Crystallization and preliminary X-ray
crystallographic analysis of the XoGroEL
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Along with the co-chaperonin GroES, the chaperonin GroEL plays an essential role in enhancing protein folding or refolding and in protecting proteins against misfolding and aggregation in the cellular environment. The *XoGroEL* gene (*XOO_4288*) from *Xanthomonas oryzae* pv. *oryzae* was cloned and the protein was expressed, purified and crystallized. The purified XoGroEL protein was crystallized using the hanging-drop vapour-diffusion method and a crystal diffracted to a resolution of 3.4 Å. The crystal belonged to the orthorhombic space group $P2_12_12_1$ with 14 monomers in the asymmetric unit, with a corresponding V_M of 2.7 Å³ Da⁻¹ and a solvent content of 54.5%.

1. Introduction

Rice was the most cultivated crop in 2011 (FAOSTAT; <http://faostat.fao.org>). Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) has damaged rice production in most rice cultivating countries. When bacterial blight breaks out, it is epidemic and causes severe crop losses of up to 50% (Verdier *et al.*, 2012). Extensive research has been carried out to understand the pathogenicity of Xoo and the rice–Xoo interaction. However, to date no effective anti-bacterials are available against Xoo; these could be crucial to prevent rice production losses. We have performed structural and functional studies of Xoo proteins that are putative drug targets or pathogenicity-related proteins (Doan *et al.*, 2014; Kim, Natarajan *et al.*, 2013; Ngo *et al.*, 2014; Natarajan *et al.*, 2012; Kim, Nguyen *et al.*, 2013; Kim *et al.*, 2011). Xoo has a type III secretion system (T3SS) to deliver its effector proteins into the rice host (Alfano & Collmer, 2004). The transferred effectors manipulate rice cellular activity to cause bacterial blight. The normal protein-folding environments are different in bacteria and eukaryotes. Chaperones are known to be involved in the effector secretion process (Parsot *et al.*, 2003). We are interested in the structure and function of the product of the *XoGroEL* gene, which encodes GroEL in Xoo.

Both prokaryotic and eukaryotic cells have evolved to have special macromolecular complexes called chaperones that capture and refold partially folded or unfolded proteins into their native state (Fenton & Horwich, 2003; Thirumalai *et al.*, 2003; Young *et al.*, 2004). Non-native proteins could inhibit the normal function of and cause damage to the cells. One of the most important chaperone complexes is the GroEL–GroES complex; it undergoes large structural changes by the cooperative binding of subunits and the subsequent hydrolysis of ATP (Piggot *et al.*, 2012). The key steps in the structural change that result in the formation of an open hydrophobic ring to an enclosed folding chamber have not yet been determined (Horwich *et al.*, 2009; Clare *et al.*, 2012).

Including the well characterized *Escherichia coli* chaperonin GroEL (Horwich *et al.*, 2007; Dyachenko *et al.*, 2013), several tens of GroEL structures have been reported and their mechanisms of action have been proposed (Saibil *et al.*, 2013). GroEL structures are homotetradecamers in which 14 identical subunits are arranged into two heptameric rings in a back-to-back manner. The cylindrical structure contains two separate central cavities. Each subunit has three domains with separate functions.

In this study, we report the cloning of the gene encoding XoGroEL and the expression, purification, crystallization and preliminary X-ray

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Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

Source	PAL 5C
Wavelength (Å)	0.97949
Detector	ADSC Q315r
Temperature of data collection (K)	100
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	200
Exposure time per image (s)	3
Resolution range (Å)	50.0–3.4
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	136.5
b (Å)	236.6
c (Å)	277.3
$\alpha = \beta = \gamma$ (°)	90.0
Total No. of reflections	488288
No. of unique reflections	121103
Completeness (%)	97.7 (94.6)
Molecules per asymmetric unit	14
V_M (Å ³ Da ⁻¹)	2.7
Solvent content (%)	54.5
$I/\sigma(I)$	11.5 (2.3)
$R_{\text{merge}}^\dagger$ (%)	15.1 (50.0)
Multiplicity	4.0 (2.9)

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

crystallographic analysis of this protein. Determination of the three-dimensional structure of XoGroEL is expected to facilitate the development of novel antibacterial drugs against Xoo.

