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Crystallization and preliminary crystallographic studies of CbsA, a secretory exoglucanase from *Xanthomonas oryzae* pv. *oryzae*

The bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* causes bacterial leaf blight, a serious disease of rice. The secreted exoglucanase CbsA is an important virulence factor of this pathogen. It belongs to the glycosyl hydrolase 6 family of proteins based on the carbohydrate-active enzyme (CAZY) classification. In this study, CbsA has been overexpressed, purified and crystallized. The crystal diffracted to a resolution of 1.86 Å and belonged to space group $P2_12_12_1$. It contained one monomer per asymmetric unit, with a solvent content of 45.8%.

1. Introduction

The plant cell wall is composed of a complex array of polysaccharides including cellulose, hemicellulose and pectin as well as lesser amounts of proteins, lipids and lignin. The cell wall serves as a formidable barrier to microbial plant pathogens. Almost all microbial plant pathogens deploy an array of hydrolytic enzymes such as cellulases, xylanases, pectinases, proteases *etc.* to degrade the plant cell wall (Béguin & Aubert, 1994).

Xanthomonas oryzae pv. *oryzae* is the causative agent of bacterial leaf blight, a serious disease which can lead to up to a 50% loss of yield in rice (*Oryza sativa*). The pathogen secretes a number of hydrolytic enzymes using a type II secretion system to degrade the rice cell wall (Ray *et al.*, 2000; Rajeshwari *et al.*, 2005). In addition to a xylanase (XynA) and a lipase/esterase (LipA), the pathogen employs exoglucanases (Rouvinen *et al.*, 1990) and endoglucanases (Davies *et al.*, 2000) for degradation of the cell wall. As part of an ongoing effort to characterize the secreted cell-wall-degrading enzymes, we have previously crystallized and solved the structure of LipA from *X. oryzae* pv. *oryzae* (Aparna *et al.*, 2007, 2009). The structural analysis showed certain unique features that have been acquired by LipA to act on the rice cell wall. In order to further explore the components of the secretome, we have undertaken a structure–function study on an *X. oryzae* pv. *oryzae* exoglucanase homologue termed CbsA.

Based on sequence analysis, the putative CbsA shows strong similarity (30–40%) to exoglucanases. It consists of an N-terminal catalytic domain (exoglucanase) and a C-terminal fibronectin type III domain (Little *et al.*, 1994). The catalytic domain belongs to the α/β protein fold, while the C-terminal domain belongs to the fibronectin fold. It is classified into the glycosyl hydrolase 6 (GH-6) family of proteins based on the CAZY classification (Cantarel *et al.*, 2009). Deciphering the structure and function of the enzyme would provide a better understanding of the host–pathogen interactions during pathogenesis.

2. Materials and methods

2.1. CbsA production

Genomic clones from a cosmid (pUFR034; DeFeyer *et al.*, 1990) library of *X. oryzae* pv. *oryzae* strain BXO43 (the wild-type strain of

this bacterium) maintained in *Escherichia coli* strain S17-1 (Rajagopal *et al.*, 1999) were screened with primers (forward, GCGAG-CTACCACTGACGCA; reverse, GTTGTAGAGCGCTTCCGCA) that amplify a 500 bp internal fragment of the *chsA* gene. A fragment of the expected size was amplified from cosmid clone pGS1 (Table 1). Another two primer pairs were designed to amplify junction fragments between the *chsA* gene and the two flanking chromosomal regions as expected from the genome sequence of *X. oryzae* pv. *oryzae* (Lee *et al.*, 2005). PCR-amplified fragments of the expected size were amplified using pGS1 cosmid DNA as a template. The identity of these fragments was confirmed by sequencing. The pGS1 plasmid was subsequently isolated and electroporated into *X. oryzae* pv. *oryzae* strain BXO43 to generate strain BXO2700. The BXO2700 strain thus had a chromosomal copy of the *chsA* gene as well as copies of the gene on the low-copy-number plasmid pGS1. CbsA (~56 kDa) was overexpressed by growing BXO2700 in peptone–sucrose (PS; Tsuchiya *et al.*, 1982) medium containing kanamycin (50 mg ml⁻¹) at 301 K for 48 h. The BXO2700 strain invokes the type II secretion system (Jha *et al.*, 2007) and overexpressed CbsA is therefore released into the medium. The culture was centrifuged at 6000 rev min⁻¹ for 15 min at 277 K to pellet the bacteria and the protein was retained in the supernatant. All purification steps were carried out at room temperature (298 K). The secreted protein in the medium was initially purified by 55% ammonium sulfate precipitation followed by centrifugation and resuspension in 0.01 M potassium phosphate buffer pH 6.6. After resuspension, dialysis was performed with 0.01 M potassium phosphate buffer pH 6.6 to remove residual ammonium sulfate and extracellular polysaccharides. Cation-exchange chromatography was performed on a Mono S column with a linear gradient from 0.01 M potassium phosphate buffer pH 6.6 (buffer A) to 0.01 M potassium phosphate buffer pH 6.6 and 1 M NaCl (buffer B). Subsequently, the protein was purified using size-exclusion chromatography (Superdex 200) to approximately 95% homogeneity in a buffer consisting of 0.02 M Tris–HCl pH 8.0, 0.02 M NaCl. The purity of the protein was checked by SDS–PAGE. The protein was concentrated using an Amicon Ultra-4 PLGC Ultracel-PL membrane (Merck Millipore, USA) 10 kDa centrifugal filter unit at 3500 rev min⁻¹ and 277 K. The concentration of the protein was estimated from its absorbance at 280 nm as determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Soon after purification, the crystallization experiment was set up.

