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Crystallization and preliminary crystallographic studies of LipA, a secretory lipase/esterase from *Xanthomonas oryzae* pv. *oryzae*

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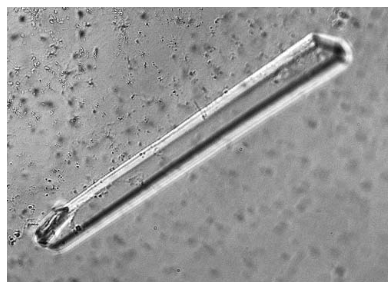
Xanthomonas oryzae pv. *oryzae* is the causal agent of bacterial leaf blight, a serious disease of rice. Several enzymes that are secreted through the type II secretion system of this bacterium play an important role in the plant–microbe interaction, being important for virulence and also being able to induce potent host defence responses. One of these enzymes is a secretory lipase/esterase, LipA, which shows a very weak homology to other bacterial lipases and gives a positive tributyrin plate assay. In this study, LipA was purified from the culture supernatant of an overexpressing clone of *X. oryzae* pv. *oryzae* and two types of crystals belonging to space group *C*2 but with two different unit-cell parameters were obtained using the hanging-drop vapour-diffusion method. Type I crystals diffract to a maximum resolution of 1.89 Å and have unit-cell parameters $a = 93.1$, $b = 62.3$, $c = 66.1$ Å, $\beta = 90.8^\circ$. Type II crystals have unit-cell parameters $a = 103.6$, $b = 54.6$, $c = 66.3$ Å, $\beta = 92.6^\circ$ and diffract to 1.86 Å. Solvent-content analysis shows one monomer in the asymmetric unit in both the crystal forms.

1. Introduction

The plant cell wall is a formidable barrier for potential pathogens. It is a complex meshwork that is primarily composed of polysaccharides such as cellulose, hemicelluloses, pectic substances *etc.* as well as lesser amounts of proteins, lipids, lignin and other constituents (Esau, 1965). In order to break down this barrier, phytopathogenic bacteria deploy a battery of cell-wall-degrading enzymes. These enzymes are therefore important virulence factors of these bacteria (reviewed in Jha *et al.*, 2005). The cell-wall-degrading enzymes secreted by microbial pathogens have been shown to be elicitors of plant defence responses (Darvill & Albersheim, 1984; Ryan & Farmer, 1991).

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight, a very serious disease of rice. This bacterium secretes several extracellular enzymes that are potentially involved in degrading rice cell walls and are important virulence factors (Ray *et al.*, 2000; Rajeshwari *et al.*, 2005; Jha *et al.*, 2007). Several of these enzymes have been characterized and shown to be a cellulase (ClsA), a putative cellobiosidase (CbsA), a xylanase (XynA) and a lipase/esterase (LipA). The ClsA, CbsA and LipA proteins have also been shown to be potent inducers of host defence responses, which are suppressed by the pathogen during infection through the action of effector proteins that are secreted through the type III secretion system (Jha *et al.*, 2007).

LipA is an ∼42 kDa protein containing the LIP domain found in secretory lipases but with a weak homology to common bacterial lipases. Bacterial secretory lipases belong to the α/β-hydrolase fold family and possess a highly conserved Ser-Asp/Glu-His catalytic triad (Jaeger *et al.*, 1999). Purified LipA gives a positive lipase/esterase assay using either tributyrin or Tween 80 as well as *p*-nitrophenyl oleate (Rajeshwari *et al.*, 2005). LipA is an important virulence factor and an elicitor of host defence responses and we are therefore interested in studying the structural features of this novel lipase/esterase.



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2. Experimental methods

2.1. Expression and purification of LipA

X. oryzae pv. *oryzae* strains were grown at 301 K in peptone–sucrose (PS) medium (Tsuchiya *et al.*, 1982). The antibiotics used in this study were rifampicin at 50 mg l^{-1} and spectinomycin at 50 mg l^{-1}. *X. oryzae* pv. *oryzae* strain BXO2001 has an insertion mutation in the *lipA* gene (Rajeshwari *et al.*, 2005). This mutation abolishes secreted lipase/esterase activity. Introduction of the *lipA* gene into BXO2001 in the broad host-range vector pHM1 (to create BXO2008) not only restores the lipase activity but also results in the overproduction of lipase. The overproduction of lipase by BXO2008 may be because the *lipA* gene in pHM1 is under the control of a constitutively expressed *lac* promoter. Wild-type *X. oryzae* pv. *oryzae* secretes the LipA protein into the extracellular medium using the type II secretion system (Rajeshwari *et al.*, 2005). The BXO2008 strain is proficient for the type II secretion system and it is expected that the overexpressed lipase is secreted into the medium through the same system. Approximately 1 mg LipA is secreted into the medium by 1 l wild-type *X. oryzae* pv. *oryzae*. The BXO2008 strain secretes approximately 25–30 mg LipA per litre of culture.

