Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction data for the carboxylesterase Est30 from *Bacillus stearothermophilus*

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Received 8 April 2003
Accepted 21 May 2003

Crystals have been grown of the carboxylesterase Est30 from *Bacillus stearothermophilus* by hanging-drop vapor diffusion using ammonium sulfate as precipitant. The crystals diffracted to better than 2.0 Å resolution. X-ray diffraction data were reduced in space group $C222_1$, with unit-cell parameters a = 55.83, b = 58.15, c = 179.65 Å. $R_{\rm merge}$ was 0.038 for 17 449 independent reflections with a completeness of 85.1%. $V_{\rm M}$ was calculated to be 2.43 Å^3 Da^{-1}, which suggested that there was one molecule of Est30 in the asymmetric unit. These crystals are suitable for structure determination.

1. Introduction

Microbial esterases show high thermal stability and have wide applications in the fields of oleochemistry, organic chemistry and the formulation of detergents, in addition to industrial uses as digestive and flavoring enzymes (Amaki *et al.*, 1992). Esterases have considerable potential as catalysts in industrial processes. These enzymes perform a number of valuable biotransformations: the resolution of racemic mixtures, synthetic reactions, the blocking or unblocking of catalytic groups in peptide chemistry and the modification of sugars (Margolin, 1993; Moher *et al.*, 1989). In addition, thermostable enzymes are valuable for industrial applications owing to their stability (Owsu & Cowan, 1989).

Two thermostable esterase genes from *Bacillus stearothermophilus* were recently isolated from the genomic libraries and were designated *est*30 and *est*50 (accession Nos. AAN81911 and AAN81910). Although both Est30 and Est50 (EC 3.1.1.1) are members of the carboxyl esterase family (Wood *et al.*, 1995; Zock *et al.*, 1994; Amaki *et al.*, 1992), Est30 has low enzymatic activity on the substrates of Est50 (unpublished data). Structural studies of Est30 could provide fundamental insights into its substrate specificity.

The high regiospecificity and stereospecificity of carboxylesterases has made them very promising biocatalysts for the production of optically pure compounds in chemical synthesis. They have applications in the synthesis of anti-inflammatory drugs, food flavors and insecticides. The crystal structure of Est30 will help to explore the potential of this enzyme for hydrolyzing specific substrates. We report the purification, crystallization and preliminary diffraction data for Est30 as the first step in determining the crystal structure.

2. Methods

2.1. Expression and purification of recombinant Est30

The coding sequence of *est*30 amplified by PCR was cloned into the pBAD-HisA vector (Invitrogen) and the resulting recombinant plasmid was designated pHE30. *Escherichia coli* Top10 (Invitrogen) harboring pHE30 was induced for 4 h by 1 m*M* arabinose in 1 l culture and then harvested at 5000*g* for 15 min at 277 K. The cell pellet was suspended in 40 ml of hypertonic solution consisting of 20 m*M* Tris–HCl pH 7.5, 2.5 m*M* EDTA and 20% sucrose. The suspension was incubated on ice for 30 min and vortexed every 10 min. Cells were removed by centrifugation at 13 000*g* for 20 min at 277 K and the cell pellet was resuspended in 60 ml of 20 m*M* Tris–HCl pH 7.5, 2.5 m*M* EDTA (hypotonic solution) and further incubated on ice for 30 min with intermittent vortexing every 10 min. Cells were removed by high-speed centrifugation at 22 000*g* for 20 min at 277 K and the supernatant applied to a Pharmacia Q-Sepharose Hiload 26/10 HP that was equilibrated with 50 m*M* Tris–HCl pH 8.0. The column was washed with 150 ml of the equilibration buffer to remove unbound proteins, followed by a linear gradient to 50 m*M* Tris–HCl pH 8.0 and 1 *M* KCl. The esterase peak eluted at 0.65 *M* KCl. Fractions containing esterase activity were pooled (32 ml), concentrated to 5 ml by ultrafiltration and applied to a Sephacryl S 200 HR column (Pharmacia, 26/60). The enzyme was eluted using 50 m*M* potassium phosphate pH 7.0 and 0.15 *M* NaCl.

