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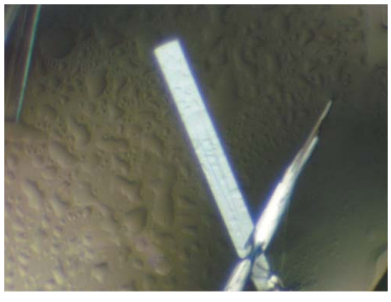

Crystallization and preliminary X-ray crystallographic studies of a Lys49-phospholipase A_2 homologue from *Bothrops pirajai* venom complexed with rosmarinic acid

PrTX-I, a noncatalytic and myotoxic Lys49-phospholipase A_2 from *Bothrops pirajai* venom, was crystallized in the presence of the inhibitor rosmarinic acid (RA). This is the active compound in the methanolic extract of *Cordia verbenacea*, a plant that is largely used in Brazilian folk medicine. The crystals diffracted X-rays to 1.8 Å resolution and the structure was solved by molecular-replacement techniques, showing electron density that corresponds to RA molecules at the entrance to the hydrophobic channel. The crystals belong to space group $P2_12_12_1$, indicating conformational changes in the structure after ligand binding: the crystals of all apo Lys49-phospholipase A_2 structures belong to space group $P3_121$, while the crystals of complexed structures belong to space groups $P2_1$ or $P2_12_12_1$.

1. Introduction

Envenomation by snakes belonging to the *Bothrops* genus is economically and socially important in Latin America as it is responsible for more than 85% of all ophidian accidents reported in the area (Ferreira *et al.*, 1992; Ribeiro *et al.*, 1998; de Oliveira, 2009). These envenomations are characterized by prominent local tissue damage arising from myonecrosis, haemorrhage and oedema (Rosenberg, 1990). These drastic local effects are not efficiently neutralized by serum therapy and may cause permanent tissue loss and amputation of the affected limb (Gutierrez & Lomonte, 1995).

Phospholipases A_2 (PLA_2s) are the main components of these venoms and in addition to their catalytic role show a broad spectrum of pharmacological activities such as neurotoxicity, myotoxicity and cardiotoxicity. Some of these activities are correlated with the enzymatic activity, while others are completely independent (Kini & Evans, 1989; Soares & Giglio, 2003). PLA_2s also affect the coagulation cascade, platelet aggregation and the inflammatory response (Kini, 1997; Andriao-Escarso *et al.*, 2002).

PLA_2 homologues with skeletal muscle-damaging activity (myotoxicity) are widely distributed in venomous snakes, with Lys49-PLA_2s being the most studied and best characterized subgroup. The loss of catalytic activity in PLA_2 homologues was initially attributed to the natural substitution D49K, as a lysine in this position impairs Ca^{2+} coordination (Maraganore *et al.*, 1984; Arni & Ward, 1996; Ward *et al.*, 1998), but other peculiarities have subsequently also been demonstrated to be involved in this phenomenon (Ward *et al.*, 2002; Lee *et al.*, 2001; dos Santos, Fernandes *et al.*, 2009). Synthetic peptides and site-directed mutagenesis studies have strongly suggested that the C-terminal region of these proteins contains the sequence that is responsible for the expression of this activity in Lys49-PLA_2s (Chioato *et al.*, 2002, 2007; Lomonte, Angulo & Calderon, 2003; Lomonte, Angulo & Santamaria, 2003; Nunez *et al.*, 2001; Ward *et al.*, 1998, 2002; Cintra-Francischinelli *et al.*, 2010).

