Cloning, expression, purification, crystallization and X-ray crystallographic analysis of PhaA from *Ralstonia eutropha*

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Polyhydroxybutyrate (PHB) is a biopolymer that is in the spotlight because of its broad applications in bioplastics, fine chemicals, implant biomaterials and biofuels. PhaA from *Ralstonia eutropha* (RePhaA) is the first enzyme in the PHB biosynthetic pathway and catalyzes the condensation reaction of two acetyl-CoA molecules to give acetoacetyl-CoA. RePhaA was crystallized using the hanging-drop vapour-diffusion method in the presence of 20% polyethylene glycol monomethyl ether 2K, 0.1 M Tris-HCl pH 8.5 and 0.2 M trimethylamine N-oxide dihydrate at 295 K. X-ray diffraction data were collected to a maximum resolution of 1.96 Å on a synchrotron beamline. The crystal belonged to space group $P2_1$, with unit-cell parameters $a = 68.38$, $b = 105.47$, $c = 106.91$ Å, $\alpha = \gamma = 90$, $\beta = 106.18^\circ$. With four subunits per asymmetric unit, the crystal volume per unit protein weight (V_M) is $2.3 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of approximately 46.2%. The structure was solved by the molecular-replacement method and refinement of the structure is in progress.

1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of various kinds of hydroxycarboxylic acids that are intracellularly accumulated as a storage material for energy and/or reducing power in bacteria (Lee, 1996; Madison & Huisman, 1999; Reinecke & Steinbüchel, 2009). To date, over 150 PHA monomers with different compositions and thermal and mechanical properties have been reported (He *et al.*, 1999). PHAs are considered to be sustainable, environmentally friendly plastics because they are synthesized by microorganisms that are grown in an aqueous solution containing sustainable resources such as starch, glucose, sucrose, fatty acids and nutrients (Lee & Choi, 1999; Ariffin *et al.*, 2010). Numerous advances in understanding the mechanism of PHA biosynthesis have been made using molecular-genetics analyses (Anderson & Dawes, 1990; Steinbüchel *et al.*, 1992; Gao *et al.*, 2013).

PHAs are polymerized by PHA synthase, which utilizes various hydroxyacyl-CoAs. Although 3-hydroxyacyl-CoAs (3HA-CoAs) have been identified as the most favourable substrates for natural PHA synthases, a broad spectrum of hydroxyacyl-CoAs, such as 4-, 5- and 6-hydroxyacyl-CoAs, are also known to be polymerized by PHA synthases (Lee, 1996; Madison & Huisman, 1999; Steinbüchel & Valentin, 1995). Recently, it has also been reported that engineered PHA synthases are even capable of accepting 2-hydroxyacyl-CoAs such as lactyl-CoA and 2-hydroxybutyryl-CoA, resulting in the synthesis of novel polyesters containing 2-hydroxyacid monomers (Park *et al.*, 2012).

Various mechanisms of PHA production have been reported (Kemnitzer *et al.*, 1993; He *et al.*, 1999; Pederson *et al.*, 2006). The biosynthetic production of PHAs leads to much higher molecular weights than chemical methods. *Ralstonia eutropha* is the representative bacterial strain used for PHA biosynthesis, and three key enzymes, β -ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase (encoded by the *phaA*, *phaB* and *phaC* genes, respectively), are involved in PHA biosynthesis (Peoples & Sinskey, 1989). The biosynthetic pathway of PHAs in *R. eutropha* was engineered in *Escherichia coli*, and can be roughly divided into two stages: monomer production and polymerization (Shiraki *et al.*, 2006). Two enzymes are involved in the production of the monomer

Figure 1

Condensation reaction of *RePhaA*. *RePhaA* is the first enzyme in PHA biosynthesis and catalyzes the condensation reaction of two acetyl-CoA molecules to give acetoacetyl-CoA in *R. eutropha*.

(*R*)-3-hydroxybutyryl-CoA. PhaA initially condenses two acetyl-CoAs, producing acetoacetyl-CoA, which is then converted to the monomer of PHA by PhaB. The polymerization step is catalyzed by PhaC to generate biodegradable plastics that can be produced at 50% dry cell weight of the maximum theoretical yield (Peoples & Sinskey, 1989). Given the potential role of PHA as a bioplastic, the ultimate goal is to improve the efficiency of each step in its biosynthesis. Although several biochemical studies have been performed to assess the roles of PhaA, PhaB and PhaC in the biosynthetic pathway, structural information on these enzymes is limited.

