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Purification, crystallization and preliminary X-ray studies of sylvaticin, an elicitin-like protein from *Pythium sylvaticum*

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Sylvaticin belongs to the elicitin family. These 10 kDa oomycetous proteins induce a hypersensitive response in plants, including necrosis and cell death, but subsequently leading to a non-specific systemic acquired resistance (SAR) against other pathogens. Sylvaticin has been crystallized using PEG 2000 MME as a precipitant agent in the presence of nickel chloride. The crystals belong to space group $C2$, with unit-cell parameters $a = 99.29$, $b = 25.67$, $c = 67.45$ Å, $\beta = 99.66^\circ$. Diffraction data were recorded to 2.1 Å resolution at a synchrotron-radiation source.

1. Introduction

Of the oomycetes, the phytopathogenic *Phytophthora* and *Pythium* spp. are distributed worldwide and cause severe damage to economically important crops in both temperate and tropical areas. They secrete small 10 kDa proteins that can induce tissue necrosis (hypersensitive-like response, HR) in some plants (particularly tobacco) followed by a systemic acquired resistance (SAR) against a wide range of pathogens. These extracellular proteinaceous elicitors constitute a class of proteins termed elicitins that can be divided into two groups depending on their pI values. The basic proteins (β-elicitins) are more necrotizing than the acidic α-elicitins (Ponchet *et al.*, 1999). All elicitins so far investigated exhibit high sequence homology (up to 80%) and lack the three residues histidine, arginine and tryptophan. The three-dimensional structure of cryptogein, a β-elicitin secreted by *Phytophthora cryptogea*, is now well known from both X-ray (Boissy *et al.*, 1996) and NMR (Fefeu *et al.*, 1997; Gooley *et al.*, 1998) analyses. Recently, the structure of β-cinnamomin from *Phytophthora cinnamomi* was reported to be nearly superimposable on that of cryptogein (Rodrigues *et al.*, 2002). In addition, all elicitins are able to load and transfer sterols (Mikes *et al.*, 1997, 1998). Two three-dimensional structures of cryptogein–sterol complexes have been determined (Boissy *et al.*, 1999; Lascombe *et al.*, 2002) and provide interesting information on how the complex is formed and stabilized. These data were of prime importance in building mutated and engineered cryptogeins which demonstrated the elicitin–sterol complex to be the active form in SAR and HR induction (Osman *et al.*, 2001).

In addition to the *Phytophthora* oomycetes, a number of *Pythium* species have been reported to secrete proteins that show high homologies with elicitins (Huet *et al.*, 1995; Panabières *et al.*, 1997). However, some of these *Pythium* strains belong to other phylogenetic clades (Wang & White, 1997) and produce additional 10 kDa elicitin-like proteins that have low sequence homology (~30–40%) and contain tryptophan or arginine residues, in contrast to elicitins (M. Ponchet & F. Panabières, unpublished work). The present study focuses on this new kind of protein with the aim of analyzing the variability among these proteins and determining their biological roles and how they recognize and transfer sterols. Until now, only oligandrin from *P. oligandrum* has been studied as it shows interesting new biological properties such as induction of SAR without associated HR symptoms (Picard *et al.*, 2000; Benhamou *et al.*, 2001; Lherminier *et al.*, 2004). Oligandrin has been crystallized (Lascombe *et al.*, 2000), but owing to perfect hemihedral twinning the structure had not yet been solved. The present report concerns preliminary data on sylvaticin, a 10 kDa protein secreted by *Pythium sylvaticum*, which is the first tryptophan- and arginine-containing elicitin.

2. Culture and purification

P. sylvaticum (strain 37 from INRA Antibes collection) was maintained on 1% malt/1% agar solid medium in 10 cm Petri dishes. 5–6 day-old cultures were used to inoculate glucose–asparagine-based liquid medium as described previously (Bonnet *et al.*, 1996). After 8 d, the culture filtrate was recovered after filtration on a GF/C filter (Whatman) and concentrated tenfold under vacuum in a rotatory evaporator. The concentrated culture filtrate was dialysed three times against ultrapure water and then adjusted to pH 4 with 5 m*M* sodium acetate/trifluoroacetic acid.

