Crystallization and X-ray diffraction analysis of an antifungal laticifer protein

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An osmotin (CpOsm) from the latex of *Calotropis procera* has been crystallized in both tetragonal and trigonal forms suitable for structure determination. Crystallographic studies of CpOsm are of great interest because limited information is available concerning the structure of latex proteins and CpOsm has previously been shown to interact with the spore membranes of some plant pathogenic fungi, thus impairing spore germination and hyphal growth. CpOsm crystals were grown using 0.1 M HEPES buffer pH 7.5, 26% PEG 4000, 0.2 M ammonium sulfate (space group $P4_3$) or using 0.1 M HEPES buffer pH 7.5, 35% MPD, 0.7 M ammonium sulfate (space group $P3_112$). X-ray diffraction data were collected to 2.17 Å ($P4_3$) and 1.80 Å ($P3_112$) resolution and molecular-replacement analyses produced initial phases for both crystal forms.

1. Introduction

Latex is a milky fluid comprising a complex mixture of secondary metabolites and proteins that is found in the cytoplasm of highly specialized cells named laticifers. These conduction-like systems represent a type of morphophysiological differentiation found in many plants that has no clear evolutionary origin (Hagel *et al.*, 2008). Latex proteins from different species have been shown to be harmful to insects and to inhibit spore germination and hyphal growth of fungi (Konno, 2011; Souza *et al.*, 2011). Because of this, latex proteins have emerged as promising tools to fight insects and phytopathogens that damage crops.

A large number of plant proteins involved in the response to pathogens have been documented. These proteins are collectively named pathogenesis-related (PR) proteins and form a large superfamily of proteins in which expression is induced under biotic and abiotic stress. The term osmotin was first used to describe a 26 kDa thaumatin-like protein (TLP) that was identified in cultured tobacco cells adapted to grow under osmotic stress (Singh *et al.*, 1987). Further studies confirmed that osmotin expression was favoured under salt-stress conditions (Onishi *et al.*, 2006). The expression of tobacco osmotins is also induced in response to pathogen attack (Woloshuk *et al.*, 1991); therefore, osmotins and other TLPs are classified as group 5 PR proteins (Van Loon *et al.*, 2006). Moreover, tobacco osmotin can inhibit hyphal growth and spore germination of a variety of plant pathogenic fungi and these properties are related to its ability to cause permeabilization of fungal plasma membranes (Abad *et al.*, 1996). These findings, which suggest that osmotins could be a novel source of resistance to fungal diseases of plants, were further strengthened by the discovery that plants experimentally infected with phytopathogenic fungi overexpressed osmotins (Hong *et al.*, 2004). Furthermore, transgenic tobacco plants possessing the thaumatin gene from *Thaumatococcus daniellii* exhibited enhanced resistance towards phytopathogens, with delayed disease symptoms (Rajam *et al.*, 2007).

To date, limited information is available regarding the structural features of latex proteins, considering the large number of latex proteins that are known. Examples of the best-studied proteins are hevein from *Hevea brasiliensis*, papain from *Carica papaya* and, more recently, the serine protease inhibitor from papaya latex (PDB entry 3s8j; Azarkan *et al.*, 2011).

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Space group	$P4_3$	$P3_112$
Wavelength (Å)	1.6080	1.6080
No. of frames	150	150
Oscillation angle (°)	1	0.5
Average mosaicity (°)	0.77	0.72
Resolution range (Å)	27.28–2.17 (2.29–2.17)	17.69–1.80 (1.90–1.80)
Unit-cell parameters (Å)	$a = 80.20$, $b = 80.20$, $c = 124.44$	$a = 95.31$, $b = 95.31$, $c = 79.24$
Unique reflections	41465 (5880)	32451 (4058)
Completeness (%)	99.5 (97.3)	84.2 (70.9)
Multiplicity	6.0 (5.8)	8.3 (6.1)
$\langle I/\sigma(I) \rangle$	17.0 (5.6)	14.9 (2.7)
$R_{\text{merge}}^\dagger$	0.082 (0.305)	0.083 (0.534)
Asymmetric unit content	Four monomers	Two monomers
Matthews coefficient (Å ³ Da ⁻¹)	2.27	2.36
Overall B factor from Wilson plot (Å ²)	24.7	27.3

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean of the i individual measurements $I_i(hkl)$ of the intensity of reflection hkl .