PhaA is the first enzyme in PHA biosynthesis and catalyzes the condensation reaction of two acetyl-CoA molecules to give acetoacetyl-CoA (Fig. 1). As a step towards elucidating the structural and biochemical properties of PhaA from *R. eutropha* (*RePhaA*), we cloned the *RePhaA* coding gene and purified the recombinant *RePhaA* protein. Diffraction-quality crystals of *RePhaA* were obtained by the hanging-drop method. The crystal diffracted well; data were collected to a resolution of 1.96 Å and the structure was determined by the molecular-replacement method. Here, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the *RePhaA* protein.

2. Materials and methods

2.1. Expression and purification of recombinant *RePhaA*

The forward and reverse primers were designed as 5'-GCGCG-CTCGAGACTGACGTTGTCATCGTATCCG-3' and 5'-GCGCG-CTCGAGTTATTGCGCTCGACTGCCAGCG-3' containing *Nde*I and *Xho*I cleavage sequences (underlined). The *RePhaA* coding gene was amplified from the *R. eutropha* chromosome by polymerase chain reaction (PCR). The PCR product was then subcloned into pProEX HTa (Life Technologies) with a 6×His tag and a rTEV protease cleavage site at the N-terminus. The resulting expression vector pProEX HTa:*RePhaA* was transformed into *E. coli* strain BL21 (DE3) and the cells were grown overnight in LB medium containing 100 µM ampicillin at 310 K. When the OD₆₀₀ reached 0.6, expression of the *RePhaA* protein was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were grown for a further 20 h at 291 K and 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 bound to Ni-NTA agarose (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 from the *RePhaA* protein by incubating with 0.5 ml 1 mg ml⁻¹ rTEV protease (Gibco). Finally, a trace amount of contaminant 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 with a molecular weight of ~160 kDa, indicating that

Figure 2

Purification of *RePhaA*. (a) Ni-NTA affinity chromatography. Molecular-weight markers are labelled on the left side of the gel in kDa and purified 6×His-tagged *RePhaA* protein is indicated on the right side of the gel. Lanes 1–12 contain molecular-weight markers, whole cell extract, pellet fraction, supernatant fraction, flowthrough, wash with 5 mM imidazole, wash with 10 mM imidazole, wash with 20 mM imidazole, wash with 30 mM imidazole, wash with 40 mM imidazole, elution with 150 mM imidazole and elution with 300 mM imidazole, respectively. Lane 13 contains molecular-weight markers. Lanes 14 and 15 indicate samples before and after treatment with rTEV protease, respectively. (b) Size-exclusion chromatography. The elution peak of *RePhaA* is indicated with an arrow. Standard size markers 1–6 indicate thyroglobulin, ferritin, aldolase, conalbumin, ovalbumin and carbonic anhydrase, with molecular weights of 669, 440, 158, 75, 44 and 29 kDa, respectively. The inset shows an SDS-PAGE of the elution peaks.

Table 1

Expression and purification of recombinant *RePhaA*.

Source organism	<i>R. eutropha</i>
DNA source	Chromosome
Forward primer	GCGCGCATATGACTGACGTTGTCATCGTATCCG
Reverse primer	GCGCGCTCGAGTTATTGCGCTCGAGTCCAGCG
Cloning vector	pProEX HTa
Expression vector	pProEX HTa
Expression host	<i>E. coli</i> BL21 (DE3) strain
Complete amino-acid sequence of the construct produced	MSYYHHHHHHYDPTTENLYFQGMDVVIVSAARTAVGKFG- GSLAKTPAPELGAUVIKAAALERAGVKEQVSEVIMGQVLT- AGSGQNPAPAAIKAGLPAMVPAMTINKVCGSGLKAVMLA- ANAIMAGDAEIVVAGGQENMSAAPHVLPGSRDGFMRGDAK- LVDTIMVGLWDVYNYQYHMGITAENVAKEYGITREAQDEF- AVGSQNKAAEAQKAGFDEEIVPVLIPQRKGDVPVAFKTD- FVRQGATLDSMSGLKPAFDKAGTVTAANASGLNDGAAAVV- VMSAAKAKELGLTPLATIKSYANAGVDPKVMGMGPVASK- RALSRAEWTPQDLMEINEAFAAALAVHQQMGWDTSKV- NVNGGAIAGHPIGASGCRILVTLHEMKRRDAKGLASL- CIGGGMGVALAVERK