2. Materials and methods

2.1. Cloning

The DNA sequence coding *XoGroEL* (*XOO_4288*) was amplified using the genomic DNA of *X. oryzae* pv. *oryzae* (Xoo; ATCC 10331) as a template via the polymerase chain reaction (PCR). This yielded a 1641 bp DNA fragment when the primers 5'-CCC CCA TAT GGC TGC TAA AGA CAT TCG TTT CGG-3' and 5'-CCC CGG ATC CTC AGA AAT CCA TGC CGC CCA T-3' were used (the bases in bold indicate *NdeI* and *BamHI* restriction sites, respectively). The fragment was double-digested with *NdeI* and *BamHI* and then ligated into pET-11a expression vector (Novagen, Republic of Korea) to generate a plasmid that harbours XoGroEL with a fragment coding for a 7×His tag at the N-terminus of the cloned target protein and a *Tobacco etch virus* (TEV) protease cleavage site before the *NdeI* site.

2.2. Overexpression and purification

The recombinant pET11a-His-TEV-XoGroEL plasmid was transformed into *E. coli* BL21(DE3) cells. The cells were grown in Luria-Bertani (LB) medium supplemented with 50 µg ml⁻¹ ampicillin at 315 K until they reached an optical density at 600 nm (OD₆₀₀) of 0.5. Overexpression of XoGroEL was induced by treating the cells with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced cells were further cultured at 288 K for an additional 20 h and then harvested by centrifugation for 30 min at 6000g (Supra 30K A1000S-4 rotor; Hanil, Seoul, Republic of Korea) at 277 K. The cell pellet was then resuspended in ice-cold lysis buffer consisting of 25 mM Tris-HCl pH 7.5, 250 mM NaCl, 3 mM β-mercaptoethanol and homogenized on ice using ultrasonication (Sonomasher; S&T Science, Republic of Korea). The crude cell extract was centrifuged for 30 min at 21 000g (Vision VS24-SMTi V508A rotor) at 277 K to remove cell

debris. The lysate was applied onto an Ni²⁺-charged resin (Ni-NTA His-Bind Resin; Novagen) to purify the XoGroEL. The XoGroEL protein was eluted with elution buffer (25 mM Tris-HCl pH 7.5, 250 mM NaCl, 250 mM β-mercaptoethanol) and dialyzed for 16 h against dialysis buffer (20 mM Tris-HCl pH 7.5, 15 mM NaCl, 3 mM β-mercaptoethanol). After dialysis, the precipitated material was removed by centrifugation for 20 min at 21 000g and 277 K. Soluble XoGroEL protein was loaded onto a 5 ml HiTrap Q FF column (GE Healthcare) equilibrated in buffer A (20 mM Tris-HCl pH 8.0, 15 mM NaCl, 3 mM β-mercaptoethanol). XoGroEL was washed and eluted with a gradient of 0–100% buffer B (buffer A with 1 M NaCl). For crystallization, purified XoGroEL was dialyzed against crystallization buffer (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 3 mM β-mercaptoethanol) and then concentrated using a Vivaspin 20 concentrator (3000 MWCO; Sartorius) to a final protein concentration of 7.8 mg ml⁻¹.

