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Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of glutamyl-tRNA synthetase (Xoo1504) from *Xanthomonas oryzae* pv. *oryzae*

Thanh Thi Ngoc Doan,[a] Sampath Natarajan,[a] Hyesoon Kim,[b] Yeh-Jin Ahn,[b] Jeong-Gu Kim,[c] Byoung-Moo Lee[c] and Lin-Woo Kang[a]*

[a]Department of Advanced Technology Fusion, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea, [b]Major in Life Science, College of Natural Sciences, Sangmyung University, 7 Hongji-dong, Jongno-gu, Seoul 110-743, Republic of Korea, and [c]Microbial Genetics Division, National Institute of Agricultural Biotechnology (NIAB), Rural Development Administration (RDA), Suwon 441-707, Republic of Korea

Correspondence e-mail: lkang@konkuk.ac.kr

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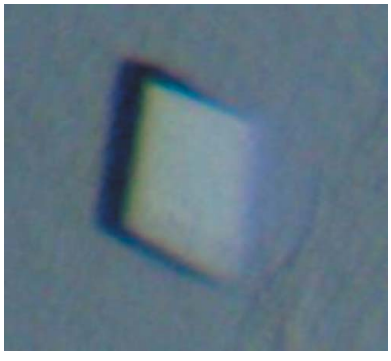
The *gltX* gene from *Xanthomonas oryzae* pv. *oryzae* (*Xoo1504*) encodes glutamyl-tRNA synthetase (GluRS), one of the most important enzymes involved in bacterial blight (BB), which causes huge production losses of rice worldwide. GluRS is a class I-type aminoacyl-tRNA synthetase (aaRS) that is primarily responsible for the glutamylation of $tRNA^{Glu}$. It plays an essential role in protein synthesis, as well as the regulation of cells, in all organisms. As it represents an important target for the development of new antibacterial drugs against BB, determination of the three-dimensional structure of GluRS is essential in order to understand its catalytic mechanism. In order to analyze its structure and function, the *gltX* gene was cloned and the GluRS enzyme was expressed, purified and then crystallized. A GluRS crystal belonging to the monoclinic space group *C*2 diffracted to 2.8 Å resolution and had unit-cell parameters $a = 186.8$, $b = 108.4$, $c = 166.1$ Å, $\beta = 96.3^\circ$. The unit-cell volume of the crystal allowed the presence of six to eight monomers in the asymmetric unit, with a corresponding Matthews coefficient (V_M) range of 2.70–2.02 $Å^3$ Da^{-1} and a solvent-content range of 54.5–39.3%.

1. Introduction

Bacterial blight (BB) results in serious rice-production losses in rice-cultivating countries. This destructive disease is caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo; Ezuka & Kaku, 2000) and resulted in a production loss worth nearly $100 million in South Korea alone in 2006. However, to date there are no effective antibacterial drugs against Xoo and it is necessary to find a drug that will halt the production loss of rice. Recently, the whole genomic sequence of Xoo has been determined (Lee *et al.*, 2005) and has provided useful information in selecting drug targets from Xoo proteins. As the first step in designing an antibacterial drug against Xoo, nearly 100 genes encoding essential enzymes (Payne *et al.*, 2004, 2007) were selected from 4538 putative Xoo genes (Lee *et al.*, 2005). As three-dimensional structures are key to *in silico* drug development, the *gltX* gene (*Xoo1504*) coding for the GluRS protein has been cloned and expressed in an *Escherichia coli* system for crystallographic studies.

