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BmajPLA$_2$-II, a basic Lys49-phospholipase A$_2$ homologue from *Bothrops marajoensis* snake venom with parasiticidal potential

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ABSTRACT

Snake venoms contain various proteins, especially phospholipases A$_2$ (PLA$_2$s), which present potential applications in diverse areas of health and medicine. In this study, a new basic PLA$_2$ from *Bothrops marajoensis* with parasiticidal activity was purified and characterized biochemically and biologically. *B. marajoensis* venom was fractionated through cation exchange followed by reverse phase chromatographies. The isolated toxin, BmajPLA$_2$-II, was structurally characterized with MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight) mass spectrometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by two-dimensional electrophoresis, partial amino acid sequencing, an enzymatic activity assay, circular dichroism, and dynamic light scattering assays. These structural characterization tests presented BmajPLA$_2$-II as a basic Lys49 PLA$_2$ homologue, compatible with other basic snake venom PLA$_2$s (svPLA$_2$), with a tendency to form aggregations. The *in vitro* anti-parasitic potential of *B. marajoensis* venom and of BmajPLA$_2$-II was evaluated against *Leishmania infantum* promastigotes and *Trypanosoma cruzi* epimastigotes, showing significant activity at a concentration of 100 μg/mL. The venom and BmajPLA$_2$-II presented IC$_{50}$ of 0.14 ± 0.08 and 6.41 ± 0.64 μg/mL, respectively, against intraerythrocytic forms of *Plasmodium falciparum* with CC$_{50}$ cytotoxicity values against HepG2 cells of 43.64 ± 7.94 and >150 μg/mL, respectively. The biotechnological potential of these substances in relation to leishmaniasis, Chagas disease and malaria should be more deeply investigated.

1. Introduction

Bothropic venoms are biochemically complex, with varying protein components, including serine proteases [1], metalloproteinases [2], L-amino acid oxidases [3], C-type lectins [4], myotoxins [5], disintegrins [6], and group II PLA$_2$s [7], which have proven to be invaluable research tools and have provided leads for development of new therapies [8–12].

Group II PLA$_2$s can display a series of actions that result in toxic effects on victims such as myotoxicity [13], neurotoxicity [14], cytotoxicity [15,16] and genotoxicity [17–19]. Several studies focusing on the biological functions of PLA$_2$s have discovered essential information of their implication in diseases such as rheumatoid arthritis, inflammation and bone erosion [20], cancer [21,22], and neurological disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and epilepsy [23,24]

There are also several studies investigating the antiparasitic effects of PLA$_2$s against the parasites that cause leishmaniasis, Cha-

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gas disease and malaria. Castillo-Vigil et al. [25] proposed that the mechanism of action of the trypanocidal activity of *B. asper* venom occurred through a combination of necrosis and apoptosis, probably mainly caused by the actions of the venom's PLA_2s due to the cell membrane's integrity being compromised.

Adade et al. [26] demonstrated that the use of *para*-bromophenacyl bromide (*p*-BPB), a PLA_2 inhibitor, caused partial inhibition of the trypanocidal activity of *C. v. viridis* venom. In three studies, PLA_2s were isolated from *B. brazili, B. mattagrossensis* and *B. moojeni* venoms and their antiparasitic effects were checked against several different species of *Leishmania* [27–29]. Additionally, a non-catalytic PLA_2 isolated from *Bothrops pauloensis* venom, BnSP-7 inhibited the proliferation of *L. amazonensis* promastigotes and amastigotes and also caused morphological changes in the parasites [30].

Among the results published against the parasite that causes malaria, a PLA_2 isolated from *Crotalus adamanteus* snake venom blocked the *in vitro* development of ookinetes, forms of the parasites *Plasmodium falciparum* and *P. gallinaceum* in the mosquito intestine [31]. Guillaume et al. [32] showed the antimalarial effects of seven PLA_2s, some from snake venoms, against intraerythrocytic forms of *P. falciparum*. These studies are corroborated by other studies with snake venoms and their PLA_2s, as well as bee venoms and their PLA_2s against *Plasmodium* spp. [33–35].

The proteins from *B. marajoensis* venom are still poorly studied. These studies include the biological characterization of the venom [36]; an investigation of the antibacterial and antiparasitic effects of a Lys49 PLA_2 isolated from the venom, called Bmar [37]; the biological characterization of an Asp49 PLA_2, Bmaj-9 [38]; the neurotoxic, myotoxic and cytolytic activity of the Asp49 PLA_2s BmjeTX-I and BmjeTX-II [39]; and the renal and cardiovascular effects of the venom and an isolated PLA_2 [40].

Based on the information presented, this study aimed to biochemically and structurally characterize a new basic phospholipase A_2 from *Bothrops marajoensis* venom and evaluate its antiparasitic activity against the protozoa that cause Leishmaniasis, Chagas disease and malaria.

