crystallization communications

Crystallization and preliminary crystallographic analysis of the ADP-ribosyltransferase HopU1

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Several Gram-negative pathogens of plants and animals and some eukaryotic associated bacteria use type III protein-secretion systems (T3SSs) to deliver bacterial virulence-associated 'effector' proteins directly into host cells. HopU1 is a type III effector protein from the plant pathogen *Pseudomonas syringae*, which causes plant bacterial speck disease. HopU1 quells host immunity through ADP-ribosylation of GRP7 as a substrate. HopU1 has been reported as the first ADP-ribosyltransferase virulence protein to be identified in a plant pathogen. Although several structures of ADP-ribosyltransferases have been determined to date, no structure of an ADP-ribosyltransferase from a plant pathogen has been determined. Here, the protein expression, purification, crystallization and preliminary crystallographic analysis of HopU1 are reported. Diffracting crystals were grown by hanging-drop vapour diffusion using polyethylene glycol 10 000 as a precipitant. Native and SAD data sets were collected using native and selenomethionine-derivative HopU1 crystals. The diffraction pattern of the crystal extended to 2.7 Å resolution using synchrotron radiation. The crystals belonged to space group $P4_3$, with unit-cell parameters $a = 92.6$, $b = 92.6$, $c = 101.6$ Å.

1. Introduction

Several Gram-negative pathogens of plants and animals and other eukaryotic associated bacteria use type III protein-secretion systems (T3SSs; He *et al.*, 2004). The T3SS is a protein structure; it is an organelle that secretes proteins that deliver bacterial virulence-associated 'effector' proteins directly into host cells using a needle-like structure that is a hallmark of the T3SS (Galan & Wolf-Watz, 2006).

Bacteria use T3SSs to modulate host physiology. In animal cells, their activities alter specific host-cell functions, including phago-cytosis, pro-inflammatory responses, apoptosis and intracellular trafficking (Mota *et al.*, 2005; Mota & Cornelis, 2005). Many type III effectors from plant pathogens suppress host immune responses (Abramovitch *et al.*, 2006; Espinosa & Alfano, 2004). Plants have evolved a sophisticated innate immune system to recognize invading pathogens and to induce a set of host defence mechanisms that result in disease resistance. To date, effectors that possess ADP-ribosyl-transferase (Fu *et al.*, 2007), tyrosine phosphatase (Bretz *et al.*, 2003), E3 ubiquitin ligase (Axtell & Staskawicz, 2003) and cysteine protease activities (Lopez-Solanilla *et al.*, 2004) have been implicated in suppression of plant innate immunity.

ADP-ribosyltransferases belong to a group of enzymes that use NAD^+ as a cofactor (Koch-Nolte & Haag, 1997). These enzymes transfer the ADP-ribose moiety onto specific targets at Arg, Cys, Asn or Glu residues. Protein ADP-ribosylation is reversible and is used for functional regulation (Mueller-Dieckmann *et al.*, 2002).

Pseudomonas syringae pv. *tomato*, the causative agent of bacterial speck disease of tomato (Whalen *et al.*, 1991), uses a T3SS to deliver effector proteins into the host cell. Fu and coworkers identified the first type III effector ADP-ribosyltransferase HopU1 from *P. syringae* and GRP7 as its substrate (Fu *et al.*, 2007). The activity of GRP7 may be disrupted after ADP-ribosylation by HopU1, which quells host innate immunity (Fu *et al.*, 2007).

Several structures of ADP-ribosyltransferases from animal pathogens have been determined. These structures and extensive

functional studies have provided a detailed understanding of the molecular mechanism of these proteins and their effects on host cells (Lopez-Solanilla *et al.*, 2004; Bretz *et al.*, 2003). However, no structure of a plant pathogen ADP-ribosyltransferase has been reported to date. The structure of HopU1 and the mechanism of its interaction with its substrate GRP7 remain unknown. As part of our ongoing studies of type III effector proteins, HopU1 was overexpressed in *Escherichia coli*, purified to homogeneity and crystallized. Here, we report the preparation, purification, crystallization and preliminary crystallographic studies of HopU1.

2. Materials and methods

2.1. Protein expression and purification

The ORF of full-length *hopu1* was engineered into modified pGEX-6P-1 vector (GE Healthcare) using *Nde*I and *Xho*I restriction sites. The construct was verified by DNA sequencing and the plasmid was transformed into *E. coli* strain Rosetta competent cells. The transformants were grown at 310 K to an OD_{600} of 0.6 in Luria broth medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol and were induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside. HopU1 was expressed as a fusion protein with a GST tag at the N-terminus. After a further 12 h incubation at 288 K, the cells were pelleted and resuspended in lysis buffer consisting of

