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Crystallization and preliminary X-ray analysis of a novel thermoalkalophilic poly(3-hydroxybutyrate) depolymerase (PhaZ7) from *Paucimonas lemoignei*

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Polyhydroxyalkanoates (PHA) are biodegradable polyesters that have attracted commercial and academic interest as environmentally friendly materials. A number of enzymes are able to degrade polyhydroxyalkanoates to water-soluble products. PhaZ7 poly(3-hydroxybutyrate) (PHB) depolymerase (EC 3.1.1.75), a 342-amino-acid hydrolase from the PHA-degrading bacterium *Paucimonas lemoignei*, has been found to possess substrate specificity for amorphous PHA. PhaZ7 was crystallized by the microdialysis method. Thin rod-like crystals were grown in low ionic strength solution and found to belong to the monoclinic space group $C2$, with unit-cell parameters $a = 225.8$, $b = 46.5$, $c = 171.3$, $\beta = 128.9^\circ$. A complete data set was collected to 2.75 Å resolution at 100 K using synchrotron radiation.

1. Introduction

Poly(*R*)-hydroxyalkanoic acids (PHAs) belong to a group of carbon- and energy-storage compounds that are accumulated intracellularly in the form of inclusion bodies by a large variety of bacteria during unbalanced growth (Madison & Huisman, 1999). PHAs are biodegradable and recyclable and have therefore attracted commercial and academic interest for many applications in industry, agriculture and medicine as environmentally friendly materials. About 150 3-hydroxyacids (HAs) have been identified as constituents of microbial polyesters (Steinbüchel & Valentin, 1995). Of these, poly(3-hydroxybutyrate) (PHB) and copolymers of 3HB and 3-hydroxyvaleric acid (3HV) have been commercialized under the trade name Biopol®. According to the number of C atoms of the monomers, PHAs are classified as either short-chain-length PHAs (PHA_{SCL}; three to five C atoms) or medium-chain-length PHAs (PHA_{MCL}; six or more C atoms; Steinbüchel & Doi, 2002). A large number of bacteria have evolved with the ability to degrade PHA polyesters to water-soluble products through the secretion of specific depolymerases that belong to the hydrolase family. Extracellular PHA depolymerases (e-PHA depolymerases) act upon extracellular PHAs, which usually are in a partially crystalline form (dPHAs). All purified dPHA depolymerases to date are specific for either $dPHA_{SCL}$ or $dPHA_{MCL}$. A number of e-$dPHA_{SCL}$ depolymerases have been isolated and their properties and specificity have recently been reviewed (Jendrossek & Handrick, 2002).

One of the most intensively studied PHA-degrading bacterium is *Paucimonas lemoignei* (formerly *Pseudomonas lemoignei*; Jendrossek, 2001). Notably, *P. lemoignei* is unable to grow on sugars, amino acids or other complex media and hence utilizes PHA_{SCL}s, their hydrolysis products and a very small number of organic acids as a carbon and energy source. At least seven isoenzymes of e-PHA depolymerase (PhaZ1–PhaZ7) have been found in *P. lemoignei*. The reason for the existence of these isoforms is as yet unknown (Jendrossek & Handrick, 2002). PhaZ7, in particular, is specific for the native amorphous form of the PHB polymer (nPHB), but is inactive against dPHB. Accordingly, PhaZ7 is the first described extracellular $nPHA_{SCL}$ depolymerase. Interestingly, the enzyme is active and stable at high temperatures (332 K) and at alkaline pH (pH 9–12). Furthermore, no detectable lipase, cutinase, amidase/

nitrilase, protease, DNAse or RNAse activity has been found, suggesting that PhaZ7 is not a conventional esterase or lipase (Handrick *et al.*, 2001). Indeed, based on its biochemical properties, PhaZ7 appears to represent the first member of a new subgroup (EC 3.1.1.75) of serine hydrolases with no significant amino-acid similarities to any other PHB depolymerases, lipases or hydrolases apart from a modified lipase-box pentapeptide sequence Ala-His-Ser_{136}-Met-Gly and short regions around a putative oxyanion pocket that contain potential candidates for the catalytic triad. Site-directed mutagenesis studies have provided further evidence to support that PhaZ7 is a serine hydrolase with a catalytic triad and an oxyanion pocket formed by His47, Ser136, Asp242 and His306 (Braaz *et al.*, 2003).

