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Identification, crystallization and preliminary X-ray diffraction analysis of esterase A from *Caulobacter crescentus* CB15, a family VIII lipolytic enzyme

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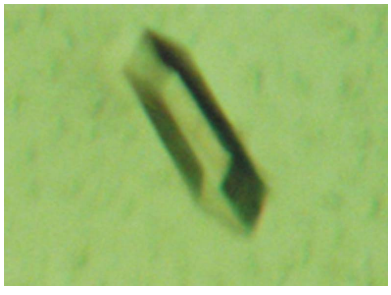

The structures and functions of family VIII lipolytic enzymes, which have moderate sequence identity to class C β-lactamases and penicillin-binding proteins, are largely unknown. Here, the X-ray crystallographic study of a family VIII esterase from *Caulobacter crescentus* CB15 (CcEstA) is described. Sequence analysis revealed that CcEstA has a conserved serine residue within the S-*X*-*X*-K motif which acts as a catalytic nucleophile. Recombinant protein containing an N-terminal His tag was expressed in *Escherichia coli* and purified to homogeneity. Functional studies showed that CcEstA acts on α- and β-naphthyl acetate as substrates. In addition, it can catalyze the hydrolysis of ketoprofen ethyl ester, a highly useful product in industrial applications. CcEstA was crystallized using a solution consisting of 1.0 *M* potassium/sodium tartrate, 0.1 *M* imidazole pH 8.0, 0.2 *M* NaCl, and X-ray diffraction data were collected to a resolution of 1.62 Å with an $R_{\rm merge}$ of 9.4%. The crystals of CcEstA belonged to space group $C222_1$, with unit-cell parameters $a = 172.23$, $b = 176.68$, $c = 47.93$ Å. Structure determination is in progress.

1. Introduction

Lipolytic enzymes such as esterases and lipases are widely employed in a number of applications in biotechnology (Schmidt *et al.*, 2009; Qian *et al.*, 2007), mostly owing to their high stability, their substrate specificity, their involvement in a broad range of reactions and the enantioselectivity of the products. These enzymes have been classified into eight families (I–VIII) based on their amino-acid sequences, tertiary structures and biological properties (Arpigny & Jaeger, 1999; Bornscheuer, 2002). In general, most lipolytic enzymes have an α/β-hydrolase fold which contains a catalytic triad consisting of a nucleophilic serine (Ser), a histidine (His) and an acidic residue (Asp or Glu). The nucleophilic serine, which can attack the electrophilic atom to form a reaction intermediate, is located within the highly conserved motif G-*X*-S-*X*-G (where *X* is any amino acid; Ollis *et al.*, 1992; Holmquist, 2000; Carr & Ollis, 2009). Unlike the enzymes in other lipolytic enzyme families, the enzymes in family VIII have the nucleophilic serine located within a conserved S-*X*-*X*-K motif. All of the enzymes belonging to this family are active towards nitrophenyl esters and triglycerides with short-chain acyl moieties and thus have been characterized as carboxylesterases. Furthermore, they show structural similarities to the class C β-lactamases in spite of functional differences, as shown in the crystal structure of EstB from *Burkholderia gladioli* (Wagner *et al.*, 2002; PDB entry 1ci8), which was the first crystal structure of a family VIII lipolytic enzyme to be determined.

To date, several esterases from family VIII have been biochemically investigated: EstA from *Pseudomonas* sp. (McKay *et al.*, 1992; AAA25813), esterase III from *P. fluorescens* (Kim *et al.*, 1994; AAC60403), ethylchrysanthemate esterase from *Arthrobacter globiformis* (Nishizawa *et al.*, 1995; AAA99492), EstA from *Streptomyces chrysomallus* (Berger *et al.*, 1998; CAA78842), EstB from *B. gladioli*

(Petersen *et al.*, 2001; AAF59826) and EstA from *Arthrobacter nitroguajacolicus* (Schütte & Fetzner, 2007; CAD61039). Furthermore, family VIII esterases have also been identified, cloned and characterized from uncultivated microorganisms (Rashamuse *et al.*, 2009, FJ025785; Kim *et al.*, 2010, GQ426329; Yu *et al.*, 2011, HQ154132). However, only one crystal structure of an esterase from this enzyme family is available (Wagner *et al.*, 2002), which makes it difficult to understand the catalytic mechanism of this enzyme family and its structural and functional relationship to the class C β-lactamases.

