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Crystallization and preliminary X-ray diffraction studies of BmooPLA$_2$-I, a platelet-aggregation inhibitor and hypotensive phospholipase A$_2$ from *Bothrops moojeni* venom

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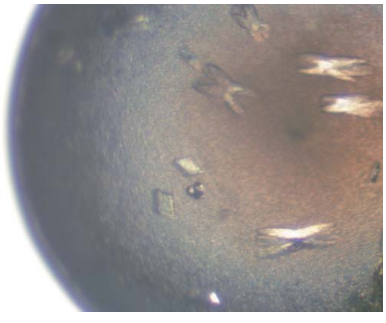
Phospholipases A$_2$ (PLA$_2$s) are enzymes that cause the liberation of fatty acids and lysophospholipids by the hydrolysis of membrane phospholipids. In addition to their catalytic action, a wide variety of pharmacological activities have been described for snake-venom PLA$_2$s. BmooPLA$_2$-I is an acidic, nontoxic and catalytic PLA$_2$ isolated from *Bothrops moojeni* snake venom which exhibits an inhibitory effect on platelet aggregation, an immediate decrease in blood pressure, inducing oedema at a low concentration, and an effective bactericidal effect. BmooPLA$_2$-I has been crystallized and X-ray diffraction data have been collected to 1.6 Å resolution using a synchrotron-radiation source. The crystals belonged to space group $C222_1$, with unit-cell parameters a = 39.7, b = 53.2, c = 89.2 Å. The molecular-replacement solution of BmooPLA$_2$-I indicated a monomeric conformation, which is in agreement with nondenaturing electrophoresis and dynamic light-scattering experiments. A comparative study of this enzyme with the acidic PLA$_2$ from *B. jararacussu* (BthA-I) and other toxic and nontoxic PLA$_2$s may provide important insights into the functional aspects of this class of proteins.

1. Introduction

Snake venoms comprise a complex mixture of pharmacological components that are able to affect several biological systems. Phospholipases A$_2$ (PLA$_2$s) are one of the main components of snake venoms and are small (∼14 kDa), stable, calcium-dependent and disulfide-rich molecules that are found in a great diversity of organisms and biological fluids (Schaloske & Dennis, 2006). This enzyme promotes the hydrolysis of the *sn*-2 acyl groups of membrane phospholipids (*sn*-3 glycerophospholipids), releasing free fatty acids and lysophospholipids such as arachidonic acid (Schaloske & Dennis, 2006; Van Deenen & De Haas, 1963).

PLA$_2$s have been classified into 15 different groups which can be divided into five types of enzyme: secreted PLA$_2$s, cytosolic PLA$_2$s, Ca^{2+}-independent PLA$_2$s, PAF acetylhydrolases and lysosomal PLA$_2$s (Schaloske & Dennis, 2006). The snake-venom PLA$_2$s from the Elapidae and Viperidae (pit viper) families, which are the most important when considering envenomation and its effects, belong to the IA and IIA/IIB groups, respectively. In addition to their catalytic action, a wide variety of pharmacological activities have been described for snake-venom PLA$_2$s, such as pre-synaptic (Chang *et al.*, 1977; Westerlund *et al.*, 1993) or post-synaptic (Bon *et al.*, 1979) neurotoxicity, myotoxicity (Gutiérrez *et al.*, 1991), cardiotoxicity (Fletcher *et al.*, 1981) and bactericidal (Páramo *et al.*, 1998), platelet-aggregation inhibition (Yuan *et al.*, 1993), oedema-forming (Lloret & Moreno, 1993), haemolytic (Condrea *et al.*, 1981), anti-coagulative (Rosenberg *et al.*, 1983), convulsive (Fletcher *et al.*, 1980) and hypotensive (Huang, 1984) effects. Some of these activities are correlated with the enzymatic activity, while others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2004). Despite the number of data that are available, the mechanisms involved in these biological and pharmacological activities remain poorly known. Envenomation resulting from snake bites is an important public health problem in rural areas of tropical and subtropical countries in



