Characterization, crystallization and preliminary X-ray diffraction analysis of an (*S*)-specific esterase (*pfEstA*) from *Pseudomonas fluorescens* KCTC 1767: enantioselectivity for potential industrial applications

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The structures and reaction mechanisms of enantioselective hydrolases, which can be used in industrial applications such as biotransformations, are largely unknown. Here, the X-ray crystallographic study of a novel (*S*)-specific esterase (*pfEstA*) from *Pseudomonas fluorescens* KCTC 1767, which can be used in the production of (*S*)-ketoprofen, is described. Multiple sequence alignments with other hydrolases revealed that *pfEstA* contains a conserved Ser67 within the S-X-X-K motif as well as a highly conserved Tyr156. Recombinant protein containing an N-terminal His tag was expressed in *Escherichia coli*, purified to homogeneity and characterized using SDS-PAGE, MALDI-TOF MS and enantioselective analysis. *pfEstA* was crystallized using a solution consisting of 1 M sodium citrate, 0.1 M CHES pH 9.5, and X-ray diffraction data were collected to a resolution of 1.9 Å with an R_{merge} of 7.9%. The crystals of *pfEstA* belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 65.31$, $b = 82.13$, $c = 100.41$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Microbial enzymes such as esterases and lipases can catalyze a large number of stereospecific hydrolysis reactions for the production of enantiomeric compounds (Quin & Schmidt-Dannert, 2011; Tang & Zhao, 2009; Ogawa & Shimizu, 1999). Use of these enzymes in industrial processes is highly attractive owing to their high performance, mild reaction conditions and low production costs. Accordingly, new enzymes with high activity and selectivity are continuously being screened from a variety of microorganisms and environmental samples (Singh *et al.*, 2009; Asano, 2002). Recently, biocatalytic methods using esterases/lipases have been employed to prepare (*S*)-ketoprofen ($\text{C}_{16}\text{H}_{14}\text{O}_3$), which could replace chemical methods such as chromatographic separation, diastereomeric crystallization or asymmetric synthesis (Liu *et al.*, 2004; Patel, 2003). Interestingly, the pharmacological effects of (*S*)-ketoprofen include the inhibition of prostaglandin production, while the (*R*)-enantiomer has been implicated in analgesic effects (Kasprzyk-Hordern, 2010; Panico *et al.*, 2005).

To date, numerous enzymes for the enzymatic hydrolysis of ketoprofen have been reported: lipase OF from *Candida rugosa* (Zhu *et al.*, 2005), a lipase from *Mucor javanicus* (Kato *et al.*, 2000), an esterase (PF1-K) from *Pseudomonas* sp. (Sathishkumar *et al.*, 2010), Est3 from *Sulfolobus solfataricus* P2 (Kim & Lee, 2004), a lipase from *Acinetobacter* sp. ES-1 (Lee *et al.*, 2004), a lipase from *Carica papaya* (Ng & Tsai, 2005), lipase A from *Bacillus subtilis* (Ni *et al.*, 2011), EstA and EstB from *Picrophilus torridus* (Hess *et al.*, 2008), Est-AF from *Archaeoglobus fulgidus* (Kim *et al.*, 2008), lipase B (CALB) from *Candida antarctica* (Wang *et al.*, 2010) and CcEstA from *Caulobacter crescentus* CB15 (Ryu *et al.*, 2012). In addition, a number of enzymes have also been identified from metagenomic libraries for the preparation of (*S*)-ketoprofen (Kang *et al.*, 2011; Yoon *et al.*, 2007). However, the molecular basis of the reaction mechanism and the enantioselectivity of these enzymes remains largely unexplored,

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making it difficult to improve the enzymes to give improved performances.

Here, we report the characterization, crystallization and preliminary crystallographic analysis of an (*S*)-specific esterase (*pfEstA*) from *Pseudomonas fluorescens* KCTC 1767. In a previous report, this enzyme was identified by consecutive steps of activity screening, the identification of positive strains and gene mining (Choi *et al.*, 2003). Furthermore, chiral resolution of the rac-ketoprofen ethyl ester for the production of (*S*)-ketoprofen has been investigated using this enzyme (Kim *et al.*, 2003). Therefore, structural studies of *pfEstA* will not only provide a molecular basis for the stereospecificity of this enzyme, but will also provide us with an enzymatic platform towards high specific activity for industrial applications.

