SeungBum Kim,^{a,‡} Sangbum
Joo,^{b,‡} Sangyoung Yoon,^{a,‡}
Sungsoo Kim,^b Jongkook Moon,^a
Yeonwoo Ryu,^a Kyeong Kyu
Kim^{b*} and T. Doohun Kim^{a*}

^aDepartment of Molecular Science and
Technology, Graduate School of
Interdisciplinary Program, Ajou University,
Suwon, Republic of Korea, and ^bDepartment of
Molecular Cell Biology, Sungkyunkwan
University School of Medicine, Suwon,
Republic of Korea

‡ These authors contributed equally to this
work.

Correspondence e-mail: kkim@med.skku.ac.kr,
doohunkim@ajou.ac.kr

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Purification, crystallization and preliminary crystallographic analysis of Est-Y29: a novel oligomeric β -lactamase

β -Lactam antibiotics such as penicillins and cephalosporins have a four-atom ring as a common element in their structure. The β -lactamases, which catalyze the inactivation of these antibiotics, are of great interest because of their high incidence in pathogenic bacteria. A novel