Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of the co-chaperonin XoGroES from *Xanthomonas oryzae* pv. *oryzae*

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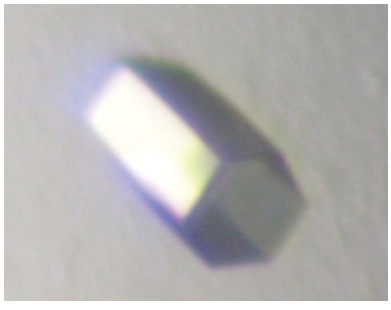
Received 10 August 2010
Accepted 28 September 2010



Bacterial blight (BB), a devastating disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), causes serious production losses of rice in Asian countries. Protein misfolding may interfere with the function of proteins in all living cells and must be prevented to avoid cellular disaster. All cells naturally contain molecular chaperones that assist the unfolded proteins in folding into the native structure. One of the well characterized chaperone complexes is GroEL–GroES. GroEL, which consists of two chambers, captures misfolded proteins and refolds them. GroES is a co-chaperonin protein that assists the GroEL protein as a lid that temporarily closes the chamber during the folding process. *Xoo4289*, the GroES gene from Xoo, was cloned and expressed for X-ray crystallographic study. The purified protein (XoGroES) was crystallized using the hanging-drop vapour-diffusion method and a crystal diffracted to 2.0 Å resolution. The crystal belonged to the hexagonal space group $P6_1$, with unit-cell parameters a = 64.4, c = 36.5 Å. The crystal contains a single molecule in the asymmetric unit, with a corresponding V_M of 2.05 Å^3 Da^{-1} and a solvent content of 39.9%.

1. Introduction

Rice is one of the most important staple foods for human consumption, especially in Asian countries, and bacterial blight (BB), a devastating disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), is one of the most serious bacterial diseases found in rice-growing countries and results in huge production losses all over the world. In 2006, agricultural reports indicated that in South Korea alone BB caused rice-production losses worth more than 100 million US dollars. To date, still there are no antibacterials that are effective against this disease and it is essential to find an agent against Xoo to halt rice-production losses. The genomic sequence of Xoo has been determined (Lee *et al.*, 2005), providing valuable information for the selection of antibacterial drug-target proteins. As the first step in initiating drug development against Xoo, 95 genes coding essential enzymes have been selected as candidate drug targets (Payne *et al.*, 2004, 2007) from among the 4538 putative Xoo genes (Lee *et al.*, 2005). The selected target genes have been systematically cloned and expressed in *Escherichia coli* to obtain supplies of the target enzymes for atomic resolution structure determination and protein–drug interaction studies using X-ray crystallographic methods. The Xoo gene coding for GroES, *Xoo4289*, was cloned and expressed in *Escherichia coli* for this purpose.

Protein misfolding in living cells may lead to malfunctioning of the cell machinery. Not only do misfolded proteins fail to perform their biological functions, but they also tend to interact with other biomolecules and disrupt the normal activity of the cell. To avoid this misfolding of proteins, both prokaryotic and eukaryotic cells have developed 'chaperone' protein complexes that capture misfolded proteins and chaperone their refolding to active proteins, thereby preventing cellular mischief (Fenton & Horwich, 2003; Thirumalai *et al.*, 2003; Young *et al.*, 2004). Chaperone proteins, which are ubiquitous in nature, control the refolding of proteins denatured by various stresses such as heat shock (Fenton & Horwich, 2003). One of the

most important chaperone complexes is the GroEL–GroES complex. It consists of two types of protein: the chaperonin GroEL (~60 kDa) and its co-chaperonin GroES (~10 kDa). GroEL is a tetradecameric oligomer consisting of two cylindrical chambers, with *cis* and *trans* rings joined together in a symmetrical fashion at the bottom. GroES forms a dome-shaped heptameric cap. Sigler *et al.* (1998) were the first to analyze the action of this chaperone complex structurally. Initially, the hydrophobic inner surface of the 'open-barrel' GroEL is proposed to capture target misfolded proteins. In the next important step, the GroEL barrel is capped by heptameric GroES. The non-native protein is now trapped inside a hydrophilic folding cavity and the protein is allowed to refold, supported by the hydrolysis of ATP to ADP. Upon binding of ATP to the *trans* ring the GroES cap is released, thereby releasing the folded protein.