2.2. Crystallization and X-ray data collection

Purified protein was dialyzed into Tris buffer pH 8.0 and concentrated to 4.6 mg ml^{-1}. Est30

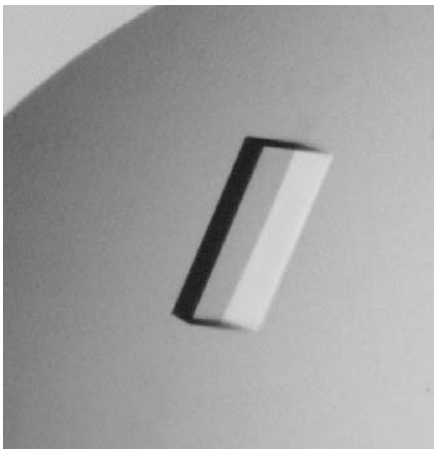

Figure 1
Crystal of Est30 grown in 50% ammonium sulfate, 10% MPD, 0.1% sodium azide, 100 m*M* HEPES pH 7.5 at room temperature. The size of this crystal is approximately 0.3 × 0.17 × 0.05 mm.

protein was crystallized by hanging-drop vapor diffusion at 297 K using 1 µl of protein and 1 µl of mother liquor. The initial crystallization screen used Hampton Research Crystal Screen 1 (Hampton Research, CA, USA; Jancarik & Kim, 1991) and crystal clusters were observed in 2.4 *M* ammonium sulfate and 100 m*M* Tris pH 8.5. The optimum conditions for crystal growth used 45–49% ammonium sulfate, 100 m*M* Tris buffer pH 7.5–8.1 or 100 m*M* HEPES pH 7.5, 8–12% methylpentanediol (MPD) and 0.1% sodium azide.

For X-ray data collection, crystals were mounted on a nylon loop and frozen directly in liquid nitrogen at 90 K. No cryoprotectant was used. X-ray diffraction data were collected on a MAR CCD165 detector at the SER-CAT beamline of the National Synchrotron Light Source at the Advance Photon Source. The crystal-to-detector distance was 150 mm. The oscillation angle was 0.2° and the rotation range was 180° for the complete data set. X-ray diffraction data were measured to 1.99 Å resolution using a wavelength of 1 Å. Data integration used *DENZO* and the intensity data scaling used *SCALEPACK* from the *HKL*2000 program suite (Otwinowski & Minor, 1997).

Table 1
Data collection and processing statistics.

Outer-shell statistics (2.07–1.99 Å) are given in parentheses.

Space group	$C222_1$
Unit-cell parameters (Å)	a = 55.83, b = 58.15, c = 179.65
No. reflections with $I > 3\sigma(I)$	16997 (1182)
$\langle I/\sigma(I)\rangle$	19.4 (7.6)
Independent reflections	17449 (1388)
Resolution (Å)	1.99 (2.07–1.99)
$R_{\rm merge}$†	0.038 (0.112)
Completeness (%)	85.1 (69.4)
Redundancy	4.8 (2.2)

† $R_{\rm merge} = \sum |I - \langle I\rangle| / \sum I$.

3. Results and discussion

The recombinant Est30 from *B. stearothermophilus* was expressed and purified for crystallographic analysis. The total yield of pure esterase was 6 mg per litre of culture. The purified Est30 was judged to be in excess of 95% homogeneous. Under the optimal crystallization conditions, Est30 crystals appeared after 2 or 3 d and reached maximum dimensions of 0.6 × 0.15 × 0.05 mm after one week. An example is shown in Fig. 1.

The crystallographic statistics are summarized in Table 1. The crystal volume per protein mass ($V_{\rm M}$) was calculated to be 2.43 Å^3 Da^{-1} (Matthews, 1968) and the solvent content was 0.49, which suggested there was one molecule of Est30 in the asymmetric unit. These crystals are suitable for structure determination.

The Est30 structure cannot be solved by molecular replacement owing to the lack of an available structure with homologous protein sequence. Multiple isomorphous data or anomalous dispersion data will be needed to obtain phase information. Halide soaks were not successful. Other heavy-atom soaks will be tested. However, Est30 has two cysteine and ten methionine residues. Therefore, SeMet-enriched protein is being prepared for phase determination using the multiwavelength anomalous diffraction (MAD) method. The high-resolution crystal structure of Est30 will provide fundamental insights into the substrate specificity of this thermostable enzyme. The structure will assist in the development of the enzyme as an industrial biocatalyst for the production of optically pure compounds in chemical synthesis.

X-ray data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID (or 22-BM) beamline at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Science under Contract No. W-31-109-Eng-38. This work was supported in part by the National Science Foundation Grant INT9713644.

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