Glutamyl-tRNA synthetase (GluRS), a class I-type aminoacyl-tRNA synthetase, is primarily responsible for the glutamylation of $tRNA^{Glu}$. Aminoacyl-tRNA synthetases (aaRSs) play an essential role in protein synthesis by producing charged tRNAs. GluRS is part of the 'minimal set' of 17 aaRSs that are found in every living organism and its presence is essential for the viability of cells. AaRSs catalyze the esterification of a particular tRNA with its cognate amino acid. All 20 aminoacyl-tRNA synthetases can be divided into two classes: I and II. The class I enzymes contain a characteristic Rossmann-fold catalytic domain and are mostly monomeric (Sugiura *et al.*, 2000). Class II enzymes are mostly dimeric or multimeric and share an antiparallel β-sheet fold flanked by α-helices (Perona *et al.*, 1993). The enzyme GluRS belongs to the class I type of aaRS characterized by a Rossmann fold in the catalytic domain and two specific



ATP-binding marker sequences such as HIGH and KMSKS motifs (Eriani *et al.*, 1990). To date, only two crystal structures of GluRS enzymes have been reported; these are from *Thermus thermophilus* (Nureki *et al.*, 1995) and *Thermosynechococcus elongatus* (Schulze *et al.*, 2006). These structures are also of the class I type with Rossmann-fold catalytic domains. These synthetase structures can be used as target proteins for the screening of antibacterial drugs. Therefore, the crystallographic study of these GluRS structures will be helpful in the high-throughput chemical compound screening approach to finding novel drugs.

In this paper, we report the cloning of the *gltX* (*Xoo1504*) gene from Xoo, the expression of the GluRS protein and its purification and crystallization screening. The purified GluRS was crystallized and preliminary X-ray crystallographic studies have been carried out. The study of the three-dimensional structure of GluRS (Xoo1504) is expected to help us to determine the molecular basis of the enzymatic reaction mechanism; its atomic resolution structure may be useful in the design of a potential drug against Xoo.

2. Methods and results

2.1. Cloning

The *gltX* gene was amplified using the genomic DNA of *X. oryzae* cells (Xoo KACC10331 strain) and the sense and antisense primers 5′-GGG GGG **CAT ATG** GCC TGC CGC ACC CGT TTT GCC C-3′ and 5′-GGG GG**G GAT CC**T CAG GCC GCG CCA ATC TTG GTA A-3′, respectively, which were designed based on the genomic report (Lee *et al.*, 2005). The amplified gene was inserted into *Nde*I- and *Bam*HI-digested expression vector pET11a (Novagen); six histidine residues were added to the N-terminus of the gene product in order to facilitate protein purification.

2.2. Overexpression and purification

The recombinant plasmid was transferred into *E. coli* BL21 (DE3) pLysS and cultures were grown in Luria–Bertani medium supplemented with 50 µg ml^{-1} ampicillin at 310 K until the OD_{600} reached 0.6; the cultures were then induced with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and incubated for an additional 20 h at 288 K. The cultured cells were harvested by centrifugation at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) for 10 min at 277 K. The 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 lysed by ultrasonication (Sonomasher, S&T Science, Korea) on ice. The crude lysate was centrifuged at 13 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) for 30 min at 277 K and the supernatant containing the recombinant GluRS protein was loaded onto a column packed with Ni^{2+}-chelating affinity resin (Novagen) which had previously been equilibrated with lysis buffer. Affinity purification was performed at 277 K. All unbound proteins were removed using lysis buffer containing 30 m*M* imidazole. The target GluRS was eluted with lysis buffer containing 200 m*M* imidazole. To remove the imidazole from the eluted protein solution, it was dialysed against buffer *A* (25 m*M* Tris–HCl pH 7.5, 3 m*M* *β*-mer-