Here, we report the identification, crystallization and preliminary crystallographic analysis of esterase A from *Caulobacter crescentus* CB15 (CcEstA), which belongs to the family VIII esterases, in an initial attempt to solve its crystal structure. Structural study of CcEstA will not only provide a molecular basis for the substrate specificity of the family VIII lipolytic enzymes, but will also provide us with a platform to engineer these enzymes for numerous industrial applications.

2. Experimental procedures

2.1. Cloning, expression and purification of CcEstA

The gene coding for CcEstA was amplified by PCR from the chromosomal DNA of *C. crescentus* CB15 (Microbank of Microbial Genomics and Application Center, Daejon, Republic of Korea). The following primers were used: forward, 5′-CA**GGATCC**ATGACTGACATCACCGGCGT-3′ (*Bam*HI site in bold), and reverse, 5′-CC**AAGCTT**CTAGAGGCTGGCGTACGCCG-3′ (*Hin*dIII site in bold). This amplification yielded an ∼1.2 kb product which included the full *estA* gene. The PCR product was inserted into the pQE30 vector (Qiagen, Hilden, Germany) and the recombinant plasmid (pQE30-*estA*) was used to express CcEstA in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, California, USA). Use of the pQE30 vector produced a fusion protein with 12 additional residues (MRGSHHHHHHGS) added to the N-terminal region. After DNA sequencing, transformed *E. coli* cells were grown in LB medium