Here, we report the crystallization and preliminary crystallographic analysis of PhaZ7 (MW 36 209 Da, 342 amino-acid mature protein) from *P. lemoignei*. No structures of homologous proteins are currently available and the structure determination will be pursued using experimental phasing methods. The structure will provide insights into the enzymatic mechanism of PHA biodegradation and may reveal novel features related to the enzyme's architecture.

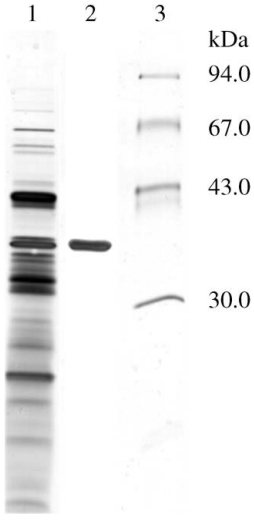
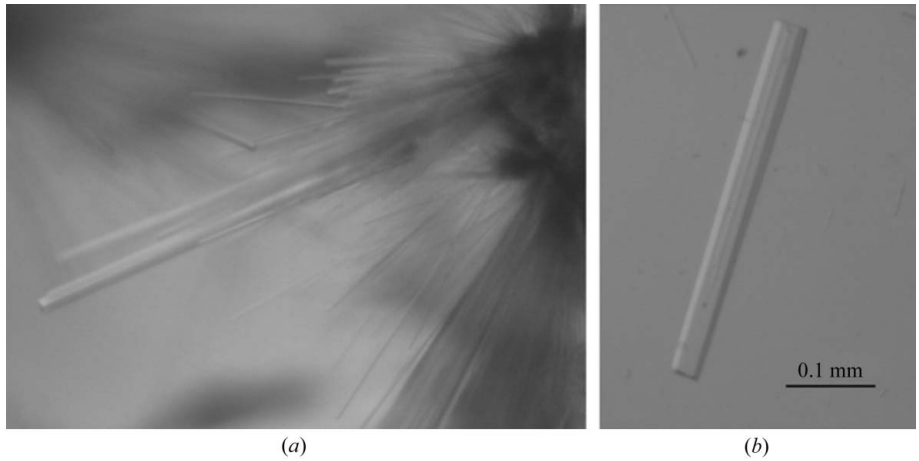


Figure 1
12% SDS–PAGE showing the purity of PhaZ7. Lane 1, *P. lemoignei* supernatant; lane 2, purified PhaZ7; lane 3, molecular-weight markers.

2. Materials and methods

2.1. Crystallization

PhaZ7 from *P. lemoignei* was isolated as described previously (Handrick *et al.*, 2001). Briefly, a succinate-grown culture of *P. lemoignei* (10 l) was harvested by centrifugation after 80 h of growth and the cell-free culture fluid was concentrated by ultrafiltration and ammonium sulfate precipitation. The protein fraction of the 85% ammonium sulfate saturated solution was dissolved and passed through a CM-Sepharose CL-6B column. Proteins were eluted with an NaCl gradient (0–200 m*M*) and fractions containing PhaZ7 were subsequently applied onto a Mono-P column (Amersham Pharmacia). The purification scheme results in homogeneous preparations (>98%) as judged by SDS–PAGE (Fig. 1). Approximately 10 mg PhaZ7 was typically obtained from a 10 l *P. lemoignei* culture. The protein concentration was estimated spectroscopically from the absorbance at 280 nm (Wetlaufer, 1962), using a calculated molar coefficient of 0.73 for 1.0 mg ml^{-1} protein solution. Prior to crystallization trials, the purified protein was first concentrated to ~10 mg ml^{-1} in 10 m*M* 1,3-diaminopropane–HCl (DAP–HCl) buffer pH 10.5 using a Centricon 10 kDa cutoff device (Amicon). As the protein samples contained high concentrations of Pharmalyte® polybuffer (Amersham Pharmacia) used for the elution of the protein from the Mono-P column, the next step included addition of NaCl to 1 *M* to facilitate the removal of Pharmalyte from the protein solution, followed by concentration of the protein in the Centricon device. This procedure was repeated several times. In the final run, only buffer was added to the Centricon's compartment in order to gradually lower the NaCl concentration in the protein sample prior to crystallization. At this stage, however, it was noticed that the protein started to precipitate. Examination of the precipitate under a light microscope revealed the presence of tiny needle-like crystals. To obtain crystals suitable for X-ray analysis, a crystallization protocol was established using the microdialysis technique. Protein samples were concentrated to 1.5–3.0 mg ml^{-1} and 20 µl protein was loaded into the chamber of microdialysis buttons (HR3-320) from Hampton Research. Each button was subsequently sealed using a double layer of 10 kDa cutoff dialysis membrane. The buttons were placed into different ionic strength reservoir solutions of NaCl or LiCl in 10 m*M* DAP–HCl buffer pH 10.5 and incubated at 277 K. Thin rod-shaped crystals that grew in clusters appeared after two weeks in reservoir conditions containing 150 and 250 m*M* NaCl or LiCl. Careful



