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Crystallization and preliminary X-ray diffraction analysis of a class II phospholipase D from *Loxosceles intermedia* venom

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Phospholipases D are the major dermonecrotic component of *Loxosceles* venom and catalyze the hydrolysis of phospholipids, resulting in the formation of lipid mediators such as ceramide-1-phosphate and lysophosphatidic acid which can induce pathological and biological responses. Phospholipases D can be classified into two classes depending on their catalytic efficiency and the presence of an additional disulfide bridge. In this work, both wild-type and H12A-mutant forms of the class II phospholipase D from *L. intermedia* venom were crystallized. Wild-type and H12A-mutant crystals were grown under very similar conditions using PEG 200 as a precipitant and belonged to space group $P12_11$, with unit-cell parameters $a = 50.1$, $b = 49.5$, $c = 56.5$ Å, $\beta = 105.9^\circ$. Wild-type and H12A-mutant crystals diffracted to maximum resolutions of 1.95 and 1.60 Å, respectively.

1. Introduction

Envenomation by members of the genus *Loxosceles* (brown spiders), considered to be the most dangerous form of arachnidism, is a serious public health problem in both North and South America (Santi Ferrara *et al.*, 2009). *Loxosceles* venom can cause local dermonecrosis with gravitational spreading and systemic manifestations such as thrombocytopaenia, haemolysis and acute renal failure that can lead to death (Futrell, 1992; da Silva *et al.*, 2004).

Several toxic proteins present in *Loxosceles* spp. venoms have been identified and biochemically characterized (da Silva *et al.*, 2004; Gremski *et al.*, 2010). Members of the phospholipase D family are abundant in the venoms of several *Loxosceles* spp. and contribute significantly to the typical response after envenomation (Kalapothakis *et al.*, 2007; Senff-Ribeiro *et al.*, 2008).

Phospholipases D (30–35 kDa), also referred to as dermonecrotic toxins, catalyze the hydrolysis of sphingomyelin and (lyso) glycerophospholipids, resulting in the formation of bioactive mediators such as ceramide-1-phosphate and lysophosphatidic acid which play a role in several pathological and biological responses (Van Meeteren *et al.*, 2004; Moolenaar *et al.*, 2004; Lee & Lynch, 2005). As proposed by Murakami *et al.* (2006), spider-venom phospholipases D can be classified into two classes. Members of class I possess a single disulfide bridge and contain an extended hydrophobic loop, whereas class II proteins contain an additional intra-chain disulfide bridge and display decreased catalytic activity towards phospholipids. To date, only the phospholipase D from *L. laeta* venom, a member of class I, has been structurally characterized (Murakami *et al.*, 2005), despite the clinical importance of phospholipases D in loxoscelism. Based on its crystal structure, an acid–base catalytic mechanism was proposed in which His12 and His47 play key roles and are supported by a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233 and Asn252 (Murakami *et al.*, 2005).

The recombinant dermonecrotic toxin (LiRecDT1) obtained from a cDNA library of the *L. intermedia* venom gland is able to directly induce renal injuries in mice and the haemolysis of human erythrocytes *in vitro*, suggesting that this protein is directly involved in the

the nephrotoxicity and haematological disturbances evoked during envenomation by *Loxosceles* spiders (Chaim *et al.*, 2006; Chaves-Moreira *et al.*, 2009). Mutation of the catalytic residue His12 to Ala abolishes both the nephrotoxic effect of LiRecDT1 in mice and the haemolysis of human erythrocytes (Kusma *et al.*, 2008; Chaves-Moreira *et al.*, 2009).

The present report describes the crystallization and preliminary crystallographic analysis of recombinant wild-type (LiRecDT1) and mutant (LiRecDT1 H12A) dermonecrotic toxin from *L. intermedia* venom, which belongs to the class II phospholipases D. The structural characterization of LiRecDT1 will be essential to shed light on the structural determinants of the functional differentiation between members of the class I and class II phospholipases D.