the *RePhaA* protein, with a molecular weight of 40.2 kDa, forms a tetrameric structure. All purification experiments were performed at 277 K. The degree of protein purification was confirmed by SDS-PAGE (Fig. 2). The purified protein was concentrated to 25 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT. The final yield of protein production was 15 mg protein per litre of culture. The activity of the protein was confirmed by measuring the thiolysis activity of the protein (50 nmol min⁻¹ mg⁻¹). The reaction mixture (total volume of 1 ml) consisted of 100 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 50 μ M CoA, 50 μ M acetoacetyl-CoA and 5 μ M *RePhaA*. After pre-incubation at 30°C for 5 min, the reaction was initiated by addition of the enzyme. The decrease in acetoacetyl-CoA was measured by monitoring the decrease in absorbance at 303 nm using an extinction coefficient of 14 $\times$ 10³. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Crystallization of the purified *RePhaA* protein was initially performed with commercially available sparse-matrix screens, including Wizard I and II from Emerald Bio, Index from Hampton Research and Structure Screen 1 and 2 from Molecular Dimensions, using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 2.0 μ l protein solution (25 mg ml⁻¹ in 40 mM Tris-HCl pH 8.0, 1 mM DTT) with 2.0 μ l reservoir solution and then equilibrating the drop against 0.5 ml reservoir solution.

Figure 3

Monoclinic crystals of *RePhaA*. Crystals with the best quality were obtained using 20% polyethylene glycol monomethyl ether 2K, 0.1 M Tris-HCl pH 8.5, 0.2 M trimethylamine *N*-oxide dihydrate at 295 K.

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well
Temperature (K)	295
Protein concentration (mg ml ⁻¹)	25
Buffer composition of protein solution	40 mM Tris-HCl pH 8.0, 1 mM DTT
Composition of reservoir solution	20% PEG MME 2K, 0.1 M Tris-HCl pH 8.5, 0.2 M trimethylamine <i>N</i> -oxide dihydrate
Volume and ratio of drop	2.0 μ l protein solution, 2.0 μ l reservoir solution
Volume of reservoir (ml)	0.5

RePhaA crystals were observed using several conditions from the Index screen: (i) 20% polyethylene glycol monomethyl ether (PEG MME) 2K, 0.1 M Tris-HCl pH 8.5, 0.2 M trimethylamine *N*-oxide dihydrate, (ii) 25% polyethylene glycol 3350, 0.1 M bis-tris pH 6.5, 0.2 M ammonium acetate and (iii) 25% polyethylene glycol 3350, 0.1 M HEPES pH 7.5, 0.2 M ammonium sulfate. After several steps that improved the crystallization process using the hanging-drop vapour-diffusion method, crystals with the best quality appeared in 7 d and reached maximum dimensions of 0.2 $\times$ 0.2 $\times$ 0.2 mm using reservoir solution consisting of 20% PEG MME 2K, 0.1 M Tris-HCl pH 8.5, 0.2 M trimethylamine *N*-oxide dihydrate (Fig. 3). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

The crystals were transferred into a cryoprotectant solution consisting of 20% PEG MME 2K, 0.1 M Tris-HCl pH 8.5, 0.2 M trimethylamine *N*-oxide dihydrate, 20% (v/v) glycerol, fished out with a loop larger than the crystals and flash-cooled by immersion in liquid nitrogen at 100 K. Data were collected to a resolution of 1.96 Å on beamline 7A of the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using a Quantum 270 CCD detector (ADSC, USA) (Supplementary Fig. S1[†]). All data were indexed, integrated and scaled using the *HKL-2000* software package (Otwinowski & Minor, 1997). The crystals of *RePhaA* belonged to space group *P*₂₁, with unit-cell parameters *a* = 68.38, *b* = 105.47, *c* = 106.91 Å, α = γ = 90°, β = 106.18°. With four molecules per asymmetric unit, the crystal volume per unit protein weight (*V*_M) was 2.3 Å³ Da⁻¹, which corresponds to a solvent content of approximately 46.2% (Matthews, 1968).