The GroEL–GroES complex from *E. coli* is a well studied chaperonin system (Xu *et al.*, 1997). Isolated GroES structures have been reported from *Mycobacterium leprae* (Mande *et al.*, 1996) and *M. tuberculosis* (Roberts *et al.*, 2003). All of these GroES structures are homoheptameric and each monomer consists of a small β-barrel with a highly flexible mobile loop. The mobile loop of GroES from *E. coli* helps in forming contacts to the GroEL tetradecamer. GroES has other important functions: it acts as an immunogen (Chua-Intra *et al.*, 1998) and forms tetradecamers with divalent cations (Roberts *et al.*, 2003). In *Thermus thermophilus*, it is stable at higher temperatures of up to 353 K (Taguchi & Yoshida, 1993).

When it became known that chaperones can help in the translocation of the effector proteins of the bacterial type III secretion system, which are disease-causing agents, into particular host plant cells (Cornelis, 2006), other functions of GroEL and GroES were investigated. In our *in vitro* research, we found that XoGroEL and XoGroES were secreted along with the effector proteins after Xoo growth had been induced by rice-leaf extract (data not shown), revealing that XoGroES plays an important role in causing the disease. In this study, we report the cloning of the gene encoding XoGroES and the expression, purification, crystallization and preliminary X-ray crystallographic studies of this protein. A three-dimensional structural study of XoGroES is expected to help us to understand its function in the GroEL–GroES structure and its role in bacterial blight disease caused by Xoo.

2. Materials and methods

2.1. Cloning

The *Xoo4289* gene encoding XoGroES was amplified by PCR from genomic DNA of Xoo (ATCC10331) using the oligonucleotide primers 5′-GGG **CAT ATG** AGC ATC AAG CCG CTT CAC GAC-3′ and 5′-GG **GGA TCC** TCA GCC GAT GAC GGC CAG GAT-3′. The forward and reverse primers contained *Nde*I and *Bam*HI sites, respectively (shown in bold). The amplified PCR product was digested with *Nde*I and *Bam*HI enzymes and then cloned into modified pET11a. The modified vector contains a fragment coding for six His residues and a tobacco etch virus (TEV) protease cleavage site before the *Nde*I site in the pET11a vector (Novagen) to facilitate purification of the expressed protein. DNA sequencing (Macrogen) confirmed that the cloned *Xoo4289* gene had one point mutation (R55H).

2.2. Overexpression and purification

The pET-*Xoo4289* plasmid was transformed into *E. coli* BL21 (DE3) cells, which were grown in Luria–Bertani (LB) medium supplemented with ampicillin (50 µg ml^{-1}) and incubated at 310 K until the density reached an OD_{600} of about 0.6. Expression was induced by using 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced cells were further incubated for 20 h at 288 K and were then harvested by centrifugation at 4000 rev min^{-1} for 15 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 15 m*M* imidazole, 3 m*M* β-mercaptoethanol) and homogenized using ultrasonication on ice (Sonomasher, S&T Science, Republic of Korea). The lysate was centrifuged at 13 000 rev min^{-1} for 30 min at 277 K (Vision VS24-SMTi V508A rotor). The soluble XoGroES protein in the supernatant was purified using Ni–NTA His-bind resin (Novagen) according to the manufacturer's protocol at 277 K. After loading the supernatant onto the resin, lysis buffer containing 30 m*M* imidazole was used to wash out unbound proteins. Lysis buffer containing 200 m*M* imidazole was then used to elute the tagged XoGroES protein. The pure fractions were collected and dialyzed for 12 h at 277 K in buffer *A* (25 m*M* Tris–HCl pH 7.5, 3 m*M* β-mercaptoethanol). The protein was subjected to a TEV protease cleavage

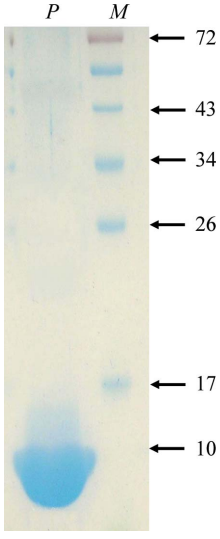


Figure 1
Purified XoGroES shown on 15% SDS–PAGE. Lane *M*, molecular-weight markers (kDa); lane *P*, protein.