2.2. Crystallization

Initial screening for crystallization was performed by the sitting-drop vapour-diffusion method with Index Screen (Hampton Research, USA) at 277 K using an in-house high-throughput crystallization facility. The crystallization drops were set up using a Mosquito robot (TTP LabTech, UK) in a 96-well MRC plate (Hampton Research). To set up the crystallization experiment, a pre-crystallization test (PCT; Hampton Research) was performed at both 277 and 293 K. The test indicated better crystallization behaviour at 277 K in a protein concentration range of 3–6 mg ml⁻¹. Therefore, we limited our screening to 277 K. Multiple protein concentrations such as 3, 5 and 6 mg ml⁻¹ were used to set up crystallization drops. The initial hit [Index screen, Hampton Research; 25% (w/v) polyethylene glycol (PEG) 3350, 0.1 M citric acid pH 3.5] was obtained after approximately 15 d. Based on this condition, further optimization was performed using the hanging-drop vapour-diffusion method. For optimization, two-dimensional gradients consisting of variation of the pH of 0.1 M citric acid in the range 3.0–6.5 (linear gradient of pH 0.5)

Table 1

Macromolecule information.

Name of protein	CbsA
Source organism	<i>X. oryzae</i> pv. <i>oryzae</i> strain BXO43
DNA source	<i>X. oryzae</i> pv. <i>oryzae</i>
Cloning vector	pUFR034
Expression vector	pUFR034 cosmid clone, pGS1
Expression host	<i>X. oryzae</i> pv. <i>oryzae</i>
Complete amino-acid sequence of the construct	MSSFTNLPCCVTSKLAVALLTGALLVPVAAASQSHV-DNPFVGASGYVNPDSKEVDSSIVKVKDVQLK-AKMQVVKSYPTVYVWLDSDIAIYGGSRNAGRSL-LOGHLNAAALQKANTPITVGLVIYDMPGRDC-HALASNGELPLTQAGLQRYKTEYIDVIASTLAN-PKYKGLRIVNIEPDSLNLVNTQSTPACGOASS-GIYEAGIKYALDKLHAIPNVYNYMDIGHSGWL-AWRSNMTPAISLYTRVVGTAAGLASADGFTIT-TANYTPLHEPNLPNPDLTIGGQPISSSTFYQWN-SVFDESTYAEVLYNAFVGAGWPSKIGFLIDTGR-NGWGGASARPTSASGNDVNTYVNSGRVDRRLH-RGNWCNQSGAGIGMPPTAAPGGHIIHAYVWGK-GGGESDGGSSKIYPNKQKGFDYCDPTYTTPD-GTLTGALPNAPIAGTWFHAFVQLVTNAYPAIG-TSTKAALQSASTDAVPTSLPTAIKGLTANAADG-KVRLSWSPVSGATGYTVQRFEEAAAAPITVAS-GLTWSSYVDQALNTGTTYKYKVTANGASGAGA-SSVTVSATPHR

and variation of the PEG 3350 concentration from 20 to 29% (w/v) (linear gradient of 1%) was performed.

2.3. Data collection and processing

Data were collected using a MAR Research MAR345dtb image-plate detector and Cu K α X-rays of wavelength 1.54 Å generated by a Rigaku MicroMax-007 HF microfocus rotating Cu-anode generator. External cryoprotectant treatment was not used as the PEG 3350 present in the crystallization drop acted as a cryoprotectant. The crystal was mounted on a nylon loop and flash-cooled in a nitrogen-gas stream at 100 K using an Oxford Cryostream system (Oxford Cryosystems). An oscillation width of 0.5° and an oscillation range of 180° with an exposure time of 120 s per image were used for data collection. Indexing was performed using *automar* (<http://www.marresearch.com/automar/>). Subsequent data processing was performed using *HKL-2000* (Otwinowski & Minor, 1997).