Recently, a 22 kDa nonglycosylated osmotin was purified from the latex of *Calotropis procera*. This protein (named CpOsm; *C. procera* osmotin) exhibits strong but differential activity against the hyphal growth of phytopathogenic fungi (Freitas, Nogueira *et al.*, 2011). It has also been demonstrated that CpOsm is capable of interacting with the negatively charged phospholipid membranes of artificial micelles, allowing the release of the internal fluid, as revealed by fluorescence spectrum analysis (Freitas, Lopes *et al.*, 2011). Furthermore, it has been shown that purified CpOsm interacts with fungal spore membranes, causing release of the cytoplasmic content, which results in spore death (Freitas, Lopes *et al.*, 2011).

With the exception of that on CpOsm and the recent work by Looze *et al.* (2009) on the study of a TLP from the latex of *C. papaya*, no information is available concerning latex osmotins. Therefore, it is important to obtain additional information on the structural features of these molecules. Furthermore, structural information about the

interaction of osmotins with fungal membrane receptors is highly desirable. However, structural data for the native protein are first needed. Therefore, we have performed crystallization assays of native CpOsm in order to establish the best conditions for the solution of its native three-dimensional structure.

2. Materials and methods

2.1. Purification of CpOsm and ESI mass-spectrometric analysis

CpOsm was purified to homogeneity from the natural latex source by ion-exchange chromatography on a Resource S column (Amersham Biosciences, Brazil), dialyzed against water and lyophilized according to the protocol reported by Freitas, Nogueira *et al.* (2011). The sample purity was assessed by SDS-PAGE according to Laemmli (1970) and ESI-MS analysis. The ESI-MS analysis was performed using a Synapt HDMS mass spectrometer (Waters, Manchester, England) coupled to a nano UPLC-ESI system. The sample was diluted to 1 mg ml⁻¹ in 0.1% (v/v) formic acid and 0.5 µl was used for reverse-phase chromatography with a gradient from 5 to 80% (v/v) acetonitrile in 0.1% (v/v) formic acid at a flow rate of 600 nl min⁻¹ in a C18 BEH column (1.7 × 100 × 100 mm). The acquired MS data were processed using a maximum-entropy technique (MaxEnt) to obtain a deconvoluted spectrum (Fig. 1). To ensure that the sample that was used in the crystallization experiments was biologically active, its antifungal activity against *Colletotrichum gloeosporioides* was evaluated according to Freitas, Lopes *et al.* (2011) (Fig. 1).

2.2. Crystallization, data collection and initial processing

Highly purified CpOsm was dissolved to 8.5 mg ml⁻¹ in 10 mM sodium phosphate buffer pH 6.0, the solution was centrifuged at 10 000g and the supernatant was used in hanging-drop crystallization trials.

Initially, 98 crystallization conditions were used to perform the hanging-drop experiments (Crystal Screen and Crystal Screen 2, Hampton Research, Riverside, California, USA). The drops were

Figure 1

Confirmation of the purity and activity of the sample. (a) The mass spectrum and (b) the antifungal activity of the native CpOsm used in the crystallization assays.

composed of equal volumes (1.5 μ l) of protein solution and reservoir solution and were equilibrated against 400 μ l reservoir solution. Crystals were grown in Linbro plates at 293 K by the vapour-diffusion method (Jancarik & Kim, 1991).

