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Structural and functional characterization of brazilitoxins II and III (BbTX-II and -III), two myotoxins from the venom of *Bothrops brazili* snake

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ABSTRACT

We report the purification and biochemical/pharmacological characterization of two myotoxic PLA_2 (BbTX-II K49 PLA_2 homologue and BbTX-III PLA_2) from *Bothrops brazili* venom. Both were purified by a single chromatographic step on reverse phase HPLC, showing $M_r \sim 14$ kDa for both myotoxins, showing high content of hydrophobic and basic amino acids as well as 14 half-cysteine residues. The BbTX-II K49 PLA_2 homologue and BbTX-III PLA_2, had a sequence of 121 amino acid residues. BbTX-II: SLFELGKMILQETGKN PAKSYGAYGCYCGVLGRGKPKDATDRCCYVHKCCYKLTGCDNKKKDRYSYSWKDKTIVCGENNPCL KELCECDKAVAICLRENLNTYNKKYRYHLKPLCKKADAC with p*I* value 8.73. BbTX-III: SLWEW GQMILKETGKNPFPYYGAYGCYCGWGGRRKPKDATDRCCFVHDCCRYKKLTGCPKTNDRYSYSRLD YTIVCGEDDPCKEICECDKAAAVCFRENLRTYNKKYMAHLRVLCKKDKPC with a p*I* value of 8.46. BbTX-III presented PLA_2 activity in the presence of a synthetic substrate and showed a minimum sigmoidal behavior, reaching its maximal activity at pH 8.0 and 35–45 °C. Maximum PLA_2 activity required Ca^{2+}. *In vitro*, BbTX-II K49 PLA_2 homologue and BbTX-III PLA_2 caused a blockade of the neuromuscular transmission in young chick biventer cervicis preparations in a similar way to other *Bothrops* species. In mice, BbTX-II K49 PLA_2 homologue and BbTX-III PLA_2 induces myonecrosis and edema-forming activity. All these biological effects induced by the BbTX-II K49 PLA_2 homologue, occur in the absence of a measurable PLA_2 activity *in vitro*, further supporting the concept of catalytic independent mechanisms exerted by Lys49 proteins.

1. Introduction

Phospholipase A_2 (PLA_2) enzymes hydrolyze glycerophospholipids at the sn-2 position of the glycerol backbone releasing lysophospholipids and fatty acids. This reaction is of particular importance if the esterified fatty acid is the arachidonic acid for its importance as substrate for eicosanoids by action of cyclooxygenases and lipoxygenases (prostaglandins and leukotrienes, respectively).

They occur ubiquitously in nature as both intracellular and extracellular forms and hydrolyze various phospholipids. They are the most studied among all phospholipases due to their pivotal role in various biological activities. Mammalian PLA_2 enzymes play important role in fertilization (Fry et al., 1992), cell proliferation (Arita et al., 1991), smooth muscle contraction (Nakajima et al., 1992; Sommers et al., 1992), hyper sensitization and chronic

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inflammatory diseases (Vadas and Pruzanski, 1986; Vadas et al., 1993) and capacity in modulate neuronal function by several mechanisms. Arachidonic acid modulates alterations of membrane fluidity and polarization states, activation of protein kinase C (PKC), stimulation of calcium ion release, activation of several important modulating enzymes and regulation of gene transcription (Farooqui and Horrocks, 2004).

Snake venom PLA_2 are secreted enzymes belonging to groups I and II (Six and Dennis, 2000). PLA_2 from the viperidae family are placed in class II and are subdivided into two main groups: D49 PLA_2, which displays an Asp residue at the position 49, with relatively high catalytic activity upon artificial substrates and K49 PLA_2 homologue, showing a Lys residue at the position 49, with low or no catalytic activity (Arni and Ward, 1996; Kini, 2003; Ownby et al., 1999; Lomonte et al., 2003). These enzymes show significant similarities in their tridimensional structures, although exhibiting different pharmacological properties, what makes them interesting targets for many researches (Ownby et al., 1999; Lomonte et al., 2003; Gutiérrez and Lomonte, 1995; Ponce-Soto et al., 2007a; Gutiérrez et al., 2008).

