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Expression, crystallization and preliminary X-ray crystallographic analysis of Xoo2316, a predicted 6-phosphogluconolactonase, from *Xanthomonas oryzae* pv. *oryzae*

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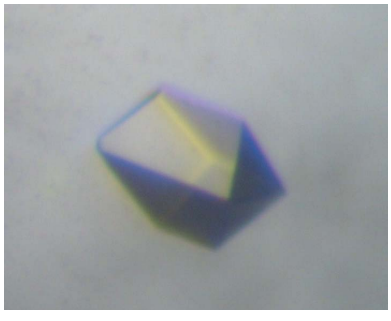

Xanthomonas oryzae pv. *oryzae* (Xoo) causes bacterial blight, which is one of the most devastating diseases of rice (*Oryza sativa* L.) in many rice-growing countries. The coding sequence of Xoo2316 (a predicted 6-phosphogluconolactonase; 6PGL) from Xoo was cloned and expressed in *Escherichia coli*. 6PGL is an enzyme that is involved in the second step of the pentose phosphate pathway, which is essential for the synthesis of nucleotide sugars and NADPH, the main source of reducing power. The protein was purified and crystallized in order to elucidate the molecular basis for its enzymatic reaction. Native crystals diffracted to 2.4 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 40.0, b = 65.1, c = 78.8 Å. A monomer exists in the asymmetric unit with a corresponding V_M of 1.93 Å^3 Da^{-1} and a solvent content of 36.5%.

1. Introduction

Rice (*Oryza sativa* L.) is an important staple for human consumption, especially in East, South and South East Asia. However, its production can be decreased greatly as a consequence of bacterial blight (BB), the most destructive bacterial disease of rice, which is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo; Ezuka & Kaku, 2000). In 2006, BB resulted in a loss in production worth 100 million dollars just in South Korea. However, to date no effective antibacterial drugs for Xoo have been developed. Recently, the whole genome sequence of Xoo was determined (Lee *et al.*, 2005; Ochiai *et al.*, 2005), providing valuable information for the management of Xoo. As the first step towards the development of antibacterial drugs against Xoo, we selected approximately 100 genes coding essential enzymes (Payne *et al.*, 2004, 2007) from 4538 putative Xoo genes (Kim *et al.*, 2008; Lee *et al.*, 2005) as drug-target protein candidates. The genes coding these drug targets have been cloned and expressed in *Escherichia coli* in order to pursue further structural study.

One of the target proteins was Xoo2316, a predicted 6-phosphogluconolactonase (6PGL; Opperdoes & Michels, 2001). A *PSI-BLAST* search showed that Xoo2316 shares 23% amino-acid sequence identity to both rice and human 6PGL (Altschul *et al.*, 1997). The 6-phosphogluconolactonase activity of Xoo2316 was confirmed by *in vitro* enzymatic assays (data not shown). In most organisms glucose is metabolized *via* two major pathways: the glycolytic pathway and the pentose phosphate pathway (PPP). The PPP has two distinct and consecutive branches: the oxidative branch and the non-oxidative branch. The oxidative branch of PPP converts glucose 6-phosphate into ribose 5-phosphate, generating two molecules of NADPH. 6PGL is the second enzyme in the oxidative branch (Zubay, 1993). The non-oxidative branch converts ribose 5-phosphate into glyceraldehyde 3-phosphate and fructose 6-phosphate. The ribose 5-phosphate provided by the PPP is required for nucleotide biosynthesis and NADPH acts as a strong reducing agent within the cell (Zubay, 1993). The biosynthesis of nucleotides and a balance between oxidative and reductive power in a cell are essential for bacterial cell growth.

This study describes the cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of Xoo2316. Three-dimensional structural studies of Xoo2316 will elucidate the molecular basis for its enzymatic reaction as a 6PGL and be useful for the design of a potential antibacterial drug against Xoo.

2. Methods

2.1. Cloning

The coding sequence of Xoo2316 was amplified *via* the polymerase chain reaction (PCR) using bacterial cells (Xoo KACC10331 strain) as a template. The sequences of the forward and reverse oligonucleotide primers designed based on the published genome sequence (Lee *et al.*, 2005) were as follows: 5′-GGG GGG **CAT ATG** AAT CTG CAG GAC AAC CCG CGC A-3′ and 5′-GGG GG**G GAT CC**T CAA GGA CAC CAA TGC ACC CGC A-3′, respectively. The bases in bold designate *Nde*I and *Bam*HI restriction sites. The amplicon was double-digested with the two DNA-restriction enzymes and inserted into a modified pET11a vector (His-TEV-pET11a) which was engineered to obtain additional residues consisting of a 6×His tag and a tobacco etch virus (TEV) protease cleavage site before the *Nde*I site in the pET11a vector (Novagen) in order to facilitate purification of the expressed protein.

