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Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of β-ketoacyl-ACP synthase III (FabH) from *Xanthomonas oryzae* pv. *oryzae*

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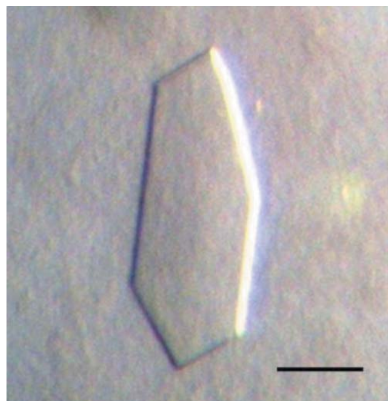
The bacterial β-ketoacyl-ACP synthase III (KASIII) encoded by the gene *fabH* (*Xoo4209*) from *Xanthomonas oryzae* pv. *oryzae*, a plant pathogen, is an important enzyme in the elongation steps of fatty-acid biosynthesis. It is expected to be one of the enzymes responsible for bacterial blight (BB), a serious disease that results in huge production losses of rice. As it represents an important target for the development of new antibacterial drugs against BB, determination of the crystal structure of the KAS III enzyme is essential in order to understand its reaction mechanism. In order to analyze the structure and function of KAS III, the *fabH* (*Xoo4209*) gene was cloned and the enzyme was expressed and purified. The KASIII crystal diffracted to 2.05 Å resolution and belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 69.8$, $b = 79.5$, $c = 62.3$ Å. The unit-cell volume of the crystal is compatible with the presence of a single monomer in the asymmetric unit, with a corresponding Matthews coefficient V_M of 2.27 $Å^3\,Da^{-1}$ and a solvent content of 45.8%.



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1. Introduction

Bacterial blight (BB) is a destructive disease caused by the plant-pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo) that results in huge production losses of rice worldwide, particularly in Asian countries (Ezuka & Kaku, 2000). Based on agricultural reports from South Korea, BB resulted in a huge production loss of rice worth more than 100 million dollars in 2006 alone. To date, no effective drugs have been developed against this disease and it is essential to find a drug against Xoo in order to halt rice-production losses. Lee *et al.* (2005) have recently determined the whole genomic sequence of Xoo, which provides the necessary information for the selection of drug-target enzymes. As the first step in initiating the development of an antibacterial drug against Xoo, almost one hundred genes, coding essential enzymes (Payne *et al.*, 2004, 2007) selected as target proteins for drug candidates, have been chosen from the 4538 putative genes (Lee *et al.*, 2005). As three-dimensional structures are crucial for drug development, *fabH* (*Xoo4209*) was cloned and the KASIII enzyme was expressed in *Escherichia coli*.

The bacterial fatty-acid synthesis type II pathway contains three ketoacyl-acyl carrier protein synthases (KASs), namely KAS I, KAS II and KAS III. The enzymes KAS I and KAS II are encoded by the genes *fabB* and *fabF*, respectively, and are involved in the condensation of malonyl-ACP with a growing acyl-ACP chain to form β-ketoacyl-ACP, which is a substrate for β-ketoacyl-ACP reductase (*fabG*). KAS III initiates chain elongation in type II fatty-acid synthesis and is also an essential enzyme for bacterial viability. Elongation of the chain by two C atoms in type II fatty-acid synthesis occurs by Claisen condensation of malonyl-acyl carrier protein (ACP) with acyl-ACP (Heath *et al.*, 2001; Khandekar *et al.*, 2003). KAS III initiates this chain-elongation process by specifically using acetyl-CoA over acyl-CoA, whereas KAS I and II utilize CoA thioesters as primers rather than acyl-ACPs.

The structures of the *fabH*-encoded KAS III enzymes from various species such as *Escherichia coli* and *Mycobacterium tuberculosis* have been determined and have been studied extensively. As they are the essential enzymes in bacteria and differ significantly from those involved in human fatty-acid synthesis, various bacterial FabH enzymes have been studied as potential antibacterial targets (Kaneda, 1963; Choi *et al.*, 2000; Khandekar *et al.*, 2000; He & Reynolds, 2002). FabH is a homodimeric structure with a monomeric molecular weight of nearly 35 kDa (Han *et al.*, 1998; Khandekar *et al.*, 2003). The substrate specificity of the various FabH enzymes appears to be the determining factor in the biosynthesis of branched- or straight-chain fatty acids in the type II fatty-acid synthesis pathway (Kaneda, 1963; Choi *et al.*, 2000). To date, only the crystal structure of *E. coli* FabH has been solved in the presence of substrate (acetyl-CoA) (Qiu *et al.*, 2001; Davies *et al.*, 2000).