Three purification/separation steps were performed using successively (i) a Macroprep High S (Biorad) cation-exchange column (elution with a gradient of NaCl to 1 *M* at pH 4), (ii) a Macroprep High Q (Biorad) anion-exchange column pre-equilibrated with 5 m*M* acetate (elution with 50 m*M* sodium acetate pH 4) and (iii) a Synchroprep C4 (Synchrom) column (elution with an acetonitrile gradient to 40% in 50 m*M* sodium formate pH 7). The fractions containing sylvaticin were pooled, briefly evaporated under vacuum to eliminate acetonitrile, extensively dialysed against ultrapure water and then freeze-dried. All purification stages were followed by SDS–PAGE and HPLC as previously described (Le Berre *et al.*, 1994). 5 l of initial culture filtrate usually leads to 150 mg of sylvaticin at homogeneity.

3. Crystallization

Prior to crystallization assays, all solutions were filtrated on 0.22 µm cellulose acetate filters and the protein stock solution (20 mg ml^{-1}) was centrifuged at 10 000 rev min^{-1} for 15 min. Crystallization experiments were performed at 292 K using the hanging-drop vapour-diffusion method. In initial trials, droplets were made up of equal amounts (1 µl) of reservoir solution and protein solution (20 mg ml^{-1} in 50 m*M* sodium acetate buffer pH 4.7). The drops were equilibrated against 0.7 ml of reservoir solution in the well. Hampton Research Crystal Screen kits I and II were used to select the best starting conditions. Microcrystalline material appeared in only one condition, with PEG 2000 MME as precipitating agent and nickel chloride as additive. These conditions were further optimized by varying the precipitant concentrations, the nature and pH of the buffer and the concentration of the protein solution. The best crystals were obtained using the following reservoir composition: 19–21% PEG 2000 MME, 0.01 *M* $NiCl_2$, 0.1 *M* Tris–HCl buffer pH 7.5 and a protein concentration of 40 mg ml^{-1}. For subsequent experiments, the volume of the droplets was increased to 4 µl (2 µl reservoir solution and 2 µl protein solution). Crystals appeared within 10–15 d as bunches of very thin plates (Fig. 1). The dimensions of these stacked plates could reach about 0.5 mm in length, but they never grew more than 0.02 mm thick. Further attempts to obtain single and/or thicker crystals were attempted either by adding various organic compounds to the crystallization drops or by trying micro-seeding techniques. These attempts did not improve the crystal characteristics. In such a situation, harvesting crystals was a difficult task because the piles of crystals have to be carefully separated into individual pieces, a work up that always leads to significant loss of material.

Figure 1
Sylvaticin crystals.

4. Data collection and processing

The native crystals were transferred into a cryoprotectant solution containing 20% PEG 2000 MME, 0.01 *M* $NiCl_2$, 0.1 *M* Tris–HCl pH 7.5 and 20% glycerol before being picked up and frozen in a 100 K nitrogen-gas Cryostream attached to the goniometer. The first X-ray diffraction experiments were carried out using a MAR Research image-plate detector mounted on an Enraf–Nonius rotating-anode generator (Cu $K\alpha$ radiation). Subsequently, a complete data set was collected at the wiggler beamline W32, LURE facility, Orsay (Fourme *et al.*, 1992) at 100 K to a resolution of 2.1 Å.

The data were indexed, integrated and merged using the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) (see Table 1). We used the program *Matthews Probability Calculator* (Kantardjieff & Rupp, 2003) to estimate the unit-cell content. There are two possibilities: the asymmetric unit contains either one or two molecules. In the first case, the Matthews coefficient (Matthews, 1968) is 4.07 Å^3 Da^{-1} and the solvent content 69.8%; in the second case, the values were 2.04 Å^3 Da^{-1} and 39.6%, respectively. Preliminary calculations favour a monomer as deduced from (i) the self-rotation function (*POLARRFN* program; Collaborative Computational Project, Number 4, 1994) calculated with standard parameters (Fig. 2*a*) and (ii) the native Patterson map (*FFT* program) which features no translational peak, thus excluding any non-crystallographic axis fortuitously aligned along the *b* axis. However, the situation becomes less clear when optimizing the parameters, resolution and radius of integration in the self-Patterson function (Fig. 2*b*). Under these conditions, a broad peak appears at coordi-

Table 1
Data-collection statistics.