The BXO2008 strain was grown to saturation in 1 l PS medium at 301 K. LipA was purified from the BXO2008 culture supernatant as described previously (Jha *et al.*, 2007) using cation-exchange chromatography. The peaks containing pure LipA were pooled and loaded onto a 24 ml Superose-12 gel-filtration column (GE Pharmacia, USA) pre-equilibrated with 10 m*M* potassium phosphate buffer pH 6.0. The purified protein was dialyzed against 20 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl and concentrated to 5 mg ml^{-1} using a 10 kDa Amicon Ultra-15 filtration device (Millipore, USA). Protein concentration was determined using BCA reagent (Pierce, USA).

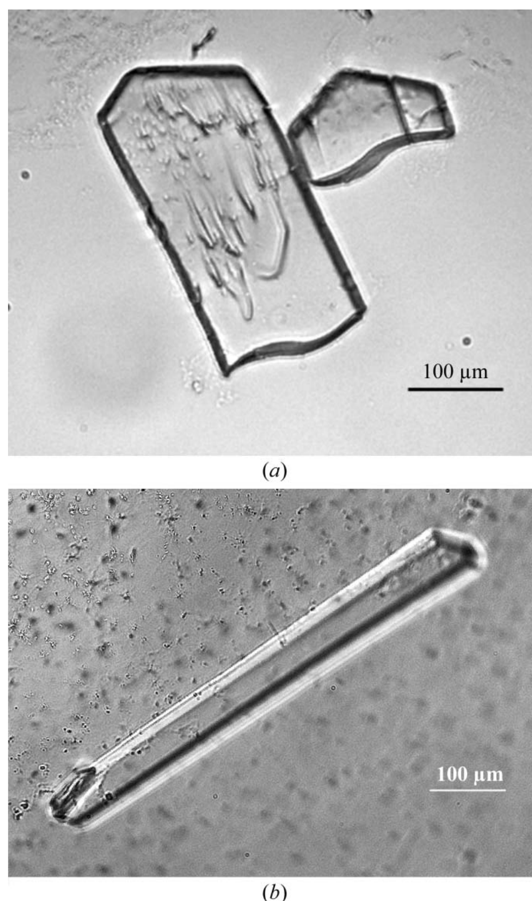

Figure 1
Crystals of LipA. (*a*) Plate-shaped type I crystals. (*b*) Rod-shaped type II crystals.

Table 1
Crystallographic statistics.

Values in parentheses are for the highest resolution shell.

	Type I	Type II
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	a = 93.1, b = 62.3, c = 66.1, β = 90.8	a = 103.6, b = 54.3, c = 66.3, β = 92.6
Unit-cell volume (Å^3)	384213.4	375639.2
Resolution (Å)	25.0–1.89 (1.96–1.89)	25.0–1.86 (1.93–1.86)
Observations	67204	108949
Unique reflections	27328 (2264)	30943 (2979)
Completeness (%)	90.1 (75.5)	99.0 (96.2)
Redundancy	2.5 (2.2)	3.5 (3.2)
R_{merge} (%)	5.6 (20.9)	3.8 (11.6)
$I/\sigma(I)$	18.4 (3.3)	11.3 (3.7)
V_M (Å^3 Da^{-1})	2.45	2.40
Solvent content (%)	49.7	48.5
Molecules per ASU	1	1

2.2. Crystallization

Crystal Screens I and II (Hampton Research, USA) were used to screen for initial crystallization conditions. The drops were set up using the hanging-drop vapour-diffusion method by mixing equal volumes (2 µl) of protein solution and reservoir solution at 298 K. Small needles and clusters were obtained within 24 h in Crystal Screen I condition Nos. 28, 37 and 41, and Crystal Screen II condition Nos. 35 and 38. Crystals that diffracted well were obtained after refining the initial conditions with PEG 400 and PEG 6000 as precipitants and 0.10 *M* MES buffer pH 6.0–7.0.