Asia, Africa, Oceania and Latin America and is considered as a neglected tropical disease by the World Health Organization (WHO). Recent statistics from the WHO estimate that at least 421 000 envenomations and 20 000 deaths occur worldwide from snakebites each year, but warn that these figures may be as high as 1 841 000 envenomations and 94 000 deaths (World Health Organization, 2010). Despite the majority of deaths from snakebites occurring in South and South East Asia and sub-Saharan Africa (Kasturiratne *et al.*, 2008), these accidents are also an important health problem in Latin America as they may cause permanent tissue loss and amputation of the affected limb (Gutiérrez & Lomonte, 1995). Snakebites from snakes of the *Bothrops* genus are responsible for more than 85% of all ophidian accidents reported in Latin America (Fundação Nacional de Saúde, 2001; de Oliveira, 2009) and display drastic local tissue damage among other biological effects (Gutiérrez & Lomonte, 1995).

Many noncatalytic homologues of PLA_2s (Lys49-PLA_2s) have been purified from *Bothrops* snake venoms and structurally and functionally characterized (Watanabe *et al.*, 2005; Soares *et al.*, 2004; Magro *et al.*, 2003; Lee *et al.*, 2001; Arni *et al.*, 1999; da Silva-Giotto *et al.*, 1998; de Azevedo *et al.*, 1999). Despite the isolation of several catalytic bothropic PLA_2s (Selistre *et al.*, 1990; Lomonte *et al.*, 1990; Gutiérrez & Lomonte, 1995; Mancuso *et al.*, 1995; Santos-Filho *et al.*, 2008), little is known about the mechanism of action of these PLA_2s, probably owing to their low concentration in the venom, which leads to experimental difficulties in structural and functional studies. In contrast, the acidic PLA_2s have high catalytic activity and other relevant pharmacological effects and are important models for structure–function relationship studies between toxic and nontoxic snake-venom PLA_2s. The unique bothropic structure available for this class of PLA_2 is that of BthA-I (an acidic PLA_2 from *B. jararacussu* venom), which has been described in two conformational states: monomeric and dimeric (Magro *et al.*, 2004). The structure of BthA-I complexed with the inhibitor *p*-bromophenacyl bromide has also been solved (Magro *et al.*, 2005).

BmooPLA$_2$-I is an acidic, nontoxic and catalytic phospholipase A_2 isolated from *B. moojeni* snake venom which exhibits an inhibitory effect on platelet aggregation, causes an immediate decrease in blood pressure, inducing oedema at a low concentration, and has an effective bactericidal effect (data not shown).

In order to better understand the structure–function relationship of these bothropic proteins, we describe the crystallization, X-ray diffraction data collection and molecular-replacement solution of BmooPLA$_2$-I.

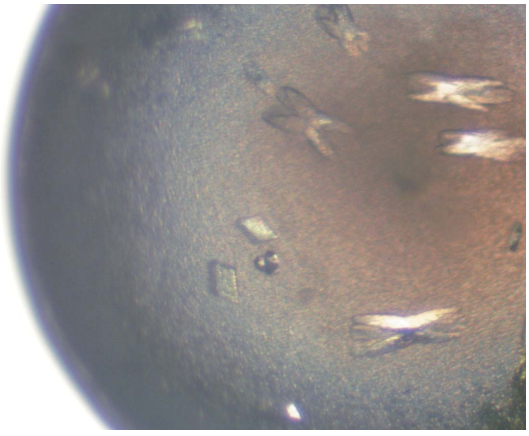


Figure 1
Crystals of BmooPLA$_2$-I from *B. moojeni*.

Table 1
X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell. Data were processed using the *HKL* suite (Otwinowski & Minor, 1997).

Unit-cell parameters (Å)	a = 39.7, b = 53.2, c = 89.2
Space group	$C222_1$
Resolution (Å)	50–1.60 (1.66–1.60)
Unique reflections	12806 (1249)
Completeness (%)	99.2 (97.8)
$R_{\rm merge}$† (%)	3.3 (16.5)
Radiation source	Synchrotron (MX1 station, LNLS)
Data-collection temperature (K)	100
$\langle I/\sigma(I)\rangle$	45.3 (7.7)
Multiplicity	5.7 (5.3)
Matthews coefficient $V_{\rm M}$ (Å^3 Da^{-1})	1.75
Molecules in asymmetric unit	1
Solvent content (%)	29.7

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl)\rangle$ is the mean intensity of that reflection. It was calculated for reflections with $I > -3\sigma(I)$.

