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Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of 3-dehydroquinate synthase, Xoo1243, from *Xanthomonas oryzae* pv. *oryzae*

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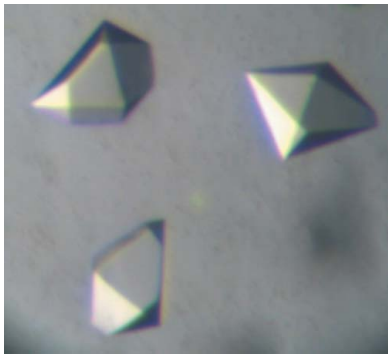
The disease bacterial blight results in serious production losses of rice in Asian countries. The *aroB* gene encoding dehydroquinate synthase (DHQS), which is a potential antibiotic target, was identified from the plant-pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo). DHQS plays an essential role in the synthesis of aromatic compounds in the shikimate pathway. The *aroB* gene (Xoo1243) was cloned from Xoo and the corresponding DHQS protein was subsequently overexpressed in *Escherichia coli*. The purified protein was crystallized using the hanging-drop vapour-diffusion method and yielded crystals that diffracted to 2.5 Å resolution. The crystals belonged to the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 118.2$, $c = 98.2$ Å. According to a Matthews coefficient calculation, the crystal contained two molecules in the asymmetric unit, with a corresponding V_M of 2.06 Å^3 Da^{-1} and a solvent content of 40.4%.

1. Introduction

Bacterial blight (BB) is one of the most devastating bacterial diseases found in rice-cultivating countries and results in serious production losses all over the world, especially in Asia. Based on agricultural reports from South Korea, BB resulted in huge production losses of rice worth more than 100 million dollars in 2006 alone. Dehydroquinate synthase (DHQS) is an essential enzyme encoded by the gene *aroB* that plays a role in the synthesis of aromatic compounds *via* the shikimate pathway. It converts 3-deoxy-D-arabinoheptulosonate (DAHP) to dehydroquinate (DFQ) (Bentley, 1990); this pathway is present in all organisms except mammals (Bentley, 1990). Inactivation of enzymes in the shikimate pathway results in attenuation of virulence and loss of viability in a large number of bacterial species (Gunel-Ozcan *et al.*, 1997; Parish & Stoker, 2002). DHQS itself is of interest because it apparently catalyzes five individual reactions, alcohol oxidation, phosphate ω-elimination, carbonyl reduction, ring opening and intramolecular aldol condensation, in a single active site as well as being a drug target (Srinivasan *et al.*, 1963; Widlanski *et al.*, 1989).

The shikimate pathway contains many enzymes that can be considered to be targets for the design of drugs against bacterial diseases. DHQS is the second enzyme in this pathway. As expected, the *aroB* gene from the genomic sequence of *Xanthomonas oryzae* pv. *oryzae* (Xoo) encodes DHQS, which is one of the enzymes important for BB and is expected to be an attractive target for the development of antibacterial drugs against BB. Based on the literature, the DHQS enzyme is an NAD^+-dependent metalloenzyme. It has been cloned and characterized from various bacterial sources such as *Escherichia coli* (Frost *et al.*, 1984), *Bacillus subtilis* (Yazdi & Moir, 1990), *Mycobacterium tuberculosis* (de Mendonca *et al.*, 2007) and *Corynebacterium glutamicum* (Han *et al.*, 1999). To date, only two DHQS structures have been characterized by X-ray crystallographic methods: these are the DHQS enzymes from *Staphylococcus aureus* (Nichols *et al.*, 2004) and *Thermus thermophilus* (Sugahara *et al.*, 2005).



In this study, we report the cloning of the *aroB* gene and the expression, purification, crystallization and preliminary X-ray crystallographic studies of the target protein DHQS from Xoo1243. A three-dimensional structural study of this DHQS is expected to help us to elucidate the molecular basis of the enzymatic reaction mechanism of the catalytic process. The atomic resolution structure of the DHQS protein may be useful for designing potential drugs against Xoo.