Peru has a rich and diverse herpetofauna that includes venomous snake species of the families Elapidae (16 species of *Micrurus* and the pelagic sea snake *Pelamis platurus*) and Viperidae (15 species) (Campbell and Lamar, 2004). Snakebite envenomations represent a public health problem in this country. The vast majority of snakebites in Peru are inflicted by species of the genus *Bothrops* (family Viperidae) (Zavaleta and Salas, 1996). *B. atrox*, *B. brazili*, and *B. bilineatus* are distributed in the tropical rainforests located in the eastern part of the country, whereas *B. barnetti* and *B. pictus* are found in the western dry coastal regions (Campbell and Lamar, 2004; Zavaleta and Salas, 1996; Fan and Cardoso, 1995).

Bothrops brazili is commonly known as "Jergón Shushupe". It is a poisonous snake that belongs to the family Viperidae and it is distributed in South America, being their presence reported in Brazil, Colombia, Ecuador, Guyana, Peru, Suriname and French Guiana (Campbell and Lamar, 2004).

In this work we have isolated and characterized two novel myotoxic PLA_2 from *Bothrops brazili* venom, a K49 (BbTX-II) homologue and a D49 (BbTX-III), with the following pharmacological activities: *in vitro* neurotoxicity on young chicken biventer cervicis preparations, inflammatory activities in the footpad of mice, *in vivo* myotoxicity and *in vitro* cytotoxicity.

2. Material and methods

2.1. Reverse phase HPLC

The brazilitoxins II and III, myotoxins (BbTX-II and -III) from *Bothrops brazili* venom was purified by reverse phase HPLC according to method described by Ponce-Soto et al. (2007b). Briefly, 5 mg of whole venom was dissolved in 250 μl of buffer A (0.1% TFA) and centrifuged at 4500 × *g* and the supernatant was then applied on the analytical reverse phase HPLC μ-Bondapack C-18, previously equilibrated with buffer A for 15 min. The elution of the protein was then conducted using a linear gradient of buffer B (66.5% Acetonitrile in buffer A) and the chromatographic run was monitored at 280 nm of absorbance. After elution the fraction was lyophilized and stored at 40 °C.

2.2. Electrophoresis

Tricine SDS-PAGE in a discontinuous gel and buffer system was used to estimate the molecular mass of the proteins, under reducing and non-reducing conditions (Schagger and Von Jagow, 1987).

2.3. MALDI-TOF mass spectrometric analysis (MS)

The molecular mass of isolated brazilitoxins II and III (BbTX-II and III), myotoxins from *Bothrops brazili* venom was analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF apparatus (Applied Biosystems, Foster City, CA, USA). 1 μl of sample in 0.1% TFA was mixed with 2 μl of the matrix sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid). The matrix was prepared with 30% acetonitrile and 0.1% TFA and its mass analyzed under the following conditions: accelerate voltage 25 kV, the laser fixed in 2890 μJ/com2, delay 300 ns, and linear analysis mode (Ponce-Soto et al., 2006).

2.4. PLA_2 activity

PLA_2 activity was measured using the assay described by Cho and Kezdy (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 $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μl of substrate (3 mM), 20 μl of water and 20 μl of PLA_2 (1 mg/ml) in a final volume of 260 μl. After adding of brazilitoxins II and III, respectively (20 μg), the mixture was incubated for up to 40 min at 37 °C, with the reading of absorbance at intervals of 10 min. The enzyme activity, expressed as the initial velocity of the reaction (V_o), was calculated based on the increase of absorbance after 20 min. The brazilitoxins II and III obtained from RP-HPLC on the C18 column was chosen by studying the kinetic parameters. The pH and optima temperature of the PLA_2 were determined by incubating the enzyme in four buffers of different pH values (4–10) and in Tris–HCl buffer, pH 8.0, at different temperatures, respectively. The effect of substrate concentration (10, 5, 2.5, 1.0, 0.5, 0.3, 0.2 and 0.1 mM) on enzyme activity was determined by measuring the increase of absorbance after 20 min of incubation in Tris–HCl buffer, pH 8.0, at 37 °C. All assays were done in triplicate and the absorbances at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA).

2.5. Amino acid analysis

Amino acid analysis was performed on a Pico-Tag Analyzer (Waters Systems) as described by Hendrickson and Meredith (1984). The brazilitoxins II and III (BbTX-II and III), sample (30 μg) was hydrolyzed at 105 °C for 24 h, in 6 M HCl (Pierce sequencing grade) containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μl of derivatization solution (ethanol:triethylamine:water:phenylisothiocyanate, 7:1:1:1,