With this in mind, *fabH* from the plant-pathogenic bacterium Xoo, which causes bacterial blight, has been cloned, expressed and purified in order to find an antibacterial drug against Xoo. As three-dimensional structures are key in identifying suitable drugs, the purified KAS III encoded by *fabH* (*Xoo4209*) was crystallized and a preliminary crystallographic study was conducted. Its structure may aid in efforts to identify an antibacterial drug against Xoo.

2. Methods and results

2.1. Cloning

The *fabH* (*Xoo4209*) gene was amplified using the genomic DNA of *X. oryzae* (Xoo KACC10331 strain); the oligonucleotides used were 5′-GGG GGG **CAT ATG** CTC TTC CAG AAT GTC TCC ATC-3′ for the sense primer and 5′-GGG GGG **GGA TCC** TCA CCA AAC CAC TTC CGC CAT-3′ for the antisense primer. These primers were designed based on the genomic report for *X. oryzae* (Lee *et al.*, 2005). The PCR-amplified fragment was digested with *Nde*I and *Bam*HI and then cloned into the vector pET11a (Novagen), encoding a polypeptide with an N-terminal hexahistidine tag to facilitate protein purification.

2.2. Overexpression and purification

FabH protein was expressed in *E. coli* BL21 (DE3) host-strain cells. Expression was performed in Luria–Bertani medium supplemented with 50 µg ml^{-1} ampicillin, which was incubated at 310 K until the OD_{600} reached ~0.6. FabH expression was induced by the addition of 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) for an additional 12 h at 288 K. For the preparation of soluble fractions, cultured cells were pelleted by centrifugation at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) for 20 min at 277 K and then resuspended in 50 ml ice-cold lysis buffer containing 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 15 m*M* imidazole, 3 m*M* β-mercaptoethanol. The cells were then lysed by ultrasonication (Sonomasher, S&T Science, Republic of Korea) on ice. The crude lysate was centrifuged at 15 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) for 30 min at 277 K and the clarified supernatant containing soluble protein was collected by centrifugation. All purification steps were performed at 277 K. FabH protein with an N-terminal hexahistidine tag was purified using Ni–NTA His-bind resin. Once all unbound protein had been washed from the column using 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 3 m*M* β-mercaptoethanol and 30 m*M* imidazole, FabH protein was eluted from the column using 200 m*M* imidazole with 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 3 m*M* β-mercaptoethanol. The eluted protein was extensively dialysed against buffer *A* (25 m*M* Tris–HCl pH 7.5, 3 m*M* β-mercaptoethanol, 20% glycerol) for 12 h at 277 K and then subjected to a TEV protease cleavage reaction to cleave the six additional His-tag residues from the protein [50:1(*w*:*w*) protein:TEV at 277 K for 16 h]. The mixture was again loaded onto an Ni–NTA resin affinity column to remove the protease and uncleaved protein. The flowthrough containing the target FabH was further purified on an UNO6 Q (Bio-Rad) anion-exchange column using buffer *B* (25 m*M* Tris–HCl pH 7.5, 20% glycerol, 3 m*M* β-mercaptoethanol, 1 *M* NaCl) as an elution reagent. The purity of the protein was estimated using SDS–PAGE at every step and the protein was found to be nearly homogeneous (Fig. 1) at 35 kDa after the second purification step (anion-exchange column). The protein solution was then concentrated to 4 mg ml^{-1} for crystallization screening.

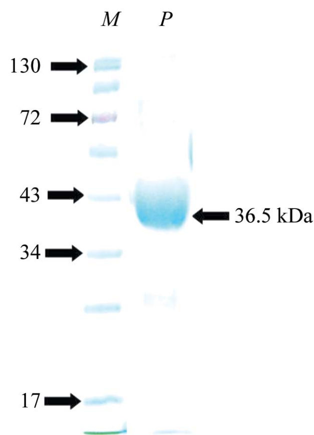

Figure 1
SDS–PAGE analysis of KAS III (Xoo4209) during purification. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane *P*, purified KAS III.