2. Material and methods

2.1. Venom and authorization

Bothrops marajoensis venom was obtained from a pool of adult specimens, acquired from the BioAgents Serpentarium (Batatais – SP) and donated by FMRP-USP, Brazil. The dehydrated venom was maintained refrigerated (4 °C) at the Amazonian Venom Bank at the Center for Studies of Biomolecules Applied to Health, CEBio-UNIR/FIOCRUZ-RO; authorization: CGEN/CNPq 010627/2011-1.

2.2. Biochemical characterization

2.2.1. Ion exchange chromatography

A 400 mg sample of *B. marajoensis* venom was solubilized in 2 mL of 50 mM ammonium bicarbonate (AMBIC), pH 8.0 (Solution A) and centrifuged at 7000 *xg* for 5 min. The supernatant was fractionated on a CM-Sepharose column (90 cm × 1.5 cm) equilibrated with AMBIC. A linear gradient of 0–100% 500 mM AMBIC, pH 8.0 (Solution B) was used for 600 min under a flow of 2.5 mL/minute, in an Akta Purifier (GE) chromatography system. Elution was monitored using 215 and 280 nm filters and the fractions were collected manually. The samples were lyophilized and stored in refrigerators at −20 °C [41].

2.2.2. Reverse phase chromatography

Fraction 11 from the ion exchange chromatography was diluted in 0.1% trifluoroacetic acid (TFA) (Solution A) and subjected to reverse phase chromatographic fractionation in a C18 column (25 cm × 0.45 cm – Discovery) previously equilibrated with solution A and eluted under a 0–70% gradient of ACN 99.9% (v/v) and 0.1% TFA (v/v) (Solution B) for 5 column volumes (one column volume contains 3.98 mL) under a flow rate of 1 mL/minute. Elution of the sample was monitored at 280 nm. Samples were manually collected, lyophilized and stored at −20 °C [41].

2.2.3. Electrophoresis in SDS-PAGE

SDS-PAGE, 12.5% (m/v), was performed in a discontinuous pH system under reducing conditions with adjustments [42]. 10 μg of toxin was mixed with 4% SDS (m/v), 0.2% Bromophenol Blue (m/v), 20% glycerol (v/v) in 100 mM Tris pH 6.8 and heated for 5 min at 90 °C. 0.2 M Dithiothreitol (DTT) was also added. After electrophoresis, the polyacrylamide gel was fixed in a solution of 40% methanol (v/v) and 7% acetic acid (v/v) for 30 min and then immersed in 0.08% Coomassie Brilliant Blue G-250® (m/v), 8.0% aluminum sulfate (m/v), 1.6% phosphoric acid (m/v) and 20.0% methanol (v/v) for 30 min. Excess dye was removed by soaking in a bleach solution containing 4% ethanol and 7% acetic acid (v/v) in water. Image scanner® (GE Healthcare Lifescience) was used to document the gel.

2.2.4. Molecular mass determination

The molecular mass of the isolated protein was determined by mass spectrometry [43] using MALDI equipment with two TOF analyzers (AXIMA TOF^2 Shimadzu Biotech), previously calibrated using Sigma molecular mass standards. An aliquot containing 1 μg of the sample was solubilized in 0.1% TFA (v/v), co-crystallized with a sinapinic acid saturated solution (ionization matrix) and applied on a metal plate. The instrument was operated in linear mode. Ions were generated by irradiation with a nitrogen laser with fixed wavelength on 337 nm. Signals were captured at 500 MHz and the obtained data was processed, using the software Launchpad.

2.2.5. Isoelectric point determination

The sample was solubilized in a hydration solution containing 7 M urea, 2 M thiourea, 2.0% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (m/v), 0.5% immobilized pH buffer gradient (IPG buffer®) (v/v), and 1.0% bromophenol blue (m/v); and applied on 7 cm polyacrylamide strips with a pH gradient from 3.0 to 10.0 immobilized non-linearly (Immobiline DryStrip®). The isoelectric focusing [44] was performed in an IPGphor III System® (GE Healthcare Life Science) according to the manufacturer's instructions. After isoelectric focusing, the strip containing the protein was placed on the upper portion of the 12.5% SDS-PAGE gel (m/v) to perform one-dimensional electrophoresis as described in 2.2.3. The gel image was obtained with the aid of an Image Scanner III®.

2.2.6. Circular dichroism

Circular dichroism experiments were performed in a Jasco J-815 spectropolarimeter (JASCO Inc., Tokyo, Japan). The solubilized toxin was isolated in 0.5 mg/mL of 50 mM AMBIC, pH 9. Measurements were recorded in the spectral range of 190–260 nm at 20 °C with an optical path length of 0.05 mm, a velocity of 100 nm/minute, a bandwidth of 2 nm and 1 s of response time. Twenty spectra were acquired, averaged and corrected for the buffer solution (baseline) and then normalized to residual molar ellipticity [θ]. Deconvolution of the CD spectra was performed with the Dichroweb on-line server [45] using the CDSSTR algorithm [46] with set Reference [4].

2.2.7. Dynamic light scattering

Dynamic light scattering was performed with the isolated toxin solubilized in 50 mM AMBIC, pH 9 at a concentration of 1 and 2 mg/mL. The sample was filtered using a PVDF membrane with