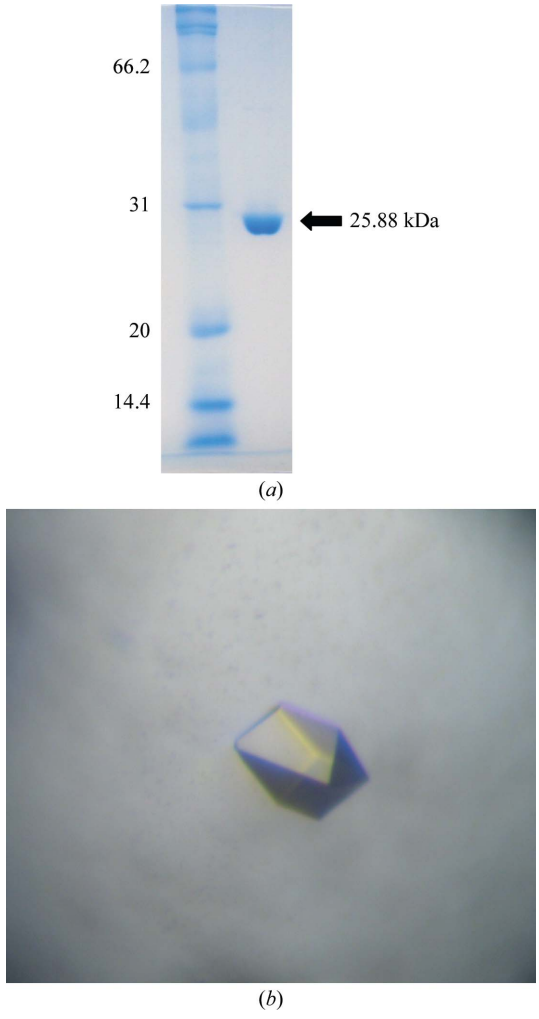

Figure 1
12% SDS–PAGE of purified Xoo2316. The left lane contains molecular-weight markers (kDa). (*b*) Crystals of Xoo2316 grew to dimensions of 0.1 × 0.1 × 0.15 mm in a week.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

	Native data set	MAD data set		
Unit-cell parameters (Å)	a = 39.7, b = 65.1, c = 78.8	a = 39.9, b = 78.9, c = 194.7		
Wavelength (Å)	1.23985	0.97953	0.97967	0.98747
Space group	$P2_12_12_1$	$P2_12_12_1$		
Resolution (Å)	39.8–2.4 (2.5–2.4)	50.0–2.5 (2.6–2.5)	50.0–2.5 (2.6–2.5)	50.0–2.5 (2.6–2.5)
No. of observations	122030	76565	80649	79228
No. of unique reflections	8815	20803	20830	20625
Completeness (%)	99.9 (99.6)	91.3 (89.7)	91.2 (93.3)	92.8 (91.9)
$R_{\rm merge}$†	4.6 (14.0)	9.8 (28.8)	11.4 (30.9)	8.8 (24.4)
$I/\sigma(I)$	53.6 (18.5)	15.4 (4.1)	16.7 (4.8)	19.0 (5.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

2.2. Overexpression and purification

The His-TEV-pET11a expression vector containing the coding sequence of Xoo2316 was introduced into *Escherichia coli* BL21 (DE3) cells. The cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin. Protein expression was induced by the addition of 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside and the cells were cultured at 288 K for an additional 16 h. Cultured cells were harvested by centrifugation for 10 min at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) and 277 K. The cell pellet was then resuspended in ice-cold lysis buffer [25 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 20% glycerol, 5 m*M* dithiothreitol (DTT)] and homogenized using ultrasonication (Sonosmasher, S&T Science, Korea). The lysate was centrifuged for 30 min at 12 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) and 277 K. Only 10% of the expressed Xoo2316 was soluble (data not shown). The supernatant containing soluble Xoo2316 was applied onto Ni–NTA His-Bind resin (Novagen) and affinity purification was performed according to the manufacturer's protocol at 277 K. The column was washed using the sonication buffer including 20 m*M* imidazole. The 6×His-tagged Xoo2316 protein was then eluted using lysis buffer containing 200 m*M* imidazole and treated with TEV protease overnight at 277 K to remove the 6×His tag. After TEV cleavage, three additional residues (Glu-Gly-His) remained before the native N-terminus of Xoo2316. The resulting protein solution was dialyzed for 3 h at 277 K in buffer *A* (25 m*M* Tris–HCl pH 7.5, 20% glycerol, 5 m*M* DTT) and further purified using an UNO6 Q column (Bio-Rad). The homogeneity of the purified protein was assessed by SDS–PAGE (Fig. 1). The protein solution in buffer *A* was concentrated further using a Centri-Prep (Millipore) to a final concentration of 8 mg ml^{-1} as determined by the Bradford method.