3. Results and discussion

A self-rotation function was calculated with *MOLREP* (Vagin & Teplyakov, 2010) to 4.0 Å resolution with a radius of integration of 48.18 Å. The crystallographic peaks for *P*₂₁ along the *b* axis are in the χ = 180° section. Two perpendicular twofold NCS axes are visible at θ = 69.3°, φ = 180° and θ = 20.7°, φ = 0.0° (Supplementary Fig. S2). The two NCS axes are also oriented perpendicular to the crystallographic twofold axis, which is in agreement with the presence of a tetramer in the asymmetric unit. We attempted the molecular-replacement method of phase determination and a solution was found using the structure of β -ketothiolase from *R. eutropha* (*ReBktB*; PDB entry 4nzs; E.-J. Kim, H. Son, S. Kim & K.-J. Kim, unpublished work) with side chains converted to Ala as a search model. *RePhaA* has an amino-acid sequence identity of 39% to *ReBktB*. *MOLREP* (Vagin & Teplyakov, 2010) located four polyalanine-model molecules in the asymmetric unit. The resulting solution had a correlation coefficient and an *R* factor of 0.482 and 56.3%, respectively. After rigid-body

[†] Supporting information has been deposited in the IUCr electronic archive (Reference: RL5083).

Table 3

Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	Beamline 7A, PAL
Wavelength (Å)	0.97954
Temperature (K)	100
Detector	Quantum 270 CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1.0
Total rotation range (°)	100
Exposure time per image (s)	1
Space group	$P2_1$
a, b, c (Å)	68.38, 105.47, 106.91
α, β, γ (°)	90, 106.18, 90
Mosaicity (°)	0.6
Resolution range (Å)	50.00–1.96 (1.99–1.96)
Total No. of reflections	657607
No. of unique reflections	93153
Completeness (%)	94.9 (98.3)
Multiplicity	6.7 (5.8)
$\langle I/\sigma(I) \rangle$	48.2 (20)
$R_{\text{r.i.m.}}$ (%)	12.8 (25.4)
Overall B factor from Wilson plot (Å ²)	22.5

refinement using *REFMAC5* (Murshudov *et al.*, 2011) from the *CCP4* suite (Winn *et al.*, 2011) in the resolution range 50–1.96 Å, R and R_{free} were 47.7 and 48.2%, respectively, with a correlation coefficient of 0.68. The initial electron-density map, which was of good quality with the main chain well defined by electron density, allowed us to build a three-dimensional *RePhaA* model. Crystallographic model building and refinement of the structure to 1.96 Å resolution are in progress. The data statistics are summarized in Table 3.

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References

- Anderson, A. J. & Dawes, E. A. (1990). *Microbiol. Rev.* **54**, 450–472.
- Ariffin, H., Nishida, H., Hassan, M. A. & Shirai, Y. (2010). *Biotechnol. J.* **5**, 484–492.
- Gao, X., Jian, J., Li, W.-J., Yang, Y.-C., Shen, X.-W., Sun, Z.-R., Wu, Q. & Chen, G.-Q. (2013). *Appl. Microbiol. Biotechnol.* **97**, 9099–9109.
- He, W. N., Zhang, Z. M., Hu, P. & Chen, C. Q. (1999). *Acta Polym. Sin.*, pp. 709–714.
- Kemnitz, J. E., McCarthy, S. P. & Gross, R. A. (1993). *Macromolecules*, **26**, 1221–1229.
- Lee, S. Y. (1996). *Biotechnol. Bioeng.* **49**, 1–14.
- Lee, S. Y. & Choi, J. (1999). *Waste Manag.* **19**, 133–139.
- Madison, L. L. & Huisman, G. W. (1999). *Microbiol. Mol. Biol. Rev.* **63**, 21–53.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst. D* **67**, 355–367.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Park, S. J., Lee, S. Y., Kim, T. W., Jung, Y. K. & Yang, T. H. (2012). *Biotechnol. J.* **7**, 199–212.
- Pederson, E. N., McChalicher, C. W. J. & Srien, F. (2006). *Biomacromolecules*, **7**, 1904–1911.
- Peoples, O. P. & Sinskey, A. J. (1989). *J. Biol. Chem.* **264**, 15298–15303.
- Reinecke, F. & Steinbüchel, A. (2009). *J. Mol. Microbiol. Biotechnol.* **16**, 91–108.
- Shiraki, M., Endo, T. & Saito, T. (2006). *J. Biosci. Bioeng.* **102**, 529–534.
- Steinbüchel, A., Hustede, E., Liebergesell, M., Pieper, U., Timm, A. & Valentin, H. (1992). *FEMS Microbiol. Rev.* **9**, 217–230.
- Steinbüchel, A. & Valentin, H. E. (1995). *FEMS Microbiol. Lett.* **128**, 219–228.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst. D* **66**, 22–25.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.