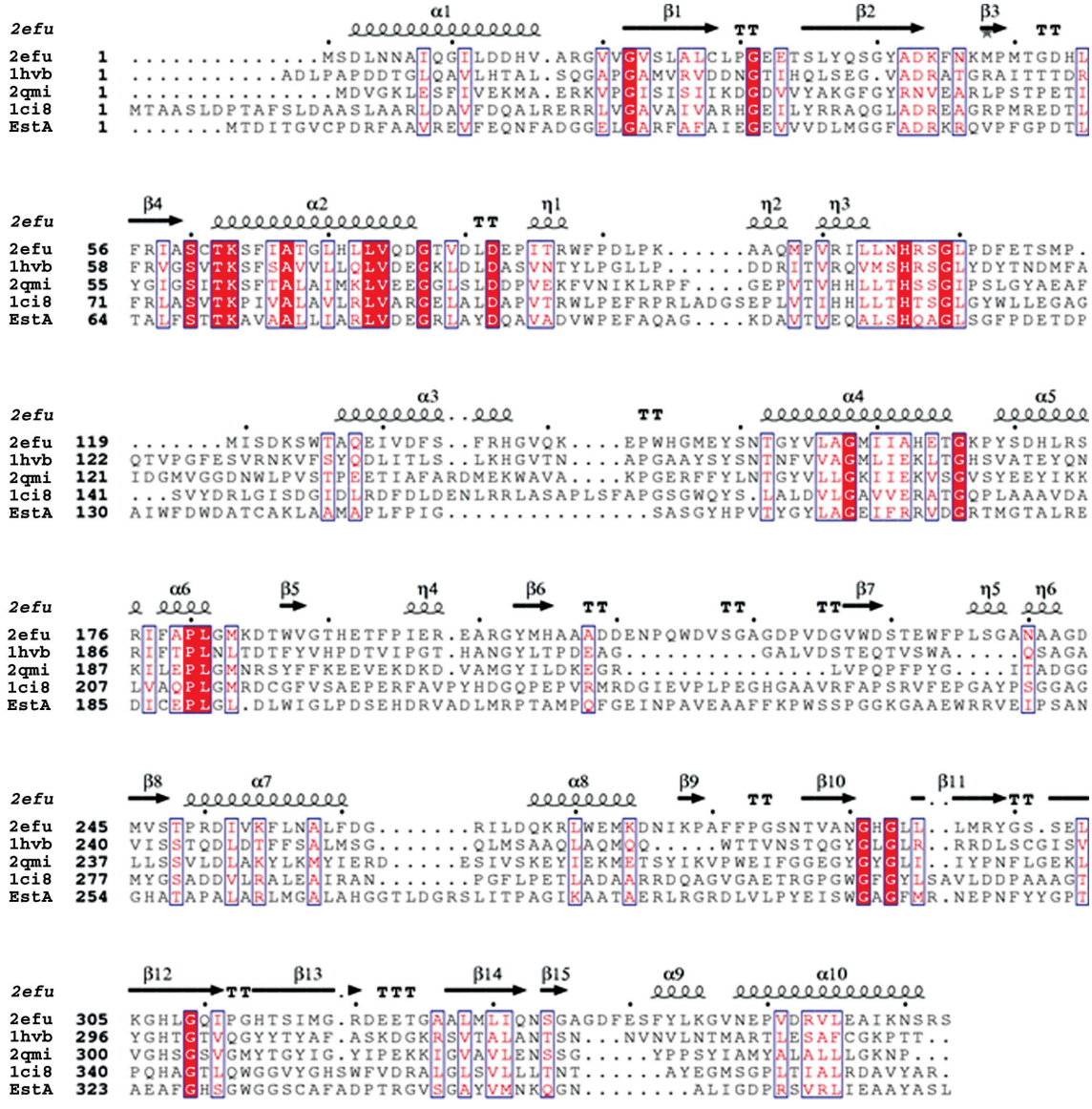

Figure 1
Multiple sequence alignment of CcEstA with four related hydrolases that were selected by a *BLAST* search using the PDB [2efu, D-amino-acid amidase from *Ochrobactrum anthropi* SV3 (Okazaki *et al.*, 2008); 1hvb, D-alanyl-D-alanine carboxypeptidase from *Streptomyces* R61 (Lee *et al.*, 2001); 2qmi, penicillin-binding protein (PBP) from *Pyrococcus abyssi* (Delfosse *et al.*, 2009); 1ci8, EstB from *B. gladioli* (Wagner *et al.*, 2002)]. Sequences retrieved from the NCBI server were aligned with *ClustalW* (Thompson *et al.*, 1994) and rendered using *ESPript* output. Identical and highly conserved residues are shown in red boxes.

containing 100 µg ml^{-1} ampicillin at 310 K; 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added when the OD_{600} reached 0.5. The culture was allowed to grow at 310 K for 4 h before the cells were collected by centrifugation (6000*g*, 10 min, 277 K). The cell pellet was then resuspended in lysis buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 20 m*M* imidazole), which was followed by sonication. The cell lysate was centrifuged at 15 000 rev min^{-1} for 20 min and the supernatant was loaded onto a HisTrap nickel-chelating column (GE Healthcare, Little Chalfont, England). The protein was washed extensively with lysis buffer containing 40 m*M* imidazole. The protein was then eluted with lysis buffer containing 250 m*M* imidazole and desalted on a PD-10 column (GE Healthcare, Little Chalfont, England) with phosphate-buffered saline (PBS) pH 7.4 (Invitrogen Corporation, Carlsbad, California, USA). The entire purification was conducted at 277 K. The purified CcEstA was concentrated to 8 mg ml^{-1} in PBS using Vivaspin concentrators (Vivascience, Massachusetts, USA) without cleavage of the N-terminal tag for crystallization purposes. The purity of the CcEstA was confirmed by SDS–PAGE. Native PAGE and SDS–PAGE were performed on a 10% polyacrylamide gel with a typical Tris-glycine buffer system. Protein concentration was determined with a Bio-Rad protein-assay kit (Bio-Rad Laboratories, Hercules, California, USA) using bovine serum albumin (BSA) as a standard (Bradford, 1976). The yield of purified protein was typically about 3.3 mg per litre of culture. The final proteins were stored without further modification at 253 K.