2. Materials and methods

2.1. Protein purification and crystallization

BmooPLA$_2$-I was isolated from *B. moojeni* snake venom by ion-exchange chromatography on a CM-Sepharose Fast Flow column (2.0 × 20 cm) previously equilibrated with 0.05 *M* ammonium bicarbonate buffer pH 7.8. The homogeneity of the toxin was assayed by SDS–PAGE (data not shown).

A lyophilized sample of BmooPLA$_2$-I was dissolved in ultrapure water to a concentration of 14 mg ml^{-1}. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening for crystallization conditions (Crystal Screen, Hampton Research). Improved crystals of BmooPLA$_2$-I were obtained using the conventional hanging-drop vapour-diffusion method (McPherson, 1982), in which 1 µl protein solution and 1 µl reservoir solution were mixed and equilibrated against a reservoir (500 µl) consisting of 32%(*w*/*v*) polyethylene glycol 1500. The crystals measured approximately 0.1 × 0.12 × 0.06 mm after 20 d at 291 K (Fig. 1).

2.2. Dynamic light scattering

Dynamic light-scattering measurements were performed with lyophilized BmooPLA$_2$-I at 283 K at a concentration of 0.5 mg ml^{-1} using a DynaPro Titan instrument (Wyatt Technology). The protein solution was prepared in ultrapure water. Data were measured 100 times and the results were analyzed using the *DYNAMICS* v.6.10 software.

2.3. X-ray data collection and processing

X-ray diffraction data were collected from a single BmooPLA$_2$-I crystal at a wavelength of 1.430 Å (at 100 K) using a synchrotron-radiation source [MX1 station, Laboratório Nacional de Luz Sincrotron (LNLS), Campinas, Brazil] and a MAR CCD imaging-plate detector (MAR Research). The crystal was mounted in a nylon loop and flash-cooled in a steam of nitrogen at 100 K without a cryoprotectant. The crystal-to-detector distance was 60 mm and an oscillation range of 1° was used, resulting in the collection of a total of 150 images. The data were processed to 1.6 Å resolution using the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

The data-collection statistics are shown in Table 1. The data set was 99.2% complete at 1.6 Å resolution, with an $R_{\rm merge}$ of 3.3%. The

crystals belonged to the centred orthorhombic space group $C222_1$, with unit-cell parameters a = 39.7, b = 53.2, c = 89.2 Å. Calculations based on the protein molecular weight indicated the presence of one molecule in the asymmetric unit. This corresponds to a Matthews coefficient V_M (Matthews, 1968) of 1.75 Å^3 Da^{-1}, with a calculated solvent content of 29.7%. These values are within the expected range for typical protein crystals, assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume.

Dynamic light-scattering (DLS) experiments performed on BmooPLA$_2$-I indicated a mean hydrodynamic radius (R_h) of 1.8 nm and a polydispersity of 11.9%. This R_h value corresponds to a molecular weight of approximately 14 kDa and is thus equivalent to a monomer.

The crystal structure of BmooPLA$_2$-I was determined using molecular-replacement techniques implemented using the program *MOLREP* (Vagin & Teplyakov, 2010) with the coordinates of an acidic PLA$_2$ from *B. jararacussu* (PDB code 1zlb; Murakami *et al.*, 2006) as the search model. The structure presents a monomeric configuration.

In conclusion, BmooPLA$_2$-I isolated from *B. moojeni* venom was crystallized and X-ray diffraction data were collected to 1.6 Å resolution. The structure presented a monomeric arrangement. Further structural studies, including a structural comparison with other PLA$_2$s, including myotoxin I, may provide insights into the molecular basis of its pharmacological actions, including its inhibition of platelet aggregation and its hypotensive effects.

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