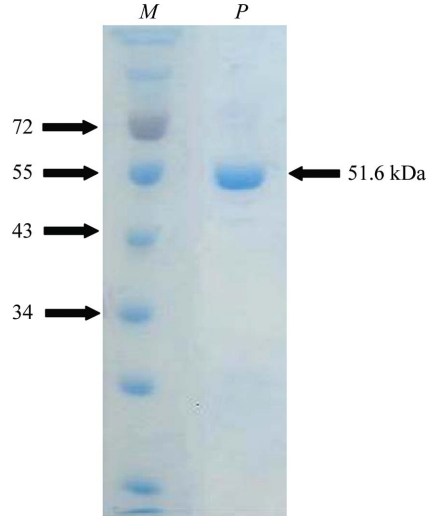
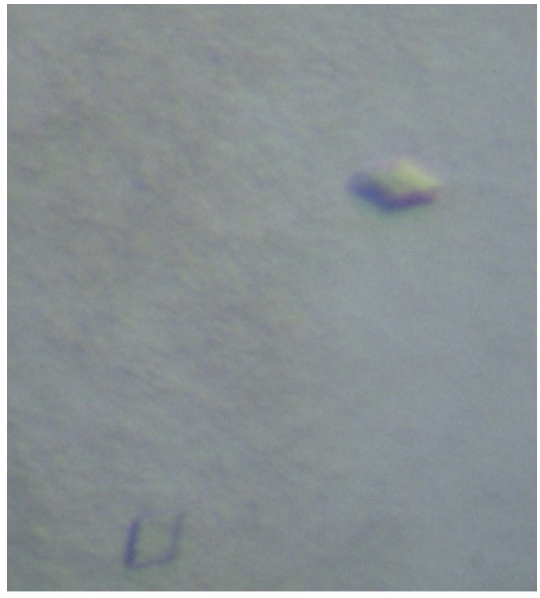
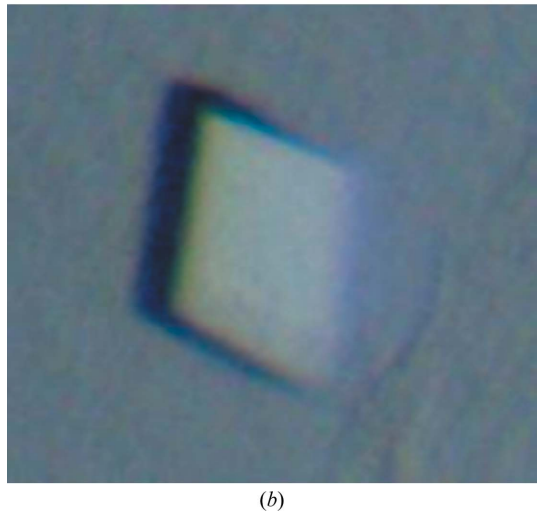


Figure 1
SDS–PAGE analysis of GluRS (Xoo1504) during purification. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane *P*, purified GluRS after UNO 6Q column (Bio-Rad).



(*a*)



(*b*)

Figure 2
(*a*) Crystallization of GluRS protein by high-throughput crystallization screening using a Hydra II e-drop automated pipetting system and Hampton Research SaltRX condition No. 96 [60%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 7.0]. (*b*) Crystal of GluRS protein from Xoo obtained by the hanging-drop vapour-diffusion method using the optimized SaltRX condition [55%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 6.5].

captoethanol) for 12 h at 277 K and then subjected to a TEV protease cleavage reaction to cleave the His-tag residues from the protein [50:1(*w*:*w*) protein:TEV protease at 277 K for 16 h]. This protein mixture was again loaded onto an Ni^{2+}-charged affinity column in order to remove the protease and uncut protein. The flowthrough containing the target GluRS was further purified using an UNO6 Q (Bio-Rad) anion-exchange column with buffer *B* (25 m*M* Tris–HCl pH 7.5, 1 *M* NaCl) as an elution reagent. The purity of the protein was examined by SDS–PAGE after every step. Finally, a single band corresponding to a molecular weight of 51.6 kDa was observed on an SDS–PAGE gel after purification (Fig. 1). The resulting protein solution was then concentrated to 7 mg ml^{-1} for crystallization screening experiments.