2.3. Crystallization and X-ray data collection

Initial crystallization screening of XoGroEL was carried out at 287 K on a submicrolitre scale by the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Hampton Research) using a Hydra II e-drop automated pipetting system (Matrix) and screening kits from Hampton Research (Index, SaltRx, Crystal Screen, Crystal Screen Cryo, Crystal Screen Lite and PEGRx), Emerald Bio (Wizard and Wizard Precipitant Synergy) and Molecular Dimensions (Morpheus). 0.5 µl protein solution was mixed with 0.5 µl reservoir solution and equilibrated against 50 µl reservoir solution. Approximately 1000 crystallization conditions were initially screened. Small thin crystals were observed after 3 d in condition No. 1 of Crystal Screen [0.02 M calcium chloride dihydrate, 0.1 M sodium acetate trihydrate pH 4.6, 30% (v/v) (±)-2-methyl-2,4-pentanediol]. Optimization of the initial crystallization conditions was carried out using the hanging-drop vapour-diffusion method. The hanging drops were manually set up with 0.9 µl protein solution and 0.9 µl reservoir solution in Nextal NCK-24 crystallization plates (Nextal Biotech, Canada). Each hanging drop was positioned over 1 ml reservoir solution. The well was sealed with a cover slip using vacuum grease. In order to obtain single protein crystals, the streak-seeding method was used (Stura & Wilson, 1991; Bergfors, 2003). Initial crystals of XoGroEL were picked up on the tip of a human hair, which was used as a seeding wand, and the tip was immediately streaked sequentially through the pre-equilibrated hanging drops. The drops were pre-equilibrated over the reservoir solution for 2 h before seeding. The original crystallization reservoir solution was optimized to a new solution (0.06 M sodium chloride dihydrate, 0.1 M sodium citrate pH 4.0, 30% (v/v) (±)-2-methyl-2,4-pentanediol) in order to improve the quality of the crystals while maintaining the protein concentration. After 10 d, single orthorhombic crystals (0.25 × 0.1 × 0.04 mm) were observed, picked up with a loop (Dual-Thickness MicroMounts; MiTeGen) and flash-cooled in liquid nitrogen. X-ray diffraction data were collected from a cryocooled crystal with a total rotation range of 200° (1° oscillations per image) and a crystal-to-detector distance of 350 mm using an ADSC Q315r detector on beamline 5C at the Pohang Accelerator Laboratory (PAL), Republic of Korea. The crystal diffracted to 3.4 Å resolution. Data were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). The final data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

The purification protocol for the recombinant form of XoGroEL consistently yielded greater than 20 mg XoGroEL per litre of culture medium. The purified XoGroEL exhibited a single band on an SDS-PAGE gel at approximately 60 kDa (Fig. 1). The optimized XoGroEL crystals grew from a reservoir consisting of 0.06 M sodium chloride dihydrate, 30% (v/v) (±)-2-methyl-2,4-pentanediol, 0.1 M sodium citrate pH 4.0 (Fig. 2). A diffraction image of an XoGroEL crystal is shown in Fig. 3. A complete set of data was collected at 3.4 Å resolution from the single crystal. The space group was determined using the auto-indexing program in *HKL-2000* (Otwinowski & Minor, 1997) and data-collection statistics are provided in Table 1. The XoGroEL crystal belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 136.5, *b* = 236.6, *c* = 277.3 Å, $\alpha = \beta = \gamma = 90^\circ$. According to the calculated Matthews coefficient of 2.7 Å³ Da⁻¹ and the calculated solvent content of 54.5% (Matthews, 1968), the crystal volume of the XoGroEL asymmetric unit is compatible with the presence of 14 monomeric molecules in the unit cell. In order to find a template structure for molecular replacement, we submitted the amino-acid sequence of XoGroEL to *SWISS-MODEL* (Arnold *et al.*,

Figure 1
Purified XoGroEL on 10% SDS-PAGE.

Figure 2
The optimized XoGroEL crystal (0.25 × 0.1 × 0.04 mm).

Figure 3
Diffraction image of the XoGroEL crystal.

2006) and a template was generated based on chain *G* of the GroEL structure from *E. coli* (PDB entry 1mnf; 77.91% sequence identity; Wang & Chen, 2003). We determined the preliminary structure of XoGroEL by molecular replacement using *Phaser* in the *CCP4* program package (McCoy *et al.*, 2007) with the search model. The initial *R* factor and *R*_{free} of the XoGroEL structure after rigid-body and initial restrained refinement were 30.5 and 37.0%, respectively. The structural details of XoGroEL will be described in a forthcoming structural paper. Our structural data for XoGroEL will be useful in the development of antibacterial drugs against Xoo.

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