In recent years, great efforts have been made with the aim of understanding the mechanism of action of snake-venom myotoxins in order to find efficient inhibitors of these proteins. One of these attempts has focused on the scientific study of plant species that are of general use in folk medicine (Mors *et al.*, 2000; Borges *et al.*, 2000, 2001; Biondo *et al.*, 2003, 2004; Januario *et al.*, 2004; Veronese *et al.*, 2005; Esmeraldino *et al.*, 2005; Oliveira *et al.*, 2005). Ticli and

coworkers studied a methanolic extract of *Cordia verbenacea* and demonstrated that this extract was able to neutralize paw oedema induced by *B. jararacussu* snake venom and by its main basic Lys49-PLA_2s (Ticli *et al.*, 2005). After the isolation of rosmarinic acid (RA) as the active component of the abovementioned extract, it was shown that RA is able to significantly inhibit the myotoxic effect induced by two basic bothropic PLA_2s (BthTX-I and BthTX-II; Ticli *et al.*, 2005). Electrophoretic analysis showed that the basis of such inhibition could not be attributed to proteolytic degradation of the myotoxic PLA_2s (Ticli *et al.*, 2005), while circular-dichroism studies showed that no significant secondary-structural changes were observed on ligand binding (Ticli *et al.*, 2005).

In this work, we report the crystallization and X-ray diffraction data collection of PrTX-I, a basic myotoxic Lys49-PLA_2 isolated from *B. pirajai* snake venom, complexed with RA. The final crystallographic model of the complex may provide insight into the mechanism(s) that lead to inhibition of the myotoxicity of snake-venom PLA_2s.

2. Materials and methods

2.1. Protein purification and crystallization

PrTX-I was isolated from *B. pirajai* snake venom as described previously (Mancuso *et al.*, 1995), while RA was purchased from Sigma–Aldrich. The lyophilized sample of PrTX-I was dissolved to a concentration of 12 mg ml^{-1} in Tris–HCl pH 7.5 buffer. The same buffer was used to dissolve the commercial RA to a 10:1 molar ratio of inhibitor:protein. The sparse-matrix method (Jancarik & Kim, 1991) was used to perform initial screening of the crystallization conditions (Crystal Screens I and II, Hampton Research). Crystals were obtained by the sitting-drop vapour-diffusion method (McPherson, 1982), combining 1 µl protein solution, 0.5 µl RA solution and 1 µl reservoir solution, and were equilibrated against 0.5 ml of the same precipitant solution (Fig. 1). After the optimization process, the best crystals were obtained at 291 K in a reservoir solution containing 20% PEG 4000, sodium citrate pH 5.6 and 20% propanol. These crystals measured approximately 0.6 × 0.1 × 0.05 mm after one month.

2.2. X-ray data collection and processing

X-ray diffraction data were collected at a wavelength of 1.427 Å (at 100 K) using a synchrotron-radiation source [the MX2 station at Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil] using a MAR CCD imaging-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at 100 K without using any cryoprotectant. The data were processed using the *HKL* program package (Otwinowski & Minor, 1997).

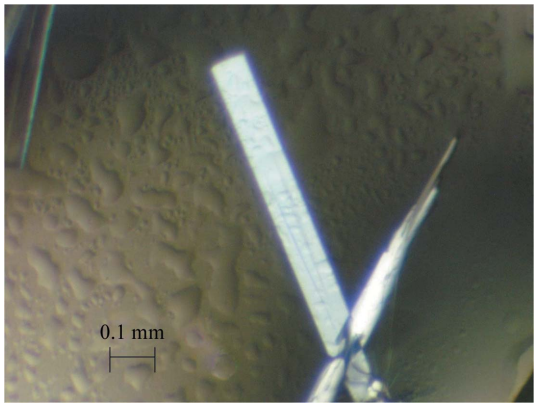

Figure 1
Crystal of PrTX-I complexed with rosmarinic acid.

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 = 49.4, b = 67.0, c = 85.5
Space group	$P2_12_12_1$
Resolution (Å)	40.0–1.80 (1.89–1.80)
Unique reflections	25698 (3766)
Completeness (%)	95.0 (98.8)
R_{merge}† (%)	6.9 (37.3)
Radiation source	Synchrotron (MX2 station, LNLS)
Data-collection temperature (K)	100
Average $I/\sigma(I)$	18.7 (2.6)
Redundancy	4.0 (3.7)
Matthews coefficient V_M (Å^3 Da^{-1})	2.6
Molecules in the asymmetric unit	2
Solvent content (%)	53.1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, 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. Calculated for $I > -3\sigma(I)$.