25 mM Tris pH 8.0, 150 mM NaCl supplemented with DNAse and protease inhibitors. Cells were lysed on ice using a French press and the solution was clarified by centrifugation at 12 000 rev min^{-1} for 25 min at 277 K. The supernatant was applied onto six Glutathione Sepharose 4B columns (1 ml resin per column; GE Healthcare) equilibrated with lysis buffer. After washing with buffer consisting of 25 mM Tris pH 8.0, 150 mM NaCl, the fusion protein was digested on the columns with PreScission (3C) protease overnight at 277 K. The molecular weight of the 3C-digested protein was 30.5 kDa, including an additional Gly-Pro-Leu-Gly-Ser-His sequence from 3C cleavage and full-length HopU1. The eluted protein was loaded onto a Source 15Q anion-exchange column (GE Healthcare) and eluted with a linear gradient of 0–0.5 M NaCl at a flow rate of 10 ml min^{-1}. The peak fractions were collected and further purified by gel-filtration chromatography on a Superdex 200 column (GE Healthcare) with buffer consisting of 10 mM Tris pH 8.0, 50 mM NaCl and 3 mM DTT. HopU1-containing fractions were concentrated to 10 mg ml^{-1} using an ultracentrifugal filter tube (Millipore) and used for crystallization.

The selenomethionine derivative of HopU1 protein was expressed using *E. coli* strain BL21 (DE3) cultured in M9 minimal medium supplemented with 100 mg l^{-1} lysine, 100 mg l^{-1} phenylalanine, 100 mg l^{-1} threonine, 50 mg l^{-1} isoleucine, 50 mg l^{-1} leucine, 50 mg l^{-1} valine and 25 mg l^{-1} selenomethionine (Acros). Expression and purification procedures were performed as for wild-type HopU1. Full incorporation of selenomethionine was verified by ESI mass spectrometry.
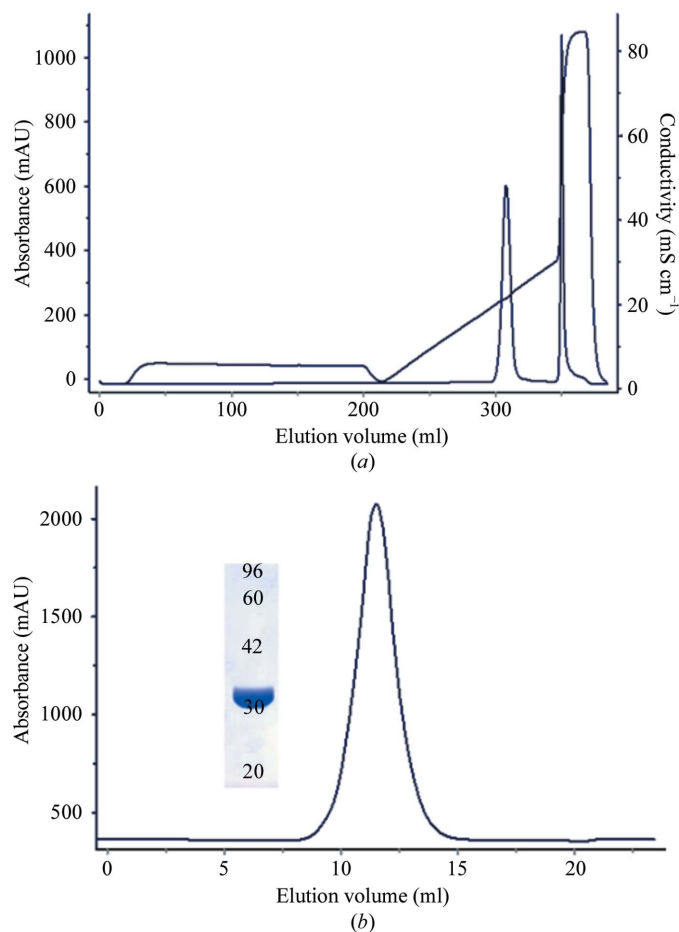
2.2. Protein crystallization

Initial crystallization trials were performed using Crystal Screen, Index, SaltRX and PEG/Ion kits from Hampton Research and Wizard I and II kits from Emerald BioSystems at 293 and 277 K. These initial screens were set up using the hanging-drop vapour-diffusion method by mixing 1 µl protein solution and 1 µl reservoir solution. Initial conditions yielding crystals were further optimized by variation of the pH, protein concentration, precipitants and additives. A total of approximately 500 conditions were set up for optimization.

2.3. Data collection and processing

All crystals were mounted in nylon loops and flash-cooled in liquid nitrogen using reservoir buffer as cryoprotectant. Data collections were carried out on Photon Factory beamline BL17A (Japan) and BL17U at SSRF (People's Republic of China) using CCD detectors.
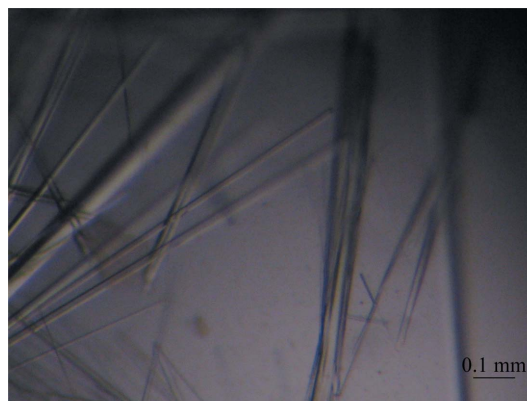

Figure 1
HopU1 protein purification. (*a*) Ion-exchange (Source 15Q, GE Healthcare) profile of HopU1 protein. (*b*) Gel-filtration (Superdex 200 10/300 GL; GE Healthcare) profile of HopU1 protein. Inset: one of the peak fractions was analyzed by SDS–PAGE with Coomassie Blue staining. Molecular weights are indicated in kDa.