X-ray diffraction data were collected at a wavelength of 1.6080 $\text{\AA}$ using a synchrotron-radiation source (MX1 station, Laboratório Nacional de Luz Síncrotron, Campinas, Brazil) and a CCD detector (MAR Research). Data were collected from CpOsm crystals at 100 K; to avoid freezing they were soaked in a cryoprotectant solution consisting of 50% reservoir solution and 50% MPD. A summary of the data-collection statistics is reported in Table 1. The crystal data were processed, indexed and integrated using *MOSFLM* and *SCALA* (Winn *et al.*, 2011; Leslie, 1992).

2.3. Model search and molecular replacement

The unit-cell content of the native crystal was analysed using the Matthews coefficient (Matthews, 1968). For initial phasing, the data from each crystal were used in a molecular-replacement procedure with the *AMoRe* program from *CCP4* (Winn *et al.*, 2011). To find a suitable protein structure for use as a search model, the N-terminal sequence of CpOsm (Freitas, Nogueira *et al.*, 2011) was compared with sequences deposited in the Protein Data Bank (PDB) using *BLASTp*. On comparing (by *BLASTp* alignment) the available N-terminal amino-acid sequence of CpOsm with the full amino-acid sequence of *Nicotiana tabacum* osmotin, the identity was 65%. Several space groups were tested for both crystal forms of CpOsm using the structure of *N. tabacum* osmotin (PDB entry 1pcv; Min

Figure 2 Crystals of CpOsm grown in two different conditions: (a) 0.1 M HEPES pH 7.5, 26% PEG 4000, 0.2 M ammonium sulfate ($P4_3$, four monomers per asymmetric unit) and (b) 0.1 M HEPES pH 7.5, 35% MPD, 0.7 M ammonium sulfate ($P3_112$, two monomers per asymmetric unit).

Figure 3 Diffraction patterns of CpOsm crystals. (a) 2.17 $\text{\AA}$ resolution, space group $P4_3$; (b) 1.80 $\text{\AA}$ resolution, space group $P3_112$.

et al., 2004) as a search model. Rotation and translation functions succeeded in obtaining the best solution, which was chosen based on the magnitude of the correlation coefficient and the *R* factor.

3. Results and discussion

3.1. Optimization of CpOsm crystals

Small irregular crystals of CpOsm appeared after one week in several crystallization conditions containing PEG and MPD as precipitants. Initially, these crystals were not suitable for X-ray diffraction experiments; however, the crystallization conditions were optimized by changing the concentrations of the precipitants and salt. The best CpOsm crystals grew after two weeks in 0.1 *M* HEPES buffer pH 7.5, 26% PEG 4000, 0.2 *M* ammonium sulfate, as well as in 0.1 *M* HEPES buffer pH 7.5, 35% MPD, 0.7 *M* ammonium sulfate, to maximum dimensions of 0.2 × 0.4 × 0.4 mm (Fig. 2).

3.2. Data collection, processing and molecular replacement

Both crystals were submitted to the same data-collection procedure. The CpOsm crystals diffracted to a maximum resolution of 2.17 Å (*P*₄₃) or 1.80 Å (*P*₃₁2) using a synchrotron-radiation source (Fig. 3). The complete data sets were indexed, integrated and scaled, and analysis of the Matthews coefficient gave the solvent contents of both forms of the crystals. A summary of the data-collection statistics is given in Table 1. Several space groups were tested for both forms of the crystals in molecular-replacement procedures and the best solutions using the *N. tabacum* structure as a model (PDB entry 1pcv; Min *et al.*, 2004) were obtained in space groups *P*₄₃ and *P*₃₁2, with *R* factors and correlation coefficients of 0.482 and 46.5 for *P*₄₃ and 0.465 and 54.9 for *P*₃₁2, respectively.

The present work shows the characteristics of CpOsm crystals and *AMoRe* provided an MR solution for both crystal forms. Molecular-replacement solutions led to acceptable crystal packing with two or four monomers per asymmetric unit. Further model building/refinement is under way and the full details of this structure are expected to be determined soon.

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