2. Materials and methods

2.1. Cloning

The target *aroB* gene was amplified from *X. oryzae* pv. *oryzae* using the polymerase chain reaction by colony PCR of the Xoo KACC10311 strain. The sequences of the forward and reverse oligonucleotide primers were designed based on the published genomic sequences 5′-GGG GGG **CAT ATG** ACA CTT CCC CGT TCC TCG CGC-3′ and 5′-GGGGGG **GGA TCC** TTA TCC CGC CAG GAT CTT CAG-3′, respectively. The highlighted bases in the primer sequences indicate *Nde*I and *Bam*HI digestion sites, respectively. The PCR-amplified DNA fragment was digested with *Nde*I and *Bam*HI enzymes and then cloned into vector pET11a (Novagen), encoding a polypeptide with an N-terminal 6×His tag to facilitate purification.

2.2. Overexpression and purification

The *aroB* gene encoding the enzyme DHQS was overexpressed in *E. coli* BL21 (DE3) cells. Expression was performed in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin, which was incubated at 310 K until the OD_{600} reached about 0.6. Protein expression was induced by the addition of 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) and the culture was maintained at 288 K for an additional 16 h. For the preparation of soluble fractions, the induced cells were harvested by centrifugation at 6000 rev min^{-1} for 30 min (Vision VS24-SMTi V5006A rotor) 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, 0.5 m*M* PMSF, 0.1 m*M* $ZnSO_4$, 20% glycerol and 3 m*M* *β*-mercaptoethanol) and then lysed by sonication (Sonomasher) on ice. The crude cell extract was centrifuged for 30 min at 15 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) at 277 K to remove cell debris. The clear supernatant, which contained only 10% soluble protein (data not shown), was collected. All subsequent purification steps were performed at 277 K. The soluble DHQS was applied onto a column containing 5 ml Ni^{2+}–NTA His-bind resin (Qiagen) for affinity purification. Once all unbound proteins had been washed from the column using 40 ml buffer *A* (25 m*M* Tris–HCl pH 7.5, 1 *M* NaCl, 20 m*M* imidazole, 20% glycerol and 3 m*M* *β*-mercaptoethanol), the target DHQS protein was eluted from the column using 250 m*M* imidazole along with 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 3 m*M* *β*-mercaptoethanol and 20% glycerol (buffer *B*). In order to remove the imidazole, the elute was extensively dialyzed against 25 m*M* Tris–HCl pH 7.5, 15 m*M* NaCl and 3 m*M* *β*-mercaptoethanol. The protein was subjected to a TEV protease cleavage reaction to cleave the six His-tag residues at the N-terminus of the protein in the same buffer using a 100:1(*w*:*w*) protein:TEV ratio at 277 K overnight. The resultant protein solution was again applied onto an Ni^{2+}–NTA resin column (Qiagen) to remove the TEV protease and any uncleaved protein. The flowthrough fraction containing cleaved DHQS protein was collected and applied onto an UNO Q6 ion-exchange column (Bio-Rad) for further purification. The homogeneity of the purified protein was examined by SDS–PAGE (Fig. 1) and it was concentrated to 8 mg ml^{-1} in 25 m*M* Tris–HCl pH 7.5, 15 m*M* NaCl and 3 m*M* *β*-mercaptoethanol for crystallization purposes.

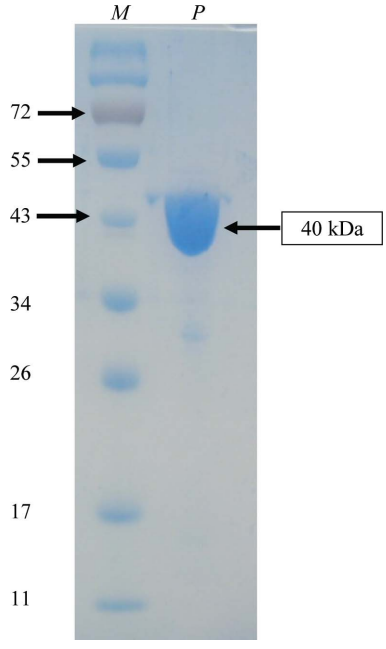


Figure 1
SDS–PAGE analysis of DHQS (Xoo1243) during purification. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane *P*, purified DHQS after passage through the UNO 6Q column (Bio-Rad).

