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Crystallization and preliminary X-ray crystallographic analysis of EstE1, a new and thermostable esterase cloned from a metagenomic library

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EstE1, a new thermostable esterase, was isolated by functional screening of a metagenomic DNA library from thermal environment samples. This enzyme showed activity towards short-chain acyl derivatives of length C4–C6 at a temperature of 303–363 K and displayed a high thermostability above 353 K. EstE1 has 64 and 57% amino-acid sequence similarity to est_{pc}-encoded carboxylesterase from *Pyrobaculum calidifontis* and AFEST from *Archaeoglobus fulgidus*, respectively. The recombinant protein with a histidine tag at the C-terminus was overexpressed in *Escherichia coli* strain BL21(DE3) and then purified by affinity chromatography. The protein was crystallized at 290 K by the hanging-drop vapour-diffusion method. X-ray diffraction data were collected to 2.3 Å resolution from an EstE1 crystal; the crystal belongs to space group $P4_12_12$, with unit-cell parameters $a = b = 73.71$, $c = 234.23$ Å. Assuming the presence of four molecules in the asymmetric unit, the Matthews coefficient V_M is calculated to be 2.2 Å^3 Da^{-1} and the solvent content is 44.1%.

1. Introduction

Esterases are ubiquitous enzymes with important physiological and biotechnological roles in the synthesis or hydrolysis of ester-containing compounds. The lipolytic enzymes, including esterases and lipases, have been widely used in industry to catalyze the stereo-specific hydrolysis, transesterification and conversion of a variety of amines as well as primary and secondary alcohols (Chen *et al.*, 1995; Bornscheuer, 2002; Bornemann *et al.*, 1992). In contrast to lipases, which display maximal activity towards water-insoluble long-chain triglycerides, esterases hydrolyse at least partly soluble fatty-acid esters with acyl chain lengths of less than ten C atoms (Jaeger *et al.*, 1999).

EstE1, a new thermostable esterase, was isolated by functional screening of *Escherichia coli* cells transformed with environmental DNA libraries constructed with the metagenomes from thermal environmental samples (Rhee *et al.*, 2004). EstE1 exhibits activity towards esters with relatively short acyl chains of C4–C6, suggesting that the enzyme is not a lipase but an esterase. EstE1 displays a high thermal stability at 353 K and its activity increases in proportion to increase in temperature up to 368 K in a reproducible manner (Rhee *et al.*, 2004). Although thermostable enzymes have considerable potential for many biotechnological and industrial applications, fewer than a dozen thermostable lipolytic enzymes have been isolated from thermophiles and hyperthermophiles (Haki & Rakshit, 2003). Of these, only three esterases from *Archaeoglobus fulgidus* (Manco *et al.*, 2000), *Sulfolobus solfataricus* (Morana *et al.*, 2002) and *Pyrobaculum calidifontis* (Hotta *et al.*, 2002) have been biochemically characterized and their cognate genes reported. Thermostable lipolytic enzymes are usually resistant to chemical denaturation. Therefore, they have been successfully applied to the synthesis of biopolymers, pharmaceuticals, agrochemicals, cosmetics and flavours (Haki & Rakshit, 2003; Jaeger *et al.*, 1999).

Bacterial carboxylesterases have been classified into eight families based on conserved sequence motifs and biological properties (Arpigny & Jaeger, 1999). The amino-acid sequence of EstE1 is significantly similar to those of the family IV enzymes, which are also known as the hormone-sensitive lipase (HSL) family (Rhee *et al.*, 2004). In particular, EstE1 shows 64 and 57% amino-acid sequence

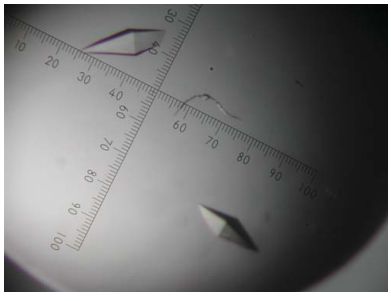

Table 1
X-ray data-collection statistics.

Wavelength (Å)	1.0000
Resolution (Å)	2.3
Space group	$P4_12_12$
Unit-cell parameters (Å, °)	a = 73.71, b = 73.71, c = 234.23, $\alpha = \beta = \gamma$ = 90
Total/unique reflections	1996840/29848
Completeness (%)	99.8 (98.8)
$R_{\rm merge}$† (%)	11.9 (32.2)
Average $I/\sigma(I)$	10.6 (4.1)

† $R_{\rm merge} = \sum |I_i - \langle I \rangle| / \sum I$, where I_i is the intensity of the ith observation and $\langle I \rangle$ is the mean intensity of the reflections.

similarity with est_{pc}-encoded carboxylesterase (Hotta *et al.*, 2002) from *P. calidifontis* and AFEST from *A. fulgidus*, respectively. Molecular structures are available for three members of the HSL family (family IV) (Wei *et al.*, 1999; De Simone *et al.*, 2000, 2001) and AFEST is one of them. The structures of these carboxylesterases show an α/β-hydrolase fold, which is found in various enzymes whose activities depend mostly on a catalytic triad formed by Ser, His and Asp residues (Ollis *et al.*, 1992; Heikinheimo *et al.*, 1999; Nardini & Dijkstra, 1999).