2. Experimental procedures

2.1. Expression and purification of *pfEstA*

The cloning of *pfEstA* from chromosomal DNA of *P. fluorescens* KCTC 1767 has been described previously (Choi *et al.*, 2003). After DNA sequencing, the recombinant plasmid (pQE30-EstA) was used to express *pfEstA* in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, California, USA). Use of the pQE30 vector produced a fusion protein with 12 additional residues (MRGSHHHHHHGS) at the N-terminus. Transformed *E. coli* cells were grown in LB medium containing

100 µg ml⁻¹ ampicillin at 310 K; 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added when the OD₆₀₀ reached 0.5. After induction for 4 h, the cells were harvested at 6000g for 10 min and resuspended in lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl) containing 20 mM imidazole. Following 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, Princeton, New Jersey, USA). After extensive washing with lysis buffer containing 40 mM imidazole, *pfEstA* was eluted with lysis buffer containing 250 mM imidazole and desalted on a PD-10 column (GE Healthcare) with phosphate-buffered saline (PBS) pH 7.4 (Invitrogen Corporation, Carlsbad, California, USA). The entire purification was conducted at 277 K. The purity of the *pfEstA* was confirmed by SDS-PAGE and the 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 final proteins were stored without further modification at 253 K.

2.2. Biochemical characterization

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed in positive-ion mode on a Voyager DE STR (Applied Biosystems, Carlsbad, California, USA) at Ajou University Center for Core Facilities. For

Figure 1

Multiple sequence alignment of *pfEstA* with four related proteins from the PDB: PDB entries 2dns (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), 1ci9 (EstB from *Burkholderia gladioli*; Wagner *et al.*, 2002) and 2bls (AmpC β-lactamase from *E. coli*; Usher *et al.*, 1998). Sequences retrieved from the NCBI server were aligned with *ClustalW* (Thompson *et al.*, 1994) and were rendered using *ESPrpt* output. Identical and highly conserved residues are shown in red boxes. The catalytic Ser in the S-X-X-K motif and the highly conserved Lys and Tyr residues are indicated by asterisks (*).

enantioselectivity analysis, *pfEstA* was reacted with (*R*) or (*S*) solutions. The (*R*) and (*S*) solutions consisted of phenol red (2 g l⁻¹), 20 mM Tris-HCl pH 8.0 and 300 mM (*R*)- or (*S*)-methyl-3-hydroxy-2-methylpropionate, respectively (Hwang *et al.*, 2010; Ivancic *et al.*, 2007).

2.3. Crystallization

The purified *pfEstA* was concentrated to 9 mg ml⁻¹ in PBS buffer using Vivaspins concentrators (Vivascience, Massachusetts, USA). Primary crystallization screening of *pfEstA* was performed by the microbatch method (Chayen *et al.*, 1990) under Al's Oil using Wizard I and II (Emerald BioSystems, Washington, USA) crystallization screening solutions at 298 K. A drop consisting of 1 µl screening solution and 1 µl protein solution was placed into each well of a Nunc 96-well Mini Tray (Nalge Nunc International, New York, USA). Crystals were initially obtained from Wizard I condition No. 34 (1.0 M ammonium phosphate dibasic, 0.1 M imidazole pH 8.0), but were very small and were not suitable for data collection. Further optimization with different concentrations of precipitants or *pfEstA* did not lead to improvement of the diffraction quality of the crystals. However, a single crystal grown from Wizard II condition No. 16 (1 M sodium citrate, 0.1 M CHES pH 9.5) was suitable for X-ray analysis without further optimization.

2.4. X-ray data collection and data processing

Crystals in a cryosolution consisting of the screening solution supplemented with 20% glycerol were flash-cooled in a nitrogen-gas stream at 100 K before data collection. Diffraction data were collected at 100 K on a Rigaku R-Axis IV⁺⁺ image-plate detector (Rigaku, Japan) using Cu K α radiation from a Rigaku rotating-anode X-ray generator operated at 50 kV and 100 mA. The crystal-to-detector distance was 160 mm and the exposure time was 8 min per frame. The crystal was rotated through a total of 360° with a 1° oscillation range per frame. The data were processed using *CrystalClear* (Pflugrath, 1999).

Table 1

X-ray data-collection and processing statistics for *pfEstA*.

Values in parentheses are for the highest resolution shell.

Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 65.31, <i>b</i> = 82.13, <i>c</i> = 100.41, $\alpha = \beta = \gamma = 90$
Wavelength (Å)	1.5418
Resolution (Å)	45.55–1.90 (1.97–1.90)
Unique reflections	42261 (4254)
Completeness (%)	97.7 (83.8)
Multiplicity	12.9 (6.5)
<i>R</i> _{merge} [†] (%)	7.9 (36.4)
$\langle I \rangle / \langle \sigma(I) \rangle$	17.3 (5.1)
Wilson <i>B</i> factor (Å ²)	30.0
Mosaicity (°)	0.85

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

3. Results and discussion

A multiple sequence alignment of *pfEstA* with four related proteins from the PDB is shown in Fig. 1. There is low sequence identity (21%) among these proteins, although the S-X-X-K consensus sequence containing the putative catalytic serine (Ser67) is highly conserved. The Ser67 in this motif is believed to act as a nucleophile, while Lys70 and Tyr156 together act as the general base in the acylation and deacylation step (Chen *et al.*, 2006, 2011). Recombinant *pfEstA* consisting of 386 residues including an N-terminal His tag and a linker was expressed in *E. coli* and purified to homogeneity for crystallization, as judged from SDS-PAGE (Fig. 2*a*). In addition, as shown in Fig. 2(*b*), the molecular mass (42.50 kDa) and purity of the *pfEstA* were further confirmed using MALDI-TOF MS.

The enantioselectivity of *pfEstA* was confirmed by a pH-shift assay using (*R*)- and (*S*)-methyl-3-hydroxy-2-methylpropionate. After incubation with *pfEstA*, the colour of the reaction mixture selectively turned yellow in the (*S*)-enantiomer-containing solution (Fig. 2*c*). In accordance, it has been shown that *pfEstA* can be effectively used for enantioselective hydrolysis of the rac-ketoprofen ethyl ester (Choi *et al.*, 2003). Future structural and functional studies will elucidate the substrate specificity and the catalytic mechanism of *pfEstA*.

A well diffracting rectangular-shaped crystal appeared from Wizard II condition No. 16 (1 M sodium citrate, 0.1 M CHES pH 9.5)

Figure 2 SDS-PAGE, MALDI-TOF MS and enantioselectivity assay of *pfEstA*. (*a*) SDS-PAGE of *pfEstA* after purification. Molecular-mass markers (left lane, labelled in kDa) were used for comparison. (*b*) MALDI-TOF mass spectrum of *pfEstA*. The $[M + H]^+$ and $[M + 2H]^{2+}$ ion peaks were found at *m/z* values of 42 500 and 21 250, respectively. (*c*) A pH-shift assay conducted with (*R*)- and (*S*)-methyl-3-hydroxy-2-methylpropionate. Con, (*S*) substrate only; 2, *pfEstA* with (*R*) substrate; 3, *pfEstA* with (*S*) substrate.

Figure 3

A *pfEstA* crystal obtained using Wizard II condition No. 16 (1.0 M sodium citrate, 0.1 M CHES pH 9.5). The crystal dimensions were $0.3 \times 0.2 \times 0.8$ mm. The scale bar represents 0.2 mm.

after two weeks. The dimensions of the crystal were approximately $0.3 \times 0.2 \times 0.8$ mm (Fig. 3). The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 65.31$, $b = 82.13$, $c = 100.41$ Å, $\alpha = \beta = \gamma = 90^\circ$. The diffraction data set was processed to 1.9 Å resolution with 97.7% completeness and an R_{merge} of 7.9%. Data-collection statistics are given in Table 1. Assuming the presence of one molecule per asymmetric unit, the Matthews coefficient (V_M) was calculated to be $3.01 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968). This V_M value is within the range commonly observed for protein crystals and corresponds to 59.1% solvent content. Systematic absences confirmed the presence of 2_1 axes along a , b and c . This suggests that the space group should be assigned as $P2_12_12_1$.

We attempted to solve the structure of *pfEstA* by molecular replacement with various programs such as *MOLREP* (Vagin & Teplyakov, 2010), *Phaser* (McCoy *et al.*, 2007) and *phenix.automr* (Adams *et al.*, 2010) using the crystal structure of D-amino-acid amidase from *Ochrobactrum anthropi* SV3 (PDB entry 2dns; Okazaki *et al.*, 2008) as a search model. However, molecular-replacement phasing was unsuccessful, probably owing to the low sequence identity (21%) between the two molecules. Therefore, the use of an experimental phasing method such as multiwavelength anomalous diffraction (MAD) will be attempted in order to solve the structure of *pfEstA*.

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