2.2. Functional assays

The *E. coli* cells were diluted with Luria–Bertani (LB) medium and plated onto LB plates containing ampicillin (100 µg ml^{-1}) such that around 50–100 colonies were visible after overnight growth at 310 K. To detect esterase activity in the plates, the LB plates were overlaid with 0.8% top agar containing Fast Blue RR (20 µg ml^{-1}) and *α*-naphthyl acetate (80 µg ml^{-1}). The *α*-naphthyl acetate was hydrolyzed to generate *α*-naphthol, which reacted with the Fast Blue RR (a diazonium salt) to form a brown diazo dye complex (Miller & Karn, 1980). The appearance of a dark brown colour around colonies within 5 min of incubation at 310 K was considered to be a positive indication of esterase activity (Ahn *et al.*, 2006; Kim *et al.*, 2012). For the ketoprofen hydrolysis assay, 1%(*v*/*v*) ketoprofen ethyl ester was included in the LB plate as the indicator substrate. Clear zones of ketoprofen ethyl ester hydrolysis around colonies were indicative of positive clones harbouring the CcEstA recombinant plasmid (Yoon *et al.*, 2007). For activity staining in native PAGE, purified CcEstA samples were mixed with an 0.2 volume of native sample buffer [62.5 m*M* Tris–HCl pH 6.8, 50% glycerol, 0.1%(*w*/*v*) bromophenol blue] and were analyzed using two native PAGE (10%) gels under nondenaturing conditions. After gel electrophoresis, one separating gel was stained with Coomassie Brilliant Blue (R-250). The other gel was washed with 10 ml 20 m*M* Tris–HCl pH 8.0 and then soaked in the same buffer (50 ml) containing *α*- or *β*-naphthyl acetate (1 mg ml^{-1}). The activity bands were developed using Fast Blue RR solution (2 mg ml^{-1}; Kim *et al.*, 2012; Hwang *et al.*, 2010).

2.3. Crystallization

Crystallization trials of CcEstA were carried out using a purified protein in PBS *via* the microbatch method (Chayen *et al.*, 1990) under Al's Oil using Wizard I and II (Emerald BioSystems, Washington, USA) as crystal screening solutions at 298 K. A drop consisting of 1 µl screening solution and 1 µl protein solution at a concentration of 8 mg ml^{-1} was placed into each well of a Nunc 96-well Mini Tray (Nalge Nunc International, New York, USA).

2.4. X-ray data collection and data processing

The crystals were transferred to a cryosolution consisting of the screening solution supplemented with 25% glycerol and were flash-cooled in a cold nitrogen-gas stream at 100 K prior to data collection. Diffraction data were collected using an ADSC Quantum 315 CCD detector on beamline PAL 4A at Pohang Accelerator Laboratory, Republic of Korea) at 100 K. The wavelength of the synchrotron X-rays was 1.000 Å and the crystal-to-detector distance was 220 mm. The exposure time was 3 s per frame. The crystal was rotated through a total of 180° with 0.5° oscillation range per frame. The data were processed using the *HKL*-2000 package (Otwinowski & Minor, 1997).