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Synchrotron	PLS, beamline 6C1
Wavelength (Å)	0.96418
Resolution range (Å)	50.0–2.5 (2.6–2.5)
Space group	$P4_32_12$
Unit-cell parameters (Å)	
$a = b$	118.2
c	98.2
Total No. of reflections	181625
No. of unique reflections	24542
Completeness (%)	99.0 (98.6)
Molecules per ASU	2
V_M (Å^3 Da^{-1})	2.21
Solvent content (%)	40.3
Average $I/\sigma(I)$	8.5 (2.2)
R_{merge}† (%)	11.0 (40.6)
$R_{p.i.m}$ (%)	2.8 (12.9)
$R_{r.i.m}$ (%)	11.3 (42.8)

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

2.3. Crystallization and X-ray data collection

Initial crystallization experiments were carried out by the sitting-drop vapour-diffusion method in 96-well Intelliplates (Art Robbins) using a Hydra II e-drop automated pipetting system (Matrix) and screening kits from Hampton Research and Emerald Biosystems. After 1 d, tiny crystals were seen in eight different conditions (Fig. 2). These initial hits were scaled up into hanging drops (1 µl protein solution + 1 µl reservoir solution) in Linbro plates. Of these, only one condition (Hampton Research Crystal Screen II condition No. 26)

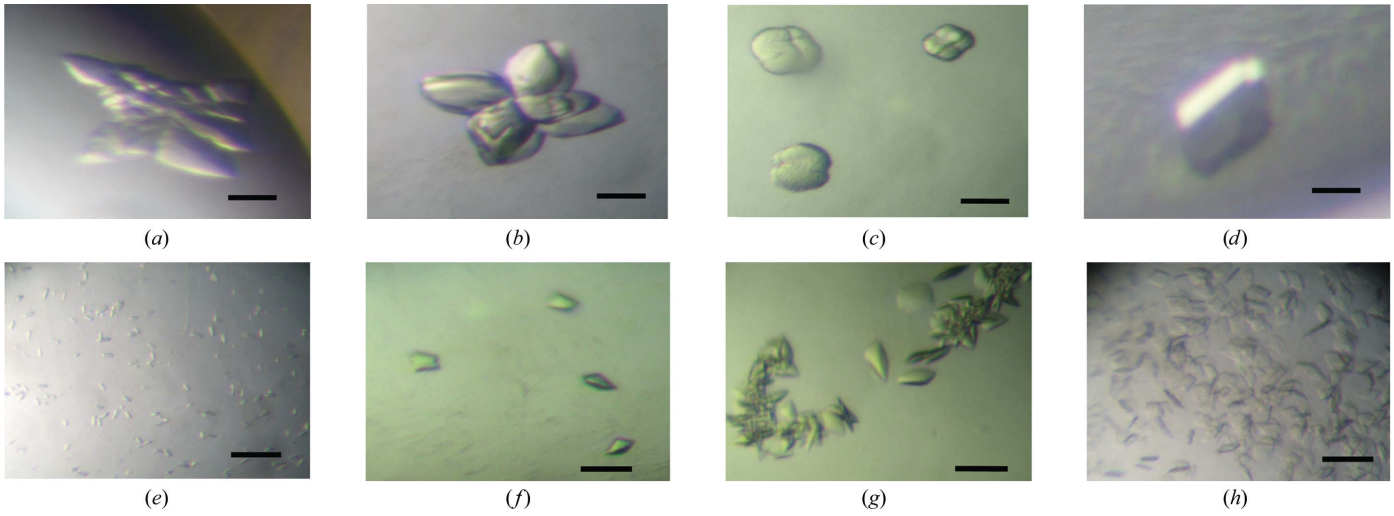


Figure 2
Crystals of DHQS from *X. oryzae* pv. *oryzae* (Xoo1243) obtained in sitting-drop setups using a Hydra automated high-throughput crystal screening machine with different screening kits. (*a*) 0.1 *M* sodium malonate pH 4.0, 12% PEG 3350 (PEG/Ion Screen condition II-1). (*b*) 0.2 *M* sodium malonate pH 6.0, 20% PEG 3350 (PEG/Ion Screen condition I-41). (*c*) 0.1 *M* Tris pH 8.5, 2.0 *M* ammonium sulfate (Index Screen condition No. 6). (*d*) 0.15 *M* caesium chloride, 15% PEG 3350 (PEG/Ion Screen condition II-45). (*e*) 1.0 *M* sodium citrate, 0.1 *M* CHES pH 9.5 (Wizard Screen II condition No. 16). (*f*) 0.2 *M* ammonium sulfate, 0.1 *M* bis-Tris pH 5.5, 25% PEG 3350 (Index Screen condition No. 66). (*g*) 0.2 *M* ammonium sulfate, 0.1 *M* MES pH 6.5, 30% PEG MME 5000 (Crystal Screen condition No. 26). (*h*) 0.02 *M* zinc chloride, 20% PEG 3350 (PEG/Ion Screen condition II-44). The scale bars represent 0.1 mm.

