Cloning, expression, purification, crystallization
and X-ray crystallographic analysis of β -ketothiolase
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Polyhydroxyalkanoates are linear polyesters that are produced by bacterial fermentation and are used as biodegradable bioplastics. β -Ketothiolase B (BktB) from *Ralstonia eutropha* (ReBktB) is a key enzyme for the production of various types of copolymers by catalyzing the condensation reactions of acetyl-CoA with propionyl-CoA and butyryl-CoA. The ReBktB protein was crystallized using the hanging-drop vapour-diffusion method in the presence of 25% polyethylene glycol 3350, 0.1 M bis-tris pH 6.5, 0.2 M lithium sulfate at 295 K. X-ray diffraction data were collected to a maximum resolution of 2.3 Å on a synchrotron beamline. The crystal belonged to space group $C222_1$, with unit-cell parameters $a = 106.95$, $b = 107.24$, $c = 144.14$ Å. With two molecules per asymmetric unit, the crystal volume per unit protein weight (V_M) is $2.54 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 51.5%. The structure was solved by the molecular-replacement method and refinement of the structure is in progress.

1. Introduction

Ralstonia eutropha H16 is a Gram-negative lithoautotrophic bacterium that inhabits soil and fresh water, and has received a significant amount of attention from the biotechnology community owing to its unique metabolic properties (Aragno & Schlegel, 1992). *R. eutropha* can utilize both organic compounds and molecular hydrogen (H_2) as energy sources, and has the ability to synthesize polyhydroxyalkanoate (PHA) polymers in the process of storing surplus organic compounds (Steinbüchel & Fuchtenbusch, 1998). For the production of PHA, *R. eutropha* uses three enzymes, β -ketothiolase (PhbA), NADPH-dependent acetoacetyl-CoA reductase (PhbB) and PHB synthase (PhbC) (Peoples & Sinskey, 1989a,b; Pohlmann *et al.*, 2006; Schubert *et al.*, 1988; Slater *et al.*, 1988). Among the 37 β -ketothiolase homologues located in the *R. eutropha* genome, two β -ketothiolases, PhbA and β -ketothiolase B (BktB), are known to play a role in PHA synthesis by catalyzing the Claisen condensation reaction of two acetyl-CoA molecules to form acetoacetyl-CoA (Walsh, 1979; Pohlmann *et al.*, 2006; Slater *et al.*, 1998).

R. eutropha BktB (ReBktB) not only catalyzes the condensation reaction between two acetyl-CoA molecules to form acetoacetyl-CoA (Fig. 1), but also catalyzes the condensation reaction between acetyl-CoA and propionyl-CoA to produce valeryl-CoA, whereas RePhbA utilizes acetyl-CoA as its sole substrate and produces acetoacetyl-CoA (Slater *et al.*, 1998). Owing to the broad enzyme specificity of ReBktB, the enzyme has been used for the biosynthesis of poly(β -hydroxybutyrate-co- β -hydroxyvalerate) (PHBV) or longer chain copolymers (Hayward *et al.*, 1988a,b,c). Furthermore, it has also been reported that ReBktB can catalyze the condensation reaction between acetyl-CoA and butyryl-CoA to synthesize 3-ketohexanoyl-CoA, which can be further used for the production of hexanoate or *n*-hexanol (Machado *et al.*, 2012; Dekishima *et al.*, 2011). As a step towards elucidating the structural and biochemical properties of ReBktB, we cloned the gene coding for ReBktB and purified the recombinant ReBktB protein. Crystals of ReBktB of diffraction quality were obtained by the hanging-drop method. The crystal diffracted well: data were collected to a resolution of 2.3 Å and the structure was determined by the molecular-replacement method.

Figure 1

Claisen condensation reactions of *ReBktB*. *ReBktB* catalyzes the production of acetoacetyl-CoA, valeryl-CoA and 3-ketohexanoyl-CoA using acetyl-CoA, propionyl-CoA and butyryl-CoA as substrates.

Here, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the *ReBktB* protein.

2. Expression and purification of recombinant *ReBktB*

The forward and reverse primers were designed as 5'-GCGCGCA-TATGtgccaccggcgagctgg-3' and 5'-GCGCGCTCGAGTCAGATACGCTCGAAGATGGCGG-3' to introduce *Nde*I and *Xho*I cleavage sites, respectively. The gene coding for *ReBktB* was amplified from the *R. eutropha* H16 chromosome by polymerase chain reaction (PCR). The PCR product was then subcloned into pPROEX Hta (Invitrogen) resulting in a 6×His tag followed by a TEV protease (rTEV) cleavage site at the N-terminus. The resulting expression vector pPROEX Hta:*ReBktB* was transformed into *Escherichia coli* B834 strain and the cells were grown overnight in LB medium containing 100 µM ampicillin at 310 K. At an OD₆₀₀ of 0.6, *ReBktB* protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 20 h at 291 K the cells were harvested by centrifugation at 4000g for 15 min at 277 K. The cell pellet was resuspended in buffer A (40 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 13 500g for 25 min and the lysate

was applied onto an Ni-NTA agarose column (Qiagen). After washing with buffer A containing 18 mM imidazole, the bound proteins were eluted with 300 mM imidazole in buffer A. The 6×His tag was removed by treatment with rTEV, such that the resulting recombinant protein had extra amino acids Gly-Ala in front of the first methionine. Finally, the trace amount of contaminants was removed by size-exclusion chromatography using a Superdex 200 prep-grade column (320 ml, GE Healthcare) equilibrated with buffer A containing 1 mM dithiothreitol (DTT). The protein eluted at a molecular weight of ~160 kDa, indicating that the *ReBktB* protein with a molecular weight of 41 kDa forms a tetrameric structure. All purification experiments were performed at 277 K. The purity of the final protein was assessed by SDS-PAGE (Fig. 2). The purified protein was concentrated to 20 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT.