2.3. Crystallization and X-ray diffraction

Crystallization was initiated at 283 K using the Hydra II e-drop automated pipetting system (Matrix) on 96-well Intelli-Plates (Art Robbins) using the screening kits Crystal Screen HT, Index HT and SaltRx HT (Hampton Research). Initially, very thin needle-like crystals were observed and reproduced in hanging drops made up of 1 µl protein solution mixed with 1 µl reservoir solution. Each hanging drop was then positioned over 1 ml reservoir solution. The well was sealed with a cover slip using vacuum grease. After optimization, thick crystals were obtained using a reservoir solution containing

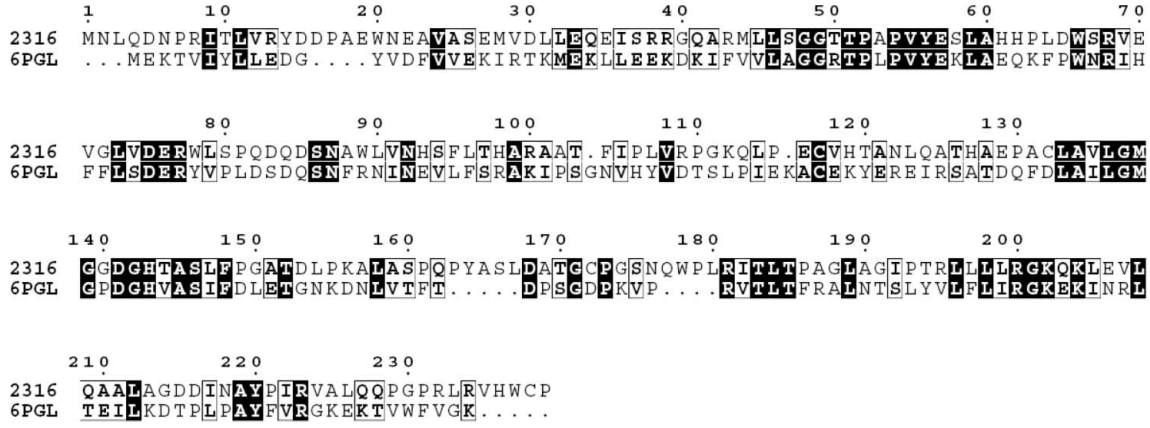

Figure 2
Sequence alignment between Xoo2316 and 6PGL from *T. maritima*.

0.1 *M* bis-tris pH 5.5, 25%(*w*/*v*) PEG 3350 and 0.2 *M* ammonium acetate over a period of one week (Fig. 1). The fully grown crystals were flash-cooled in liquid nitrogen with 25%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data were collected at 100 K using 1° oscillations with a crystal-to-detector distance of 251 mm and a Bruker Proteum 300 CCD on beamline 6C1 at the Pohang Light Source (PLS), South Korea. A native data set was collected to 2.4 Å resolution and a MAD data set was collected to 2.5 Å resolution. Each data set was integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

3. Results and discussion

The crystallographic space group $P2_12_12_1$ was derived by auto-indexing (Otwinowski & Minor, 1997) for both the native and MAD data sets, with unit-cell parameters a = 39.7, b = 65.1, c = 78.8 Å and a = 39.9, b = 78.9, c = 194.7 Å, respectively. Data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), there is one molecule in the asymmetric unit for the native data set, with a solvent content of 36.5%, and there are two or three molecules in the asymmetric unit for the MAD data set, with solvent contents of 58.6% or 37.9%, respectively. When the *SOLVE*/*RESOLVE* programs were used to determine SeMet sites, no SeMet sites were identified using the MAD data set (data not shown). However, molecular replacement (MR) using *Phaser* from the *CCP*4 program package (McCoy *et al.*, 2007) using 6-phosphogluconolactonase from *Thermotoga maritima* (PDB code 1vl1; 30% sequence identity; Fig. 2) as a search model was successful and showed three monomers in the asymmetric unit for the MAD data set and a monomer for the native data set. The MR solution provided informative $2F_o - F_c$ and $F_o - F_c$ electron-density maps for improving the model. The structural details will be described in a separate paper. Our structural data for Xoo2316 will provide an insight into its enzymatic mechanism and will be useful for developing antibacterial drugs against Xoo.

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