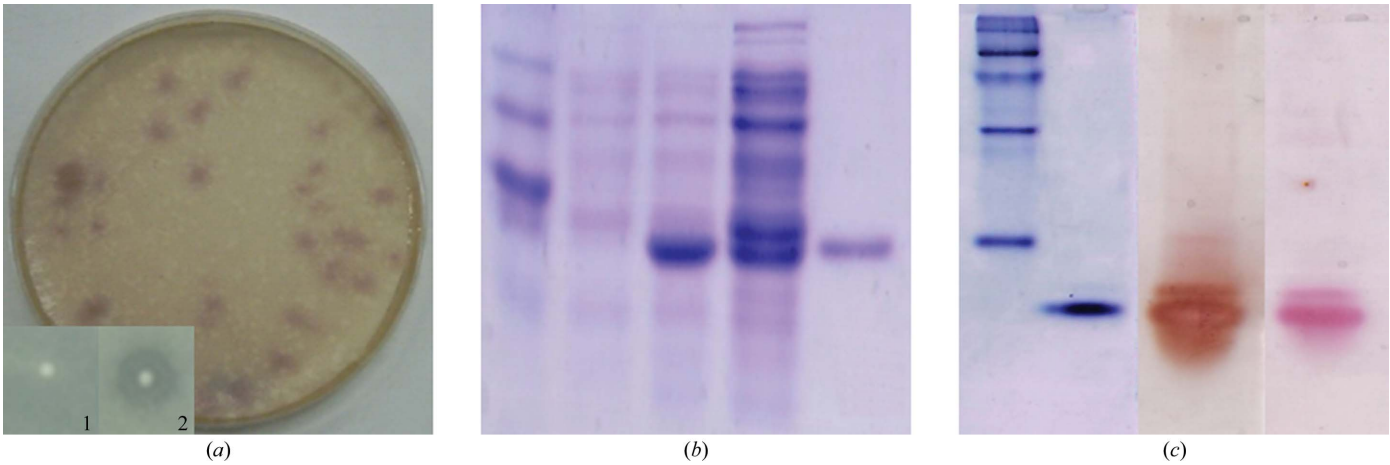

Figure 2
Functional assay, SDS–PAGE and activity staining of CcEstA. (*a*) Hydrolysis activity of *E. coli* harbouring *estA* plasmid against *α*-naphthyl acetate. Inset, the hydrolysis of ketoprofen ethyl ester was shown by a clear circle around a positive clone (2), while a control (1) showed no sign of change. (*b*) SDS–PAGE analysis of protein samples during purification. From left to right: molecular-weight markers (20, 30, 40, 50, 75 and 100 kDa), *E. coli* crude extracts before induction, crude extracts 3 h after induction, supernatants after sonication and purified CcEstA. (*c*) Native PAGE of CcEstA. From left to right: molecular-weight standards (66, 140, 232 and 440 kDa), Coomassie Brilliant Blue staining, Fast Blue RR/*α*-naphthyl acetate and Fast Blue RR/*β*-naphthyl acetate.

Table 1
X-ray data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 172.23, b = 176.68, c = 47.93, $\alpha = \beta = \gamma$ = 90.00
Wavelength (Å)	1.000
Resolution (Å)	50.00–1.62 (1.68–1.62)
Total reflections	610863 (42055)
Unique reflections	89626 (7378)
Completeness (%)	95.7 (79.7)
R_{merge}† (%)	9.4 (36.5)
Mean $I/\sigma(I)$	24.7 (2.8)
Wilson B factor (Å^2)	22.0
Crystal mosaicity (°)	0.54

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results and discussion

CcEstA, which is made up of 374 amino acids with a calculated pI value of 4.9, has been demonstrated to contain a β-lactamase domain (pfam00144, Cd length 327, $E = 1.04 \times 10^{-63}$) from a domain search against the CDD database (Marchler-Bauer & Bryant, 2004). CcEstA also harbours an S-T-T-K sequence (residues 68–71) corresponding to the S-*X*-*X*-K motif conserved in family VIII lipolytic enzymes (Arpigny & Jaeger, 1999), class C β-lactamases (Knox *et al.*, 1996) and penicillin-binding proteins (PBPs; Joris *et al.*, 1988). Ser68 in the S-*X*-*X*-K motif is thought to function as a nucleophile, while Tyr157 acts as a general base; these work together to form an intermediate during catalysis (Wagner *et al.*, 2002; Negoro *et al.*, 2007).

Despite sequence similarities to class C β-lactamases, family VIII lipolytic enzymes are typically devoid of activity against β-lactam substrates (Elend *et al.*, 2006; Rashamuse *et al.*, 2007; Ogino *et al.*, 2004; Petersen *et al.*, 2001; Nishizawa *et al.*, 1995). Two sequence motifs, Y-A-N and K-T/S-G, that are typically found in class C β-lactamases are lacking in the family VIII esterases, which partially explains their deficiency in β-lactamase activity. Multiple sequence alignment of CcEstA with the four sequentially related hydrolases in the PDB revealed that it only shares the motif essential for functionality and has low sequence identity (Fig. 1). For example, the sequence identity between CcEstA and EstB from *B. gladioli*, which also belongs to the family VIII esterases, is only 17.9%.