Figure 1

Purification of CbsA using a three-step purification protocol, as shown by 12% SDS–PAGE analysis. Lanes 1–5 contain CbsA after cation-exchange (Mono S) chromatography, lanes 6–8 show other proteins after cation-exchange (Mono S) chromatography, lane M contains molecular-weight marker (labelled on the right in kDa) and lane 9 contains purified CbsA after size-exclusion chromatography.

Table 2
Crystallization conditions.

Method	Hanging-drop vapour diffusion
Plate type	Iwaki 24-well plate
Temperature (K)	277
Protein concentration (mg ml ⁻¹)	5
Buffer composition of protein solution	0.02 M Tris-HCl pH 8.0, 0.02 M NaCl
Composition of reservoir solution	22% (w/v) PEG 3350, 0.1 M citric acid pH 3.5
Volume and ratio of drop	2 µl protein and 2 µl reservoir, 1:1
Volume of reservoir (µl)	750

Table 3
Data-collection statistics.

Values in parentheses are for the outer shell.

Diffraction source	Rigaku MicroMax-007 HF Cu
Wavelength (Å)	1.54
Temperature (K)	100
Detector	MAR345dtb
Crystal-to-detector distance (mm)	150
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	120
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 46.14, <i>b</i> = 90.72, <i>c</i> = 99.78, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.9
Resolution range (Å)	50–1.86 (1.93–1.86)
Total No. of reflections	226399 (17961)
No. of unique reflections	33791 (3151)
Completeness (%)	97.8 (96.6)
Multiplicity	6.7 (5.7)
$\langle I/\sigma(I) \rangle$	25.8 (5.4)
$R_{\text{r.i.m}}^\dagger$ (%)	4.5 (19.0)
R_{merge} (%)	4.2 (17.3)
Overall <i>B</i> factor from Wilson plot (Å ²)	17.4

$^\dagger R_{\text{r.i.m}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

3. Results and discussion

CbsA was overexpressed in *X. oryzae* pv. *oryzae* strain BXO2700 in secreted form in PS medium. The protein was purified to homogeneity using ammonium sulfate precipitation followed by cation-exchange and size-exclusion chromatography. Analysis of the purified CbsA protein band revealed that it was close to a 45 kDa marker protein, which prompted us to check whether the protein had been truncated (Fig. 1). MS/MS tandem mass-spectrometric analysis of the protein band revealed that all of the fragments corresponded to the

Figure 2
Crystal of CbsA obtained by the hanging-drop vapour-diffusion method at 277 K. The protein crystallized in space group *P*2₁2₁2₁.

N-terminal catalytic CbsA domain (data not shown). The truncation could have arisen from the activity of one or more *X. oryzae* pv. *oryzae* proteases that were present in the extracellular medium. Mutations in the genes for these secreted proteases might result in accumulation of the full-length protein. The secreted pectate lyase Pell of *Erwinia chrysanthemi* has previously been shown to be truncated owing to the activity of a secreted protease (Shevchik *et al.*,

Figure 3
Representative diffraction image of CbsA. (a) The crystal diffracted to 1.86 Å resolution (at the edge). (b) Enlargement of a high-resolution region.

1998; Creze *et al.*, 2008). It is interesting to note that the truncated regions of CbsA and PelI both contain a fibronectin type III domain.

Overexpression of CbsA yielded approximately 4 mg protein from 1 l culture. For crystallization of the purified CbsA, crystallization drops were set up using 2 µl of the protein at a concentration of 5 mg ml⁻¹ in 0.02 M Tris-HCl pH 8.0, 0.02 M NaCl and 2 µl reservoir solution (Table 2). The volume of reservoir solution used was 750 µl in each case. Well diffracting crystals were obtained in a crystallization condition consisting of 22%(w/v) PEG 3350, 0.1 M citric acid pH 3.5 (Fig. 2) after a duration of approximately 10–12 d. A single crystal of CbsA diffracted to a resolution of 1.86 Å (Fig. 3). The mosaicity of the crystal was 0.9°. The crystal belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 46.14, *b* = 90.72, *c* = 99.78 Å (Table 3). Attempts to solve the structure by molecular replacement are under way using cellobiohydrolase II from *Trichoderma reesei* (PDB entry 1qk0; Rouvinen *et al.*, 1990), which shares approximately 33% sequence identity with CbsA, as the search model.

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