2.3. Crystallization and X-ray diffraction data collection

Initial crystallization screening was performed using commercially available kits, namely Crystal Screens I and II, Index Screen, PEG/Ion Screen, SaltRX (Hampton Research) and Wizard Screen, at 287 K by high-throughput crystallization screening using a Hydra II

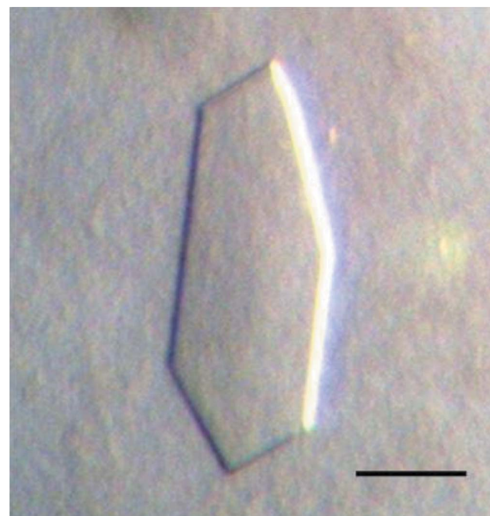

Figure 2
A crystal of KAS III protein obtained by the sitting-drop vapour-diffusion method using a condition optimized from Crystal Screen II condition G6 (0.1 *M* HEPES pH 7.2, 30% PEG 6000, 5% 2-methyl-2,4-pentanediol and 3% D-galactose). The scale bar represents 0.1 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Synchrotron	PLS, beamline 4A
Wavelength (Å)	0.96418
Resolution range (Å)	29.0–2.05 (2.1–2.05)
Space group	$P2_12_12$
Unit-cell parameters (Å)	
a	69.75
b	79.45
c	62.29
Total No. of reflections	21786
No. of unique reflections	20632
Completeness (%)	100.0 (23.2)
Molecules per ASU	1
V_M ($Å^3$ Da^{-1})	2.27
Solvent content (%)	45.8
Average $I/\sigma(I)$	7.9 (2.8)
R_{merge}† (%)	11.7 (41.5)
Oscillation angle (°)	1
Images collected	180

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

e-drop automated pipetting system (Matrix) in 96-well Intelli-Plates (Art Robbins). Crystal Screen II condition G6 produced tiny crystals in the condition 0.1 *M* HEPES pH 7.5, 10% PEG 6000, 5% 2-methyl-2,4-pentanediol. This condition was further optimized to obtain better quality crystals by changing the precipitant concentration and the buffer pH and using additives. A crystal suitable for diffraction (Fig. 2) was finally obtained using the condition 0.1 *M* HEPES pH 7.2, 30% PEG 6000, 5% 2-methyl-2,4-pentanediol with 3% D-galactose as an additive by the sitting-drop method. The crystallization setup used 1 µl protein solution mixed with 1 µl reservoir solution and equilibrated against 500 µl mother solution in the reservoir.

A crystal was frozen in liquid nitrogen with 20%(*v*/*v*) glycerol as a cryoprotectant, which was added all at once. X-ray diffraction data sets were collected to 2.05 Å resolution at 293 K from the frozen crystal using the ADSC Quantum 210 CCD detector on beamline 4A of Pohang Light Source (PLS), South Korea. The entire FabH data set was processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), respectively. The orthorhombic space group $P2_12_12$ was derived by auto-indexing (Otwinowski & Minor, 1997), with unit-cell parameters *a* = 69.8, *b* = 79.5, *c* = 62.3 Å. The completeness of the data in the highest resolution shell was very low (23.2%) even though the $I/\sigma(I)$ was good (2.8). 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 suite (Collaborative Computational Project, Number 4, 1994) and 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 36.5 kDa. The *BALBES* program (Long *et al.*, 2008) was used for molecular-replacement calculations and *REFMAC*5 (Murshudov *et al.*, 1997) was used for refinement. Our three-dimensional structural information on KAS III (Xoo4209) will provide a molecular basis for the enzymatic reaction mechanism and will be useful for developing potential antibacterial drugs against Xoo.

3. Results and discussion

Solution of the structure was obtained from these data by molecular replacement using the crystal structure of KAS III (PDB code 1hnj) from *E. coli*, which shares 23.3% sequence identity, as a search model. The R_{work} of the unrefined structure was found to be 39.2%, with a correlation coefficient of 65.3%. Examination of the molecular-replacement solution structure revealed good crystal packing with symmetry-related molecules and no clashes were found between them. The initial σ-weighted electron-density map with $2F_o - F_c$ Fourier coefficients and molecular-replacement phases was of interpretable quality almost throughout the chain. This preliminary model is currently being refined. This refined FabH structure may help us to develop antibacterial drugs against Xoo.

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