Crystallization and preliminary crystallographic analysis of poly(3-hydroxybutyrate) depolymerase from *Bacillus thuringiensis*

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Poly[(*R*)-3-hydroxybutyrate] (PHB) is a microbial biopolymer that has been commercialized as biodegradable plastics. The key enzyme for the degradation is PHB depolymerase (PhaZ). A new intracellular PhaZ from *Bacillus thuringiensis* (BtPhaZ) has been screened for potential applications in polymer biodegradation. Recombinant BtPhaZ was crystallized using 25% polyethylene glycol 3350, 0.2 M ammonium acetate, 0.1 M bis-tris pH 6.5 at 288 K. The crystals belonged to space group *P*1, with unit-cell parameters *a* = 42.97, *b* = 83.23, *c* = 85.50 Å, α = 73.45, β = 82.83, γ = 83.49°. An X-ray diffraction data set was collected to 1.42 Å resolution with an *R*_{merge} of 6.4%. Unexpectedly, a molecular-replacement solution was obtained using the crystal structure of *Streptomyces lividans* chloroperoxidase as a template, which shares 24% sequence identity to BtPhaZ. This is the first crystal structure of an intracellular poly(3-hydroxybutyrate) depolymerase.

1. Introduction

Poly[(*R*)-3-hydroxybutyrate] (PHB) is synthesized by a wide variety of bacteria and accumulates intracellularly in granule form, serving as a carbon- and energy-storage material (Anderson & Dawes, 1990). The key enzyme for PHB degradation is PHB depolymerase (PhaZ). Intracellular PhaZ acts on the accumulated native PHB during starvation periods, while extracellular PhaZ utilizes the denatured PHB released from dead bacteria (Jendrossek & Handrick, 2002). PHB and related copolymers have been commercialized as biodegradable plastics and thus many PhaZs have been screened for potential applications in polyester biodegradation (Jendrossek, 2009).

PhaZs belong to the α/β -hydrolase superfamily and contain a conserved Ser-Asp-His catalytic triad; nevertheless, these enzymes vary widely in length and show obviously distinct variations in sequence and in the structural domains that are present (Knoll *et al.*, 2009). For example, the extracellular PhaZs usually consist of a catalytic domain, a linker domain and one or two PHB-binding domains (Jendrossek & Handrick, 2002). However, there are also small PhaZs that consist of only the catalytic domain. To date, the only extracellular PhaZ crystal structures to have been solved are from *Paucimonas lemoignei* and *Penicillium funiculosum* (Hisano *et al.*, 2006; Papageorgiou *et al.*, 2008). Both are small PhaZs and possess a large number of different solvent-exposed hydrophobic residues forming a putative polymer-attachment site (Wakadkar *et al.*, 2010; Jendrossek *et al.*, 2013). A new small intracellular PhaZ from *Bacillus thuringiensis* (BtPhaZ) has been identified which is able to efficiently hydrolyze trypsin-treated PHB granules and produces 3-hydroxybutyrate (3HB) monomer as the major product (Tseng *et al.*, 2006). BtPhaZ shares no significant sequence similarity with any known protein. To understand the structural basis for PHB hydrolysis, how this enzyme produces 3HB monomer and how polymer binding occurs with BtPhaZ, we have obtained protein crystals and solved the phase problem using the molecular-replacement method.

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Table 1
BtPhaZ production information.

Source organism	<i>B. thuringiensis</i> subsp. <i>israelensis</i> ATCC 35646
DNA source	Chromosomal DNA
Forward primer	GGATCCATTAAAGCTGCAACAATGG
Reverse primer	GCCAAAGCTTCACTTCTCATTTCGGGGA
Cloning vector	pQE30
Expression vector	pQE30
Expression host	<i>E. coli</i> JM109
Complete amino-acid sequence of the construct produced	MRGSHHHHHHSIKPATMEFVSLNGETIAYQEVGRRTDIL-VLIHGNTSSQHWDLVIEKLQDQYHIYALDLRGFGQSTYN-QSIDSLQDFAEDVKLFIDELKLEKFSMLGWSMGGGVAMQF-TANHPTFVEKLLVESVGMKGYPFKKDTNGQPISVSLVK-TKEETIADQPVQIAPVLDIAIKNMNKLYYRTVWNLIIYTHNQ-PEPDREYKYLDDMLTQRNFVDVNYALITFNISDEHNGVVG-GSKQIHRKAPTLVIQGDYVVPVQVVEELAKHLPNAEL-KVLEDCGHSPFIDCLDFIKHVEDWLEQK

2. Materials and methods

2.1. Macromolecule production

BtPhaZ was expressed using the vector pEQ30 (Qiagen) in *Escherichia coli* JM109 (Tseng *et al.*, 2006). The recombinant protein contains 12 additional vector residues (MRGSHHHHHHGS) at the N-terminus. Cell pellets were resuspended in lysis buffer consisting of 20 mM Tris-HCl pH 7.0, 20 mM imidazole, 100 mM NaCl, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride and were lysed using a French press. After removal of cellular debris by centrifugation at 15 000g at 277 K for 30 min, the crude extract was applied onto a 5 ml nickel-nitrilotriacetic acid column (Qiagen). After washing with 20 and 40 mM imidazole, the protein was eluted with 200 mM imidazole and dialyzed against 20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM dithiothreitol at 277 K. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The initial crystallization screening was performed with Crystal Screen (Hampton Research) using the hanging-drop vapour-diffusion method at 295 and 288 K. Crystals were grown in 25% polyethylene glycol 3350, 0.2 M ammonium acetate, 0.1 M bis-tris pH 5.6, using a combination of 2 µl reservoir solution and 2 µl protein solution (10–12 mg ml⁻¹). Crystals appeared and reached their final dimensions of

Figure 1
A rod-shaped crystal of BtPhaZ with dimensions of 0.2 × 0.05 × 0.05 mm.