2. Materials and methods

2.1. Expression and purification

DNA corresponding to the wild-type (LiRecDT1) and mutated (LiRecDT1 H12A) forms of the mature phospholipase D was cloned into pET-14b vector (Novagen, Madison, USA) as described by Chaim *et al.* (2006) and Kusma *et al.* (2008). Both recombinant constructs were expressed as fusion proteins with a 6×His tag at the N-terminus and a 13-amino-acid linker including a thrombin site between the 6×His tag and the mature protein. pET-14b/*L. intermedia* cDNA constructs were transformed into One Shot *Escherichia coli* BL21 (DE3) pLysS competent cells (Invitrogen) and plated on LB agar plates containing 100 mg ml^{-1} ampicillin and 34 mg ml^{-1} chloramphenicol. A single colony was inoculated into 50 ml LB broth (plus antibiotics) and allowed to grow overnight at 310 K. A 10 ml portion of this overnight culture was grown in 1 l LB broth/ampicillin/chloramphenicol at 310 K until the OD at 550 nm reached 0.5. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.05 m*M* and the culture was induced by incubation for an additional 3.5 h at 303 K. Cells were harvested by centrifugation (400*g*, 7 min) and the pellet was frozen at 253 K overnight.

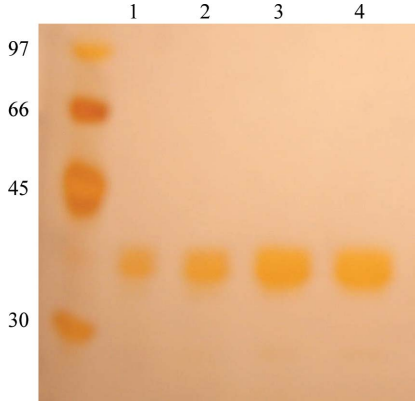

Figure 1
Silver-stained SDS–PAGE (12%) of purified samples of wild-type and H12A-mutant dermonecrotic toxin from *L. intermedia*. Lane 1, molecular-weight markers (kDa); lanes 2 and 4, purified LiRecDT1 (18 and 34 µg, respectively); lanes 3 and 5, purified LiRecDT1 H12A (18 and 34 µg, respectively).

The cell suspensions were thawed and were additionally disrupted by six 10 s cycles of sonication at low intensity. The lysed materials were centrifuged (20 000*g*, 20 min) and the supernatants were incubated with 1 ml Ni^{2+}–NTA agarose beads for 1 h at 277 K (with gentle agitation). The suspensions were loaded onto a column and the packed gel was exhaustively washed with 50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 20 m*M* imidazole until the OD at 280 nm reached 0.01. The recombinant proteins were eluted with 10 ml elution buffer (50 m*M* sodium phosphate pH 8.0, 500 m*M* NaCl, 250 m*M* imidazole) and 1 ml fractions were collected and analyzed by 12.5% SDS–PAGE under reducing conditions (Fig. 1). The fractions were pooled and dialyzed against phosphate-buffered saline (PBS). Site-directed mutagenesis did not alter the correct folding of the brown spider phospholipase D as assessed by circular-dichroism and fluorescence experiments (results not shown).

2.2. Crystallization

The wild-type and mutant proteins were initially crystallized by vapour diffusion in sitting drops using a Cartesian HoneyBee 963 system (Genomic Solutions) at 291 K. For initial screening, 1 µl protein solution at a concentration of 17 mg ml^{-1} for the wild type and of 9 mg ml^{-1} for the H12A mutant was mixed with 1 µl screening solution and equilibrated over a reservoir containing 100 µl of the latter solution. Small crystals of wild-type LiRecDT1 were observed in the condition 0.1 *M* Tris–HCl pH 8, 35%(*v*/*v*) PEG 200, which was refined by varying the PEG 200 concentration *versus* the pH using the hanging-drop method. The best wild-type LiRecDT1 crystals were observed in drops consisting of 2 µl protein solution (17 mg ml^{-1}) and 2 µl reservoir solution equilibrated over 1 ml reservoir solution [0.1 *M* Tris–HCl pH 7.5, 40%(*v*/*v*) PEG 200] (Fig. 2*a*). Crystals of the H12A mutant were grown in a very similar condition consisting of 0.1 *M* Tris–HCl pH 7.5 and 35%(*v*/*v*) PEG 200 (Fig. 2*b*).