3. Results and discussion

The data-collection statistics are given in Table 1. The crystals belong to the orthorhombic system and the data set was 95.0% complete at 1.8 Å resolution, with an R_{merge} of 6.9%.

Calculations based on the molecular weight of the protein indicated the presence of two molecules in the asymmetric unit. This corresponds to a Matthews coefficient (V_M; Matthews, 1968) of

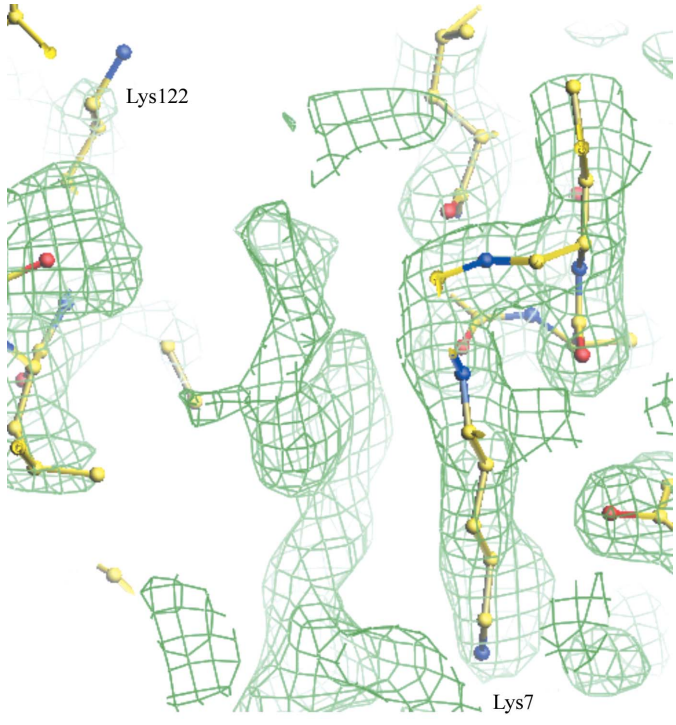

Figure 2
$2|F_{obs}| - |F_{calc}|$ electron-density map contoured at 1.0 standard deviation at the entrance to the hydrophobic channel, where electron density that corresponds to rosmarinic acid was found.

2.6 Å^3 Da^{-1} and a calculated solvent content of the crystals of 53.1%. These values are within the expected range for typical protein crystals, assuming a value of 0.74 cm^3 g^{-1} for the partial specific volume of the protein.

The crystal structure was solved by the molecular-replacement technique as implemented in the program *AMoRe* (Navaza, 1994) using the coordinates of native PrTX-I (dos Santos, Soares *et al.*, 2009) as a model and confirmed the presence of a dimer in the asymmetric unit. Electron-density maps that unambiguously correspond to RA molecules were observed at the entrance to the hydrophobic channels (Fig. 2).

A recent review of Lys49-PLA$_2$ crystallographic structures indicated that the apo structures belong to space group $P3_121$, while the complexed forms belong to space groups $P2_1$ and $P2_12_12_1$ (dos Santos, Soares *et al.*, 2009). The space-group change is a consequence of conformational changes that occur when a ligand is bound to Lys49-PLA$_2$s (dos Santos, Soares *et al.*, 2009). Since space group $P2_12_12_1$ was observed for the PrTX-I–RA complex, it is possible to suggest that inhibitor binding has led to changes in the quaternary structure of PrTX-I. After refinement of the structure, we will be able to confirm the presence of the inhibitor and the interactions that are established between RA and PrTX-I.

It has been demonstrated that RA is able to potentiate the ability of commercial equine polyvalent antivenom to neutralize the lethal and myotoxic effects of crude *B. jararacussu* venom (Ticli *et al.*, 2005). RA was also capable of neutralizing two isolated Lys49-PLA$_2$s from *B. jararacussu* snake venom in experimental models (Ticli *et al.*, 2005). Therefore, detailed structural studies of the PrTX-I–RA complex may provide new and important insights into how structural changes in the quaternary structure of Lys49-PLA$_2$s after ligand binding can lead to toxin inhibition.

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