3. Crystallization

Crystallization of the purified *ReBktB* protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald BioSystems using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 2.0 µl protein solution (20 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT) with 2.0 µl reservoir solution and then equilibrating the mixture against 0.5 ml reservoir solution. *ReBktB* crystals were observed from several crystallization screening conditions. After several steps of improvement using the hanging-drop vapour-diffusion method, crystals of the best quality appeared in 2 d and reached their maximum dimensions of 0.05 × 0.2 × 0.2 mm using 25% polyethylene glycol 3350, 0.1 M bis-tris pH 6.5, 0.2 M lithium sulfate as the reservoir solution (Fig. 3).

4. X-ray analysis

The crystals were transferred to a cryoprotectant solution consisting of 25% polyethylene glycol, 0.1 M bis-tris pH 6.5, 0.2 M lithium sulfate, 30%(v/v) glycerol, fished out with a loop larger than the crystals and flash-cooled by immersion in liquid nitrogen at 100 K. The data were collected to a resolution of 2.3 Å on beamline 7A of the Pohang Accelerator Laboratory (PAL, Pohang, Republic of Korea) using a Quantum 270 CCD detector (ADSC, USA). All data

Figure 2

SDS-PAGE of purification of recombinant *ReBktB* protein. Lanes 1 and 10 show molecular-weight markers and are labelled in kDa. Lanes 2–9 show the purification procedure of *ReBktB* using Ni-NTA chromatography. Lane 2, whole cell extract; lanes 3 and 4, supernatant and pellet fraction after centrifugation of whole cell extract, respectively; lane 5, flowthrough from Ni-NTA column; lanes 6 and 7, wash with 5 and 10 mM imidazole, respectively; lanes 8 and 9, elution with 150 and 300 mM imidazole, respectively; lane 11, collection of elution from Ni-NTA; lane 12, TEV protease treatment; lane 13, collection of size-exclusion chromatography. 6×His *ReBktB* and native *ReBktB* are indicated on the right of the gel.

Table 1

Data-collection and refinement statistics for *ReBktB*.

Values in parentheses are for the outer shell.

Beamline	7A (MXII), PAL
Wavelength (Å)	1.0
Temperature (K)	100
Oscillation (°)	1.0
Mosaicity (°)	0.81
Total rotation range	360
Space group	C222 ₁
Unit-cell parameters (Å)	$a = 106.95$, $b = 107.24$, $c = 144.14$
Resolution (Å)	50.0–2.3 (2.34–2.30)
Total reflections	179113
Unique reflections	32074
Completeness (%)	85.9 (79.9)
$R_{\text{merge}}^{\dagger}$ (%)	7.0 (32.0)
$\langle I/\sigma(I) \rangle$	25.6 (2.6)
Multiplicity	5.6 (2.6)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections.

were indexed, integrated and scaled using the *HKL-2000* software package (Otwinowski & Minor, 1997). The crystals of *ReBktB* belonged to space group C222₁. A self-rotation function was calculated with *MOLREP* (Vagin & Teplyakov, 2010) to 2.5 Å resolution with a radius of integration of 39.7 Å. A peak in the $\chi = 180^\circ$ section suggests the presence of two noncrystallographic twofold axes in the *ac* plane and perpendicular to the crystallographic twofold axis along *b* (Fig. 4). The two axes are mutually perpendicular at $\varphi = 0^\circ$, $\theta = 50^\circ$ and $\varphi = 180^\circ$, $\theta = 40^\circ$, indicating overall 222 symmetry of the tetramer. Assuming that an asymmetric unit contains two molecules of *ReBktB*, the crystal volume per unit protein weight (V_M) is $2.54 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 51.5% (Matthews, 1968).

We attempted phase determination using the molecular-replacement method and a solution was found using the PhbA thiolase from *Zoogloea ramigera* (ZrPhbA; PDB entry 1dm3; Modis & Wierenga, 2000) with side chains converted to Ala as a search model. *ReBktB* has an amino-acid sequence identity of 52% to ZrPhbA. *MOLREP* (Vagin & Teplyakov, 2010) located two polyaniline model molecules in the asymmetric unit. The resulting solution had a correlation coefficient and *R* factor of 0.421 and 59.1%, respectively. After rigid-body refinement using *REFMAC5* (Murshudov *et al.*, 2011) from the *CCP4* suite (Winn *et al.*, 2011) in the resolution range 50–2.3 Å, *R* and *R*_{free} were 51.3 and 54.2%, respectively, with a correlation coefficient

Figure 3

Orthorhombic crystals of *ReBktB*. Crystals of the best quality were obtained using 25% polyethylene glycol 3350, 0.1 M bis-tris pH 6.5, 0.2 M lithium sulfate.

Figure 4

Stereographic projection of the $\chi = 180^\circ$ section of the self-rotation function calculated using data in the resolution range 31.8–2.5 Å with an integration radius of 39.7 Å.

of 0.653. The initial electron-density map, which was of good quality with backbone atoms well defined by electron density, allowed us to build a three-dimensional *ReBktB* model. Crystallographic model building and refinement of the structure to 2.5 Å resolution are in progress. The data statistics are summarized in Table 1.

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