2.3. X-ray diffraction analysis

LiRecDT1 and LiRecDT1 H12A crystals were directly flash-cooled in a 100 K nitrogen-gas stream. X-ray diffraction data were collected on the W01B-MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil). The LiRecDT1 crystal was exposed for 60 s per 2° rotation in φ with the crystal-to-detector distance set to 100 mm. The LiRecDT1 H12A crystal was exposed for 20 s per 1° rotation in φ with the crystal-to-detector set to 69 mm. A total of 180 and 300 images were collected from the LiRecDT1 and the LiRecDT1 H12A crystals, respectively. The data were indexed,

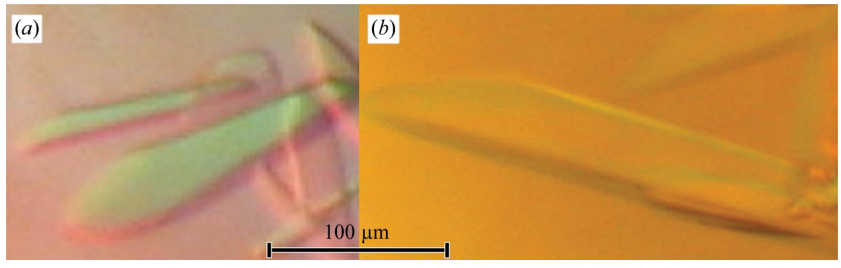

Figure 2
Microphotograph of crystals of (*a*) wild-type and (*b*) mutant dermonecrotic toxin from *L. intermedia*.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

	LiRecDT1	LiRecDT1 H12A
Data collection		
Temperature (K)	100	100
Radiation source	Brazilian Synchrotron Light Laboratory	Brazilian Synchrotron Light Laboratory
Beamline	W01B-MX2	W01B-MX2
Wavelength (Å)	1.458	1.458
Detector	MAR Mosaic 225 mm	MAR Mosaic 225 mm
Space group	$P12_11$	$P12_11$
Unit-cell parameters (Å, °)	a = 50.08, b = 49.43, c = 56.59, β = 105.88	a = 49.58, b = 49.46, c = 56.40, β = 105.56
Resolution range (Å)	30.0–2.0 (2.07–2.00)	30.0–1.60 (1.66–1.60)
R_{merge} (%)†	12.1 (49.4)	7.3 (37.0)
$\langle I/\sigma(I)\rangle$	9.3 (2.4)	19.5 (2.9)
Data completeness (%)	99.5 (98.1)	98.9 (92.4)
No. of unique reflections	18148 (1765)	34632 (3241)
Multiplicity	3.1 (2.7)	5.1 (3.3)
Data analysis		
V_M (Å^3 Da^{-1})	2.25	2.22
Solvent content (%)	45.25	44.63
Molecules per asymmetric unit	1	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 ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations I of reflection hkl.

integrated and scaled using the *DENZO* and *SCALEPACK* programs from the *HKL*-2000 package (Otwinowski & Minor, 1997). Data-processing statistics are summarized in Table 1.

3. Results and discussion

LiRecDT1 and LiRecDT1 H12A crystals diffracted to resolutions of 1.95 and 1.60 Å, respectively. Although the mutant protein crystal diffracted better than the native protein crystal, the H12A mutation did not alter the crystal packing or the unit-cell symmetry and parameters. The LiRecDT1 and LiRecDT1 H12A data sets were indexed in the monoclinic crystal system. The presence of systematic absences indicated that the crystals belonged to space group $P12_11$. Both crystals possessed highly similar unit-cell parameters (Table 1). The Matthews coefficient calculated for the LiRecDT1 crystal was 2.25 Å^3 Da^{-1}, corresponding to a solvent content of 45% (Matthews, 1968). Considering the molecular weight of 30 000 Da, one molecule is present in the asymmetric unit of both crystals. Data-processing statistics for both data sets are presented in Table 1.

The atomic coordinates of the phospholipase D from *L. laeta* venom (PDB code 1xx1; Murakami *et al.*, 2005), which displays a sequence identity of 58% with LiRecDT1, were used to generate a search model and molecular-replacement calculations were carried out using the program *MOLREP* in the resolution range 15.0–3.0 Å (Vagin & Teplyakov, 2010). A solution was obtained for one molecule in the asymmetric unit in space group $P12_11$. Analysis of the packing contacts and steric clashes clearly showed that this was the correct solution. *REFMAC*5 (Murshudov *et al.*, 1997) was used for rigid-body refinement of this solution in the resolution range 30.0–1.95 Å (excluding 5% of reflections for R_{free} calculations), resulting in a correlation coefficient of 49.2, a score of 0.675 (the score of the next highest peak was 0.338) and an R factor of 45.6% (R_{free} = 49.1%). Structure refinement and analysis are currently in progress. Determination of the LiRecDT1 H12A crystal structure will be performed using the final model of wild-type LiRecDT1.

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