Figure 2
Crystals of selenomethionine-derivative HopU1 grown in 0.1 M HEPES pH 7.3, 5% PEG 10 000, 8% ethylene glycol at 277 K.
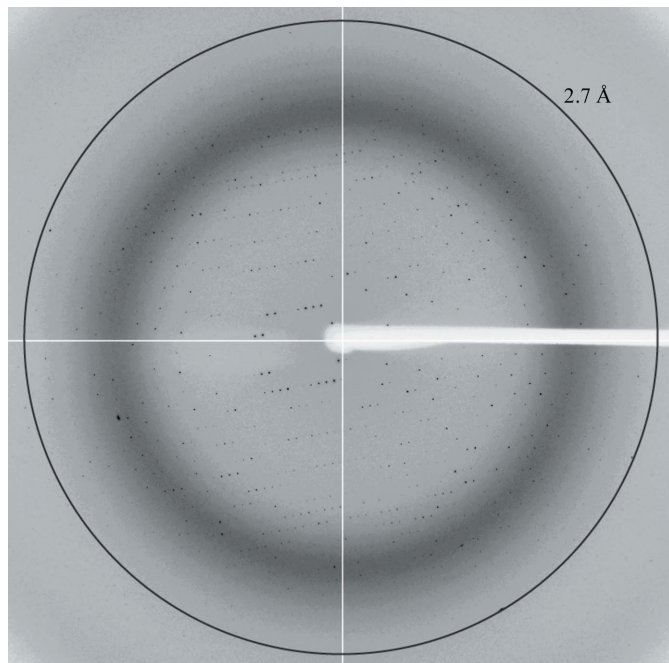
Figure 3
X-ray diffraction pattern of the crystal of selenomethionine-derivative HopU1. The ring indicates a resolution of 2.7 Å.

Data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant HopU1 protein was expressed, purified to homogeneity and used for crystallization (Figs. 1*a* and 1*b*). The purified HopU1 protein was estimated to be >98% pure (Fig. 1*b*, inset). Initial screening was performed at 293 K and the HopU1 protein precipitated out. We then set up screening at 277 K and crystals appeared in several conditions. After a further optimization screen of solution buffer pH, precipitants and additives, we obtained crystals that were suitable for diffraction. The crystals (Fig. 2) used for data collection were obtained by mixing 1 µl protein solution (10 mg ml⁻¹) with 1 µl reservoir solution consisting of 0.1 *M* HEPES pH 7.3, 5% PEG 10 000, 8% ethylene glycol at 277 K. Long rod-shaped crystals appeared after 2 d and reached their maximum dimensions one week later. Crystals of the selenomethionine derivative of HopU1 were grown under similar conditions.

As no similar structure was found in the Protein Data Bank based on amino-acid sequence similarity, we produced crystals of a selenomethionyl derivative of the HopU1 protein in order to solve the phase problem. There are nine methionines in the HopU1 sequence. Se-SAD (single-wavelength anomalous diffraction) data were collected from a single crystal at a wavelength of 0.9788 Å (Fig. 3; Hendrickson, 1991). A native data set was also collected for HopU1 from a single crystal; the diffraction extended to 2.7 Å resolution. The CCD used for data collection on SSRF BL17U was a MAR CCD

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

	HopU1	SeMet HopU1
Wavelength (Å)	0.9788	0.9788
Resolution (Å)	50.00–2.70 (2.80–2.70)	50.00–2.70 (2.80–2.70)
Space group	$P4_3$	$P4_3$
Unit-cell parameters (Å)	$a = b = 92.6, c = 101.6$	$a = b = 92.6, c = 101.6$
Completeness (%)	99.9 (99.7)	99.9 (99.8)
R_{merge} (%)†	14.2 (52.8)	14.4 (51.9)
$I/\sigma(I)$	10.5 (2.8)	13.7 (4.4)
Redundancy	7.4 (7.2)	7.5 (7.2)
Total No. of reflections	175402 (17035)	177405 (16985)
No. of unique reflections	23703 (2366)	23654 (2359)

† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th measurement of the intensity of reflection *hkl* and $\langle I(hkl) \rangle$ is the mean intensity of reflection *hkl*.

MX-225 detector. The crystals belonged to space group $P4_3$, with unit-cell parameters $a = b = 92.6$, $c = 101.6$ Å. Data-collection statistics are summarized in Table 1. Determination of the structure using the SAD phasing method is under way.

The crystal structure of HopU1 will deepen our understanding of the molecular mechanism of T3SS effector proteins in plant host cells.

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