gave crystals in the larger drop format. The crystals were optimized by varying the pH and the PEG MME 5000 concentration. The best crystals were obtained after two weeks from a reservoir containing 0.2 *M* ammonium sulfate, 0.1 *M* MES monohydrate pH 6.0 and 31%(*w*/*v*) PEG MME 5000 (Fig. 3). The crystallization is very sensitive to changes in buffer pH or PEG MME 5000 concentration. A well grown crystal (0.5 × 0.25 × 0.2 mm) was frozen in liquid nitrogen using 20%(*v*/*v*) glycerol as a cryoprotectant. X-ray diffraction data were collected from the frozen crystal using an ADSC Quantum 210 CCD detector on beamline 6C1 of Pohang Light Source (PLS), South Korea. The data revealed significant diffraction to 2.5 Å resolution. The data were integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). Auto-indexing was conducted with *DENZO* and indicated that the DHQS crystal belonged to the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 118.2$, $c = 98.2$ Å. The final statistics of data collection and processing details are summarized in Table 1.

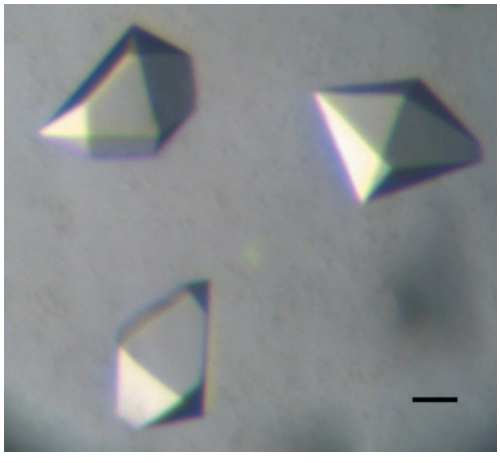


Figure 3
Crystals of DHQS from *X. oryzae* pv. *oryzae* (Xoo1243) obtained in a hanging-drop vapour-diffusion setup using 0.2 *M* ammonium sulfate, 0.1 *M* MES monohydrate pH 6.0 and 31%(*w*/*v*) PEG MME 5000. The scale bar represents 0.15 mm.

3. Results and discussion

Initial high-throughput crystallization screening of DHQS protein produced tiny crystals in eight of the conditions tested (Fig. 2). Not all of these initial leads could be scaled up successfully into hanging-drop experiments. The optimized condition [0.2 *M* ammonium sulfate, 0.1 *M* MES monohydrate pH 6.0 and 31%(*w*/*v*) PEG MME 5000, based on Hampton Research Crystal Screen II condition No. 26] resulted in well diffracting crystals (Fig. 3) and a complete diffraction data set was collected to 2.5 Å resolution from a single crystal. The systematic absences suggested that the crystal belonged to space group $P4_32_12$. The volume of the asymmetric unit of the DHQS crystal is compatible with the presence of two monomers in the unit cell, with a volume per unit molecular weight of the protein of 2.07 $Å^3\ Da^{-1}$ and a calculated solvent content of 40.4% (Matthews, 1968). A preliminary structure solution of the DHQS protein was obtained by molecular-replacement (MR) calculations using *AMoRe* (Navaza, 1994) with the crystal structure of DHQS from *T. thermophilus* (PDB code 1ujn) as a search model. The best results were obtained in space group $P4_32_12$, giving a correlation coefficient of 30.3% and an *R* factor of 51.2% for data in the resolution range 15–3.0 Å. Examination of the best MR solution structure showed good crystal packing and no clashes were found between symmetry-related molecules. The solution model was improved by rigid-body refinement in the resolution range 45.0–2.5 Å. The model was then mutated with the original amino-acid residues and was further improved (*R* factor of 32.2% and correlation coefficient of 61.4%) by restrained refinement (*REFMAC5*). The final model is currently being refined. Our structural data on DHQS will provide an insight into its enzymatic reaction mechanisms and may be useful for developing a drug against Xoo.

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