(*a*) (*b*)

Figure 2
(*a*) A typical clump of PhaZ7 crystals. (*b*) A single crystal of PhaZ7.

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the highest resolution shell (2.81–2.75 Å).

Space group	*C*2
Unit-cell parameters (Å, °)	a = 225.8, b = 46.5, c = 171.3, β = 128.9
Matthews coefficient (Å^3 Da^{-1})	2.2
Resolution (Å)	30–2.75 (2.81–2.75)
Total observations	340578
Unique reflections	35718
Data completeness (%)	96 (95)
$R_{\rm merge}$† (%)	8.0 (32.0)
Average $I/\sigma(I)$	15 (9)
Mosaicity (°)	0.24

† $R_{\rm merge} = \sum|I_j - \langle I\rangle|/\sum\langle I\rangle$, where I_j is the observed intensity of reflection j and $\langle I\rangle$ is the average intensity of multiple observations.

separation of the crystals from the clusters led to the isolation of single crystals of suitable size for X-ray diffraction studies (Fig. 2).

2.2. Data collection and processing

Diffraction data were collected using synchrotron radiation. A native data set was collected from a single crystal on the EMBL BW7A beamline at the DORIS storage ring, DESY, Hamburg. Crystals were transferred into a reservoir solution containing 30% glycerol as cryoprotectant and flash-cooled to 100 K in a nitrogen-gas cold stream using an Oxford Cryosystems device. 300 images with 0.5° rotation each were collected to a resolution of 2.75 Å at a wavelength of 0.92106 Å. Diffraction data were recorded on a MAR CCD detector with a diameter of 165 mm. Data were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The Matthews coefficient was determined with the program *MATTHEWS_COEFF* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

PhaZ7 from *P. lemognei* was successfully crystallized using the microdialysis technique. The thin rod-like crystals diffract poorly in-house on a rotating-anode generator. A native data set to 2.75 Å resolution was collected using synchrotron radiation. From the diffraction data processing, the crystals were found to be monoclinic and the space group was determined to be *C*2, with unit-cell parameters a = 225.8, b = 46.5, c = 171.3 Å, β = 128.9°. Complete data-collection and processing statistics are given in Table 1. Scaling and merging of the crystallographic data resulted in an overall $R_{\rm merge}$ of 8.0% and an $R_{\rm merge}$ in the highest resolution shell of 32.0%. The merged data set was 96% complete to 2.75 Å resolution. Assuming the presence of four molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.2 Å^3 Da^{-1} and the solvent content is 43.4%. No homologous structures are currently available in the Protein Data Bank. The structure of PhaZ7 will be determined using heavy-atom derivatives. Attempts to express the SeMet derivative have been unsuccessful (D. Jendrossek, unpublished observations). During the search for a suitable heavy atom, soaking with 10 m*M* $EuCl_3$ resulted in unit-cell changes, suggesting possible binding. Optimization of the data collection to detect the anomalous signal of $EuCl_3$ for phasing purposes is currently under way. The structure of PhaZ7 will be the first of this family of proteins and may reveal novel features related to enzymatic biodegradation mechanisms.

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