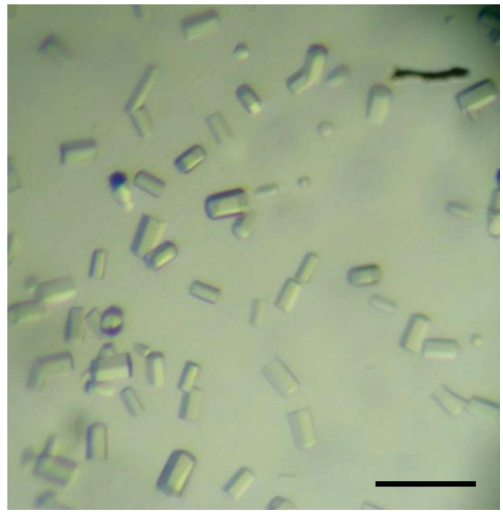


Figure 2
Crystals of XoGroES protein obtained using a sitting-drop vapour-diffusion setup with condition No. 39 of Wizard I: 20%(*w*/*v*) PEG 1000, 0.1 *M* phosphate–citrate pH 4.2, 0.2 *M* Li_2SO_4. The scale bar represents 0.1 mm.

reaction using a protein:protease ratio of 50:1 in buffer *A* at 288 K overnight to cleave the His-TEV tag at the N-terminus of the protein. The resultant protein solution was again applied onto Ni–NTA His-bind resin to remove the TEV protease and any uncut protein. The homogeneity of the purified protein was examined using SDS–PAGE. Only one band of cleaved XoGroES was visible on 15% SDS–PAGE (Fig. 1). The purified XoGroES was concentrated to 2.5 mg ml^{-1} in buffer *A* for crystallization purposes. The resultant protein product has the sequence GH attached directly to the N-terminus.

2.3. Crystallization and X-ray data collection

Initial crystallization screening was carried out on a submicrolitre scale by the sitting-drop vapour-diffusion method (0.5 µl protein solution was mixed with 0.5 µl reservoir solution and equilibrated against 70 µl reservoir solution) using a Hydra II e-drop automated pipetting system (Matrix) and screening kits from Hampton Research. After 1 d, some tiny crystals were seen in one condition [20%(*w*/*v*) PEG 1000, 0.1 *M* phosphate–citrate pH 4.2, 0.2 *M* Li_2SO_4; Fig. 2]. To obtain larger crystals, this condition was optimized by changing the PEG concentration, the type of PEG and the buffer. Well diffracting crystals were produced in 4 d using the optimized condition 16%(*v*/*v*) PEG 400, 0.2 *M* sodium citrate pH 4.1, 0.2 *M* Li_2SO_4 and 4%(*v*/*v*) acetone as an additive with the hanging-drop method (drops consisted of 1 µl protein solution mixed with 1 µl reservoir solution and were equilibrated against 1 ml reservoir solution). A crystal (0.3 × 0.1 × 0.1 mm; Fig. 3) was picked up in a loop, soaked in cryoprotectant consisting of 30%(*v*/*v*) PEG 400, 0.2 *M* sodium citrate pH 4.1 and 0.2 *M* Li_2SO_4 and frozen in liquid nitrogen. The cryoprotectant was selected after optimization of the PEG 400 concentration in which the crystal remained undamaged. X-ray diffraction data were collected from this crystal using an ADSC Quantum 210 CCD detector on beamline 4A of Pohang Light Source (PLS), South Korea. Crystal data were collected to 2.0 Å resolution and were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). The autoindexing program initially suggested *P*3 as the best space group, with unit-cell parameters a = 64.4, c = 36.5 Å and hexagonal angle $\gamma = 120^\circ$. Calculation of the self-rotation function proved that the crystal possesses twofold, threefold and sixfold symmetry. Since no molecular-replacement (MR) solution was found using this space group, the data were re-indexed using the program *POINTLESS* (Collaborative Computational Project, Number 4, 1994), which suggested that the crystal belonged to space group $P6_1$ or $P6_5$, with the same unit-cell parameters. The final statistics of data collection and processing details are summarized in Table 1.