2.3. Crystallization and X-ray diffraction (data collection)

Initial crystallization screening was performed using the commercially available kits Crystal Screens I and II, Index Screen and SaltRX (Hampton Research) at 287 K using high-throughput crystallization screening with a Hydra II e-drop automated pipetting system (Matrix) in 96-well Intelli-Plates (Art Robbins). SaltRX condition No. 96 produced tiny crystals (Fig. 2*a*) in the condition 60%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 7.0. This condition was further optimized to obtain better quality crystals by changing the precipitant concentration, the buffer pH and the protein concentration. Finally, a crystal suitable for diffraction (Fig. 2*b*) was obtained using the condition 55%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 6.5 with a protein concentration of 9 mg ml^{-1} using the hanging-drop vapour-diffusion method. The crystallization setup consisted of 2 µl protein solution mixed with 2 µl reservoir solution and the drop was equilibrated against 1 ml reservoir solution [55%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 6.5].

A crystal was frozen in liquid nitrogen using 22%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data sets were collected at 293 K to 2.8 Å resolution from the frozen crystal using an ADSC Quantum 270 CCD detector on beamline 17A at the Photon Factory (KEK), Japan. The entire data set of the GluRS was processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), respectively. The crystallographic monoclinic space group *C*2 was derived by autoindexing (Otwinowski & Minor, 1997), with unit-cell parameters $a = 186.8$, $b = 108.4$, $c = 166.1$ Å, $\beta = 96.3^\circ$. The data-collection statistics of this crystal are summarized in Table 1. The intensity data thus obtained were truncated to amplitudes using the program *TRUNCATE* (French & Wilson, 1978) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994) and were used for structure solution and refinement. In order to estimate the protein content of the asymmetric unit, the Matthews coefficient (V_M; Matthews, 1968) and solvent content were calculated based on a subunit of molecular weight 51.6 kDa. The *Phaser* program (McCoy *et al.*, 2005) was used for molecular-replacement calculations and *REFMAC*5 (Murshudov *et al.*, 1997) was used for refinement. The

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	0.96418
Resolution range (Å)	50.0–2.8 (2.9–2.8)
Space group	*C*2
Unit-cell parameters	
a (Å)	186.76
b (Å)	108.38
c (Å)	166.13
β (°)	96.26
Total No. of reflections	134353
No. of unique reflections	64406
Completeness (%)	79.9 (23.2)
Molecules per ASU	6 or 8
V_M (Å^3 Da^{-1})	2.3
Solvent content (%)	54.5–39.3
Average $I/\sigma(I)$	9.2 (1.2)
R_{merge}† (%)	9.3 (40.5)