Values in parentheses refer to the outer resolution shell.

Wavelength (Å)	0.966
Space group	*C*2
Unit-cell parameters (Å, °)	a = 99.29, b = 25.67, c = 67.45, β = 99.66
No. measured reflections	71037
No. unique reflections	9827
Resolution limits (Å)	25.6-2.12 (2.20-2.12)
Data completeness (%)	99.4
$I/\sigma(I)$	19.8 (5.9)
$R_{\rm merge}$† (%)	5.4(18.5)

† $R_{\rm merge} = \sum_{\mathbf{h}} \sum_i |\langle I(\mathbf{h})\rangle - I(\mathbf{h})| / \sum_{\mathbf{h}} \sum_i I_i(\mathbf{h})$, where $I_i(\mathbf{h})$ is the ith observation of reflection $\mathbf{h}$.

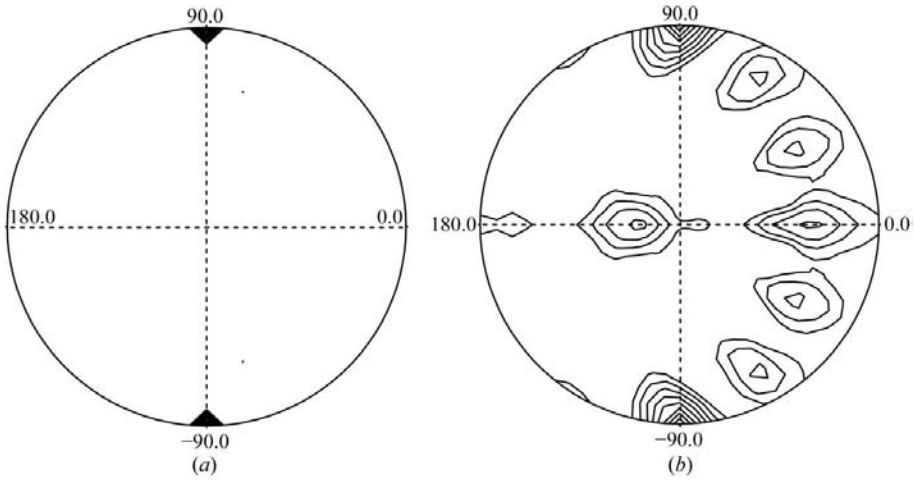

Figure 2
Self-rotation function (κ = 180°) calculated with default and optimized parameters (integration radii and resolution). Contouring is at every 10% (normalized to crystallographic axis values). (*a*) Integration radius 20–5.3 Å; (*b*) integration radius 14.6–4.0 Å.

nates ($\omega = 67$, $\varphi = 0$, $\kappa = 180^\circ$) that may reach a maximum of 40% of the normalized value of the crystallographic twofold axis, a strong indication that the structure could equally be a tight dimer. All attempts to clarify this point by density measurements were inconclusive owing to the instability of the crystals.

The first attempts to solve the structure by molecular replacement (Navaza *et al.*, 1993, 1998) with the closest models available in the elicitin family, cryptogein (Boissy *et al.*, 1996) or cinnamomin (Rodrigues *et al.*, 2002), were unsuccessful despite gross molecular similarities as deduced from sequence alignments and by the presence of the three conserved disulfide bridges. In addition, it is known that elicitins are not good candidates for binding heavy atoms (Boissy *et al.*, 1996), a problem that limits the use of MIR methods. We are currently looking at direct sulfur SAD phasing (Hendrickson & Teeter, 1981; Ramagopal *et al.*, 2003), as sylvaticin contains an unusually high number of S atoms (eight in 95 residues) compared with the average (3.2–4.3 per 100 residues) usually found in proteins.

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