, nonenzymatic component known as crotapotin (Hendon and Fraenkel-Conrat, 1971) that increases the pharmacological activity of

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PLA_2 by acting as chaperone protein for the enzyme, preventing the binding of PLA_2 to non-specific sites (Breithaupt, 1976; Habermann and Breithaupt, 1978; Choumet et al., 1996).

This toxin had been isolated from subspecies such as *Crotalus durissus terrificus* (Slotta and Fraenkel-Conrat, 1938; Toyama et al., 2003; Hernandez-Oliveira et al., 2005), *Curissus durissus collilineatus* (Ponce-Soto et al., 2002; Rangel-Santos et al., 2004), *Crotalus durissus cascavella* (Beghini et al., 2000; Beghini et al., 2004a,b; Rangel-Santos et al., 2004) and *Crotalus durissus ruruima* (Ponce-Soto et al., 2007a), and recently one fraction of *C. d. cumanensis* from Venezuela with characteristic of CB was isolated (Hernández et al., 2007). In this report, we describe the purification, biochemical, biological and preliminary structural characterization of a PLA_2 (CB) from Colombian *C. d. cumanensis* rattlesnake.

2. Materials and methods

2.1. Venom, chemicals and reagents

The venom was obtained by manual extraction of four specimens from Meta, south east region in Colombian, and maintained in captivity at the Serpentarium of the Universidad de Antioquia (Medellín, Colombia). Venoms were centrifuged at 800*g* for 15 min, and supernatants were lyophilized and stored at −20 °C until used. All chemicals and reagents used in this work were analytical, HPLC or sequencing grade.

2.2. Animals

Swiss Webster mice, 18–20 g body weight, were used for the in vivo assays. All experiments were conduced in accordance with guidelines of the Universidad de Antioquia Ethics Committee (Medellín, Colombia).

2.3. Isolation and purification of PLA_2

The PLA_2 was purified from *C. d. cumanensis* venom by molecular exclusion chromatography and reverse phase HPLC (RP-HPLC). Fifty milligram of venom were dissolved in 0.25 M ammonium bicarbonate (pH: 7.9) and applied to a column (1.8 cm × 120 cm) of Sephadex G-75, which had been pre-equilibrated with this same buffer. The proteins were eluted at a flow rate of 0.5 ml/min and elution profile was monitored at 280 nm. The fractions corresponding to main peaks were pooled, lyophilized and stored at −20 °C. Five milligrams of the fraction containing PLA_2 activity were dissolved in 0.25 M ammonium bicarbonate and applied to C-18 column. Proteins were eluted with a linear gradient (0–100%) of 66.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm and fractions were manually collected, lyophilized and stored at −20 °C.

2.4. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kézdy (1991) and Holzer and Mackessy (1996), modified for 96-well plates (Ponce-Soto et al., 2002). The standard assay mixture contained 200 μl of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μl of substrate (4-nitro-3-octanoyloxy-benzoic acid), 20 μl of water and 20 μl of PLA_2 in a final volume of 260 μl. After the addition of PLA_2 (20 μg), the mixture was incubated 40 min at 37 °C, with the absorbance being read at 10 min intervals. The optimum pH and temperature of the PLA_2 were determined by incubating the enzyme in buffers (10 mM citrate, 10 mM phosphate, 10 mM Tris, and glycine 10 mM) of different pHs (4.0–9.0) and in 10 mM Tris–HCl, pH 8.0, at different temperatures (25–45 °C). The effect of substrate concentration on enzyme activity was determined by measuring the absorbance increase after 20 min incubation in 10 mM Tris–HCl, pH 8.0, at 37 °C. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase in absorbance after 20 min. All assays were conducted in triplicate, and the absorbances at 425 nm were measured using a SpectraMax 340 multiwell plate reader (Molecular Devices).

2.5. Amino acid analysis

Amino acid analysis was performed on a Pico-Tag amino acid analyzer (Water System) as described by Heinrikson and Meredith (1984). One nanomole of PLA_2 was hydrolyzed in 6 M HCl 1% phenol at 106 °C for 24 h. The hydrolysates were reacted with 20 μl of fresh derivatization solution (methanol:triethylamine:water:phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature. After pre-column derivatization, PTC amino acids were identified on a reverse phase HPLC column by comparing their retention times to those of standard PTC amino acids (Pierce). Cysteine residues were quantified as cysteic acid.

2.6. Protein reduction, carboxymethylation and N-terminal sequencing procedure

One milligram of purified PLA_2 was dissolved in 6 M guanidine chloride (Merck) containing 0.4 M Tris–HCl and 2 mM EDTA (pH 8.1); the solution was reduced with DTT and then carboxymethylated with ^{14}C iodoacetic acid. The sequencing of the N-terminal was made using the reduced and carboxymethylated protein in a direct form. The sequence was performed in the Procise f gas–liquid protein automatic sequencer. The phenylthyoidantoin amino acid (PTH) derivatives from the protein were identified by comparing their retention times with those of the 20 phenylthyoidantoin amino acid standards, using an Applied Biosystems model 450 microgradient PTH-analyzer.

2.7. MALDI-TOF mass spectrometric (MS) analysis

The molecular mass of PLA_2 was analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). One microliter of sample on 0.1% TFA was mixed with 2 μL of the matrix (3,5-dimethoxy-4-hydroxycinnamic acid). The matrix was prepared with 30% acetonitrile and 0.1% v/v TFA and its mass analyzed under the following