2.3. Data collection and processing

An in-house MAR Research MAR345dtb image-plate detector and Cu $K\alpha$ X-rays of wavelength 1.54 Å generated by a Rigaku RU-H3R rotating-anode generator were used to collect diffraction data. The crystal was mounted on a nylon loop and flash-cooled in a nitrogen-gas stream at 100 K using an Oxford Cryostream system. No cryoprotectant was used for the type I crystal. 15% glycerol in mother liquor was used as a cryoprotectant for the type II crystal. Data were collected with an oscillation angle of 0.5° and an exposure time of 600 s for each image. A total of 120° and 180° of oscillation data was collected for the type I and type II crystals, respectively. Indexing, scaling and merging of the data were performed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results

LipA was overexpressed in the secreted form by cultures of BXO2008 grown in peptone–sucrose medium. The protein was purified to homogeneity and crystallized at a concentration of 5 mg ml^{-1} at 298 K. Needles and clusters were obtained using Hampton Research Crystal Screens I and II and the best conditions were used for expansion to obtain single mountable crystals of two types. Plate-shaped type I crystals (Fig. 1*a*) were obtained using 48% PEG 400, 0.10 *M* MES pH 6.0. A crystal of this type diffracted to 1.89 Å resolution. The unit-cell parameters were found to be a = 93.1, b = 62.3, c = 66.1 Å, β = 90.8° (Fig. 1*b*). The crystal belonged to space group *C*2 and the mosaicity of the crystal was 0.98°. The R_{merge} of the data set was 5.6% (Table 1).

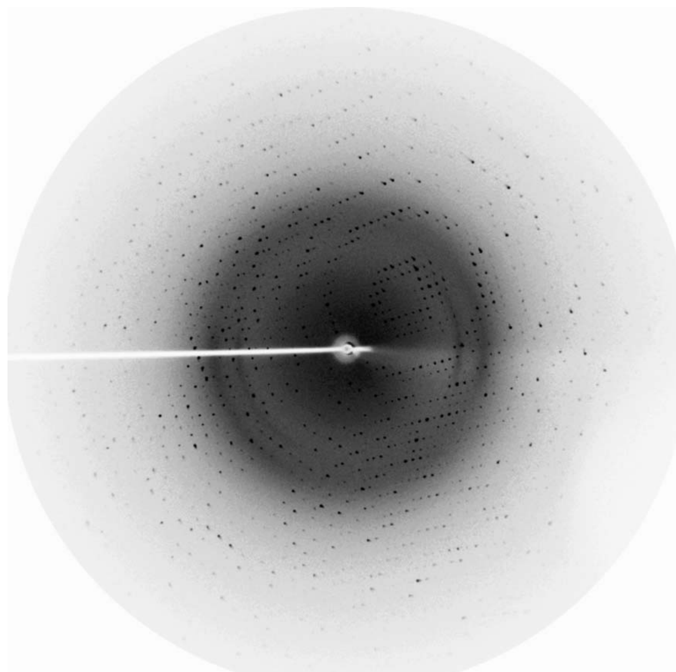

Figure 2
A diffraction image from a type II LipA crystal. The edge corresponds to a resolution of 1.86 Å.

The rod-shaped type II crystals also belonged to space group *C*2, but had different unit-cell parameters of $a = 103.6$, $b = 54.6$, $c = 66.3$ Å, $\beta = 92.6°$. The crystals were obtained from a reservoir solution containing 12% PEG 6000, 0.10 *M* MES pH 6.7 (Fig. 1*b*) and diffracted to 1.86 Å resolution (Fig. 2) with a mosaicity of 0.44°. The data set was 99% complete, with an $R_{\rm merge}$ of 3.8% (Table 1). Molecular-replacement attempts using the programs *MOLREP* (Vagin & Teplyakov, 1997) and *Phaser* (Read, 2001; Storoni *et al.*, 2004) as a part of the *CCP*4 package (Computational Collaborative Project, Number 4, 1994) with several structural homologues of LipA as search models did not yield any clear solution. This failure could be attributed to the low sequence identity of only around 20% and also the fact that α/β-hydrolase fold proteins can have very different curvatures of the core β-sheet. Since the type II crystal form is more reproducible, we are using this crystal form for a heavy-atom search in order to solve the LipA structure using the multiple isomorphous replacement method.

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