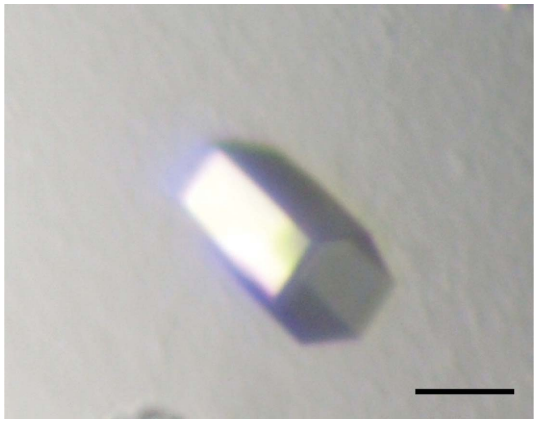


Figure 3
The XoGroES crystal (0.3 × 0.1 × 0.1 mm) chosen for crystallographic study, which was obtained using 16%(*v*/*v*) PEG 400, 0.2 *M* sodium citrate pH 4.1, 0.2 *M* Li_2SO_4 and 4%(*v*/*v*) acetone. The scale bar represents 0.1 mm

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Source	Beamline 4A, PLS
Wavelength (Å)	0.96418
Resolution range (Å)	55.8–2.0
Space group	$P6_1$
Unit-cell parameters (Å)	
$a = b$	64.4
c	36.5
Total No. of reflections	51450
No. of unique reflections	6225
Completeness (%)	99.8 (100.0)
Molecules per asymmetric unit	1
V_M (Å^3 Da^{-1})	2.05
Solvent content (%)	39.9
Average $I/\sigma(I)$	12.3 (2.8)
R_{merge}† (%)	8.4 (63.0)

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

3. Results and discussion

High-throughput crystallization screening of the XoGroES protein produced a well diffracting crystal (Fig. 3) using 16%(*v*/*v*) PEG 400, 0.2 *M* sodium citrate pH 4.1, 0.2 *M* Li_2SO_4 and 4%(*v*/*v*) acetone. A complete set of data was collected to 2.0 Å resolution from a single crystal. The data analysis and systematic absences suggested that the crystal belonged to the hexagonal space group $P6_1$ or $P6_5$. The crystal volume of the asymmetric unit of XoGroES is compatible with a single monomeric molecule in the unit cell, with a volume per unit molecular weight of the protein of 2.05 Å^3 Da^{-1} and a calculated solvent content of 39.9% (Matthews, 1968). In order to confirm the crystal symmetry, self-rotation functions were calculated at $\chi = 60$, 90, 120 and 180° to detect sixfold, fourfold, threefold and twofold axes, respectively. According to the self-rotation functions, the XoGroES crystal proved to possess twofold, threefold and sixfold symmetry. A preliminary structure solution of the XoGroES protein was obtained using MR (*MOLREP* program; Vagin & Teplyakov, 2010) with the structure of the co-chaperonin from *T. thermophilus* (PDB code 1wnr; Numoto *et al.*, 2005) as a model structure. The best MR model gave a correlation coefficient of 56.2% and an R factor of 47.4% in the resolution range 15–3.5 Å and indicated that the crystal belonged to space group $P6_1$; space group $P6_5$ did not yield an acceptable solution. Analysis of the MR model showed good crystal packing and no clashes were found between symmetry-related molecules. Once refined, our structure should provide insight into the enzymatic reaction mechanism, with the aim of supporting the development of antibacterial drugs against Xoo.

We are grateful to the staff members for their assistance at beamline 4A of Pohang Light Source (PLS), South Korea. This work was supported by the faculty fund of Konkuk University in 2008.

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