2. Materials and methods

2.1. Protein expression and purification

The EstE1 gene, which had been inserted into the T7 promoter-driven expression vector pET-22b(+) (Novagen) (Rhee *et al.*, 2004), was introduced into *E. coli* strain BL21(DE3) competent cells. The transformants were selected on LB agar plates containing 100 µg ml^{-1} ampicillin and cultured at 310 K in liquid LB media containing the same concentration of ampicillin. Production of the EstE1 protein with a histidine tag at the C-terminus was induced at a temperature of 310 K by the addition of 1 m*M* isoproryl-β-D-thio-galactopyranoside (IPTG) and the cells were cultivated for a further 12 h. The cells were harvested and resuspended with binding buffer containing 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 10 m*M* β-mercaptoethanol, 10% glycerol and then sonicated on ice. After centrifugation of the cell extracts, the supernatant was incubated at 353 K for 10 min and further centrifuged to acquire clarified cell lysates by discarding denatured proteins. The supernatant, which included the EstE1 protein, was loaded on Ni-charged Chelating Sepharose Fast Flow resin (Amersham Biosciences) that had been previously equilibrated with binding buffer. Contaminating proteins were removed by washing with a decreased concentration of imidazole. In this way, EstE1 protein was purified using the affinity chromatographic method and concentrated to about 10 mg ml^{-1} for crystallization.

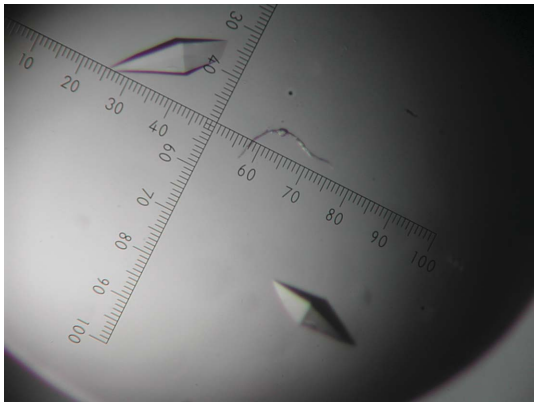

Figure 1
Crystals of EstE1 grown by the hanging-drop vapour-diffusion method. The approximate dimensions of the crystal are 0.4 × 0.2 × 0.1 mm.

2.2. Crystallization and data collection

Initial crystallization of EstE1 was performed with commercially available screening solutions (Hampton Research) using the micro-batch method at 290 K. Crystals of EstE1 were formed using various conditions and subsequent steps for the optimization of crystallization were carried out using the hanging-drop vapour-diffusion method at 290 K. X-ray diffraction data from the crystals were collected on a Bruker Proteum 300 CCD detector at the 6B beamline of Pohang Light Source (PLS), South Korea.

3. Results and discussion

Crystals suitable for data collection were obtained in drops formed of a mixture of 1.5 µl protein sample and 1.5 µl reservoir solution [0.1 *M* Bis-Tris pH 6.5, 0.2 *M* ammonium sulfate, 35%(*w*/*v*) PEG 3350] and equilibrated against 1000 µl reservoir solution for 2 d (Fig. 1). Prior to mounting, the crystals were transferred and soaked in a cryoprotectant solution containing 15% ethylene glycol, 0.1 *M* Bis-Tris pH 6.5, 0.2 *M* ammonium sulfate, 35%(*w*/*v*) PEG 3350. The data collected were processed using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). The crystal of EstE1 diffracted to approximately 2.3 Å resolution and belongs to space group $P4_12_12$, with unit-cell parameters $a = b$ = 73.71, c = 234.23 Å, $\alpha = \beta = \gamma = 90°$ (see Table 1). Assuming the presence of four molecules in an asymmetric unit, the Matthews coefficient $V_{\rm M}$ is calculated to be 2.2 Å^3 Da^{-1}, which corresponds to a solvent content of 44.1%. Molecular replacement of these crystals is in progress using the program *CNS*. The crystal structure of the hyperthermophilic carboxylesterase AFEST from *A. fulgidus* (PDB code 1jji) has been used as a molecular-replacement search model and advanced refinement is in progress.

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