An *E. coli* strain harbouring the recombinant plasmid encoding CcEstA showed hydrolytic activity against α-naphthyl acetate (Ahn *et al.*, 2006). The *estA*-positive colonies showed a dark brown colour in the presence of a substrate (Fig. 2*a*). In addition, they were able to hydrolyze a ketoprofen ethyl ester (Fig. 2*a*, inset) which was used for the preparation of a nonsteroidal anti-inflammatory drug (Yoon *et al.*, 2007). Recombinant CcEstA consisting of 386 residues, including an N-terminal His tag and a linker, was expressed in *E. coli* and purified to electrophoretic homogeneity for biochemical characterization and crystallization (Fig. 2*b*). Using SDS–PAGE, the molecular mass of CcEstA was estimated to be 41 kDa, which was consistent with the calculated molecular mass (41.37 kDa). The biochemical activity of CcEstA was also confirmed on a native PAGE gel by a brown colour at the position of the CcEstA band in the presence of α- and β-naphthyl acetate (Fig. 2*c*). Future structural and functional studies will elucidate the substrate specificity and catalytic mechanism of CcEstA.

Rectangular rod-shaped crystals appeared within two weeks using Wizard I condition No. 18 (1.0 *M* potassium/sodium tartrate, 0.1 *M* imidazole pH 8.0, 0.2 *M* NaCl) and grew to final dimensions of ~0.1 × 0.1 × 0.5 mm (Fig. 3). The crystals belonged to space group $C222_1$, with unit-cell parameters a = 172.23, b = 176.68, c = 47.93 Å. The diffraction data set was processed to 1.62 Å resolution with 95.7% completeness and an R_{merge} of 9.4%. The data-collection statistics are given in Table 1. Assuming the presence of two molecules per asymmetric unit, the Matthews coefficient (V_M) is calculated to be 2.16 Å^3 Da^{-1} (Matthews, 1968). This V_M value is within the range commonly observed for protein crystals and corresponds to 43.1% solvent content. Consistently, a strong Patterson peak at (0.5, 0.0, 0.0) with 93% of the height of the origin peak was identified, suggesting that the two molecules in the asymmetric unit are related by pseudo-translation.

We attempted to solve the structure of CcEstA by molecular replacement with programs such as *MOLREP* (Vagin & Teplyakov, 2010), *Phaser* (McCoy *et al.*, 2007) and the *Phenix AutoMR* wizard (Adams *et al.*, 2010) using the crystal structure of the *B. gladioli* esterase EstB, the first structure of a class VIII lipolytic enzyme to be obtained, as a search model. However, MR phasing was unsuccessful, presumably owing to the low sequence identity between the two molecules. We are currently attempting to solve the structure of CcEstA using an experimental phasing method such as multiple-wavelength anomalous dispersion.

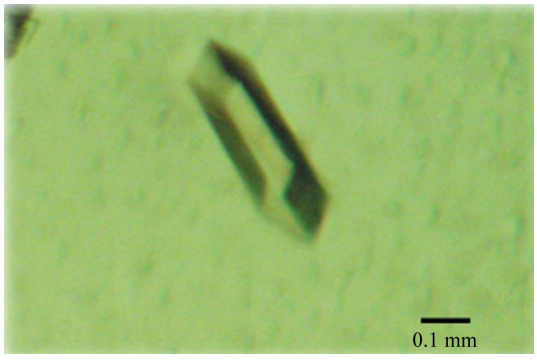

Figure 3
A CcEstA crystal obtained using Wizard I condition No. 18 (1.0 *M* potassium/sodium tartrate, 0.1 *M* imidazole pH 8.0, 0.2 *M* NaCl). The crystal dimensions were 0.1 × 0.1 × 0.5 mm.

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