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

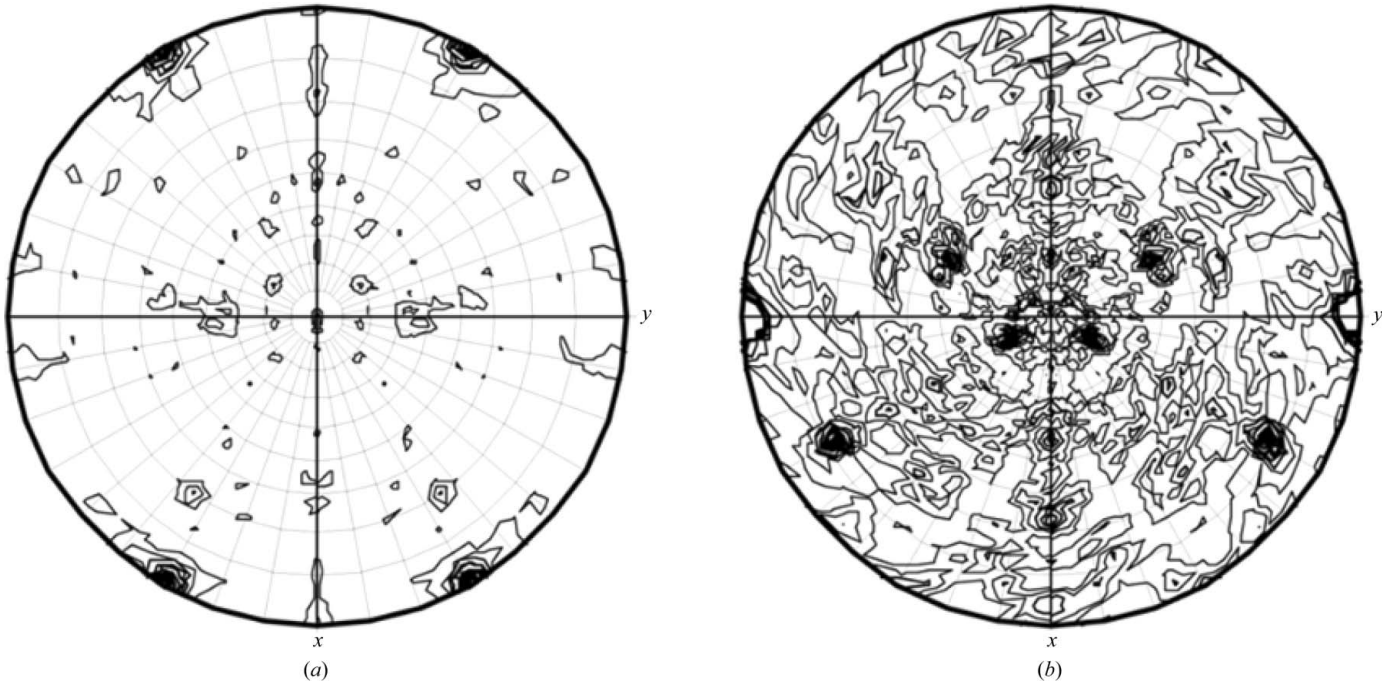


Figure 3
The $\chi = 120^\circ$ (*a*) and $\chi = 180^\circ$ (*b*) sections of the self-rotation function using data from 50 to 4 Å resolution calculated using *MOLREP* (Vagin & Teplyakov, 1997).

structural information on this GluRS (Xoo1504) will provide the molecular basis of the enzymatic reaction mechanism and will be useful in the development of drugs against Xoo.

3. Results and discussion

The target *gltX* gene (*Xoo1504*) was cloned and expressed with an N-terminal His_6 tag in *E. coli* BL21 (DE3) pLysS cells and the GluRS protein was purified. The homogeneity of the purified samples was verified at each step by SDS–PAGE analysis. The GluRS protein was crystallized by the hanging-drop method using the condition 55%(*v*/*v*) Tacsimate, 0.1 *M* bis-tris propane pH 6.5 and the harvested crystal diffracted to 2.8 Å resolution. Systematic absences in the crystal data analysis indicated that the crystal belonged to the monoclinic space group *C*2. The volume of the asymmetric unit was compatible with the presence of between six and eight monomers in the unit cell, with a volume per unit molecular weight of the protein (V_M) of 2.70–2.02 $Å^3$ Da^{-1} and a calculated solvent content of 54.5–39.3% (Matthews, 1968). Self-rotation functions were calculated at $\chi = 60^\circ$, 90°, 120° and 180° to detect twofold, threefold, fourfold and sixfold noncrystallographic symmetry. Based on this self-rotation calculation (Fig. 3), the crystal contains six monomers (a hexamer) in the asymmetric unit with 23 point symmetry. A noncrystallographic symmetry threefold lies in the *xy* plane and noncrystallographic twofolds lie in the plane normal to the triads. The triads are clearly visible in the $\chi = 120^\circ$ section and the dyads in the $\chi = 180^\circ$ section. The structure solution of this data was obtained by molecular replacement (with a *Z* score of 5.8) using the crystal structure of GluRS from *Synechococcus elongatus* (PDB code 2cfo; Schulze *et al.*, 2006), which shares 41.3% sequence similarity, as a search model. The R_{work} and R_{free} values of the unrefined structure were 42.4% and 51.3%, respectively. Examination of the best MR solution structure revealed good crystal packing and no clashes were found between symmetry-related molecules. The initial σ-weighted electron-density map with $2F_o - F_c$ Fourier coefficients and molecular-replacement phases were of interpretable quality almost throughout the chain. This preliminary model is currently being refined. Our final structural model of GluRS may help in the development of drugs against Xoo.

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