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	Hampton Research 24-well VDX plate
Temperature (K)	288
Protein concentration (mg ml ⁻¹)	10–12
Buffer composition of protein solution	20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM dithiothreitol
Composition of reservoir solution	0.1 M bis-tris pH 5.6, 0.2 M ammonium acetate, 25% polyethylene glycol 3350
Volume and ratio of drop	4 µl (1:1)
Volume of reservoir (ml)	0.3

~0.2 × 0.05 × 0.05 mm within 10–14 d at 288 K (Fig. 1). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

X-ray diffraction data were collected at 110 K with the addition of 20% glycerol as a cryoprotectant. As almost all crystals diffracted too weakly to collect data from, crystals were screened to obtain crystals suitable for data collection. Data were collected using an ADSC Q315r detector on beamline BL13B1 at the National Synchrotron Radiation Research Center, Taiwan. Diffraction data were processed to 1.42 Å resolution using *HKL-2000* (Otwinowski & Minor, 1997). The crystal belonged to space group *P1*, with unit-cell parameters $a = 42.97$, $b = 83.23$, $c = 85.50$ Å, $\alpha = 73.45^\circ$, $\beta = 82.83^\circ$, $\gamma = 83.49^\circ$. The twofold noncrystallographic symmetry in the self-rotation function map suggested that each asymmetric unit contains four protomers, giving a crystal volume per protein mass (V_M) of 2.04 Å³ Da⁻¹ and a solvent content of 39.7% (Matthews, 1968). The data statistics are summarized in Table 3.

3. Results and discussion

Since no suitable crystals of selenomethionine-incorporated protein could be obtained, we therefore performed a JCSG molecular-replacement (MR) trial even though BtPhaZ shares less than 25% sequence identity with all other proteins in the PDB database (Schwarzenbacher *et al.*, 2008). We first used the *FFAS* fold-recognition server (Rychlewski *et al.*, 2000) to identify the potential top ten homologous templates and subsequently employed the *WHAT IF* program (Vriend, 1990) to generate all-atom and mixed models as search models (Schwarzenbacher *et al.*, 2004). MR searches were performed with *MOLREP* (Vagin & Teplyakov, 2010) and the top solutions were subjected to rigid-body and restrained refinement with *REFMAC5* (Murshudov *et al.*, 2011). All MR trials gave relatively high R_{free} values and relatively low figures of merit (FOMs), indicating that these were incorrect solutions. However, using the monomeric mixed model generated from the crystal structure of *Streptomyces lividans* chloroperoxidase (PDB entry 1a88; Hofmann *et al.*, 1998) as the search model, the top MR solution contained four protomers, which showed good crystal packing without clashes between symmetry-related molecules. Unexpectedly, even with an R_{free} of 0.54 and FOM of 0.37, the model gave an interpretable electron-density map for all four protomers, in particular five α -helices and six β -strands. After several rounds of refinement with the removal of ~60 residues that did not fit the map and manual side-chain rebuilding of ~200 residues, the resulting model was used for another MR trial, leading to a better density map with an R_{free} and FOM of 0.34 and 0.73, respectively. A nearly complete atomic model was then automatically built by *ARP/wARP* (Morris *et al.*, 2003). Unexpectedly, in addition to the canonical α/β -hydrolase catalytic domain, BtPhaZ was found to possess a unique α -helical cap domain,

Table 3

Data collection and processing.

Values in parentheses are for the highest resolution shell.

Diffraction source	NSRRC 13B1
Wavelength (Å)	1.0
Temperature (K)	110
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	170
Rotation range per image (°)	0.5
Total rotation range (°)	360
Exposure time per image (s)	5
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	42.97, 83.23, 85.50
α , β , γ (°)	73.45, 82.83, 83.49
Mosaicity (°)	0.27
Resolution range (Å)	50.00–1.42 (1.47–1.42)
Total No. of reflections	753574 (58485)
No. of unique reflections	200742 (19495)
Completeness (%)	94.5 (91.9)
Multiplicity	3.8 (3.0)
$\langle I/\sigma(I) \rangle$	21.5 (2.6)
$R_{\text{merge}}^{\dagger}$ (%)	6.4 (51.6)
$R_{\text{r.i.m}}^{\ddagger}$ (%)	7.5 (63.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	20.29

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\ddagger$ The redundancy-independent merging *R* factor is estimated by multiplying the conventional R_{merge} value by the factor $[N(N-1)]^{1/2}$, where *N* is the data multiplicity.

which may represent a novel PHB-binding domain. Further structural refinement is in progress. The crystal structure of BtPhaZ will help to provide additional information that should aid in the modification of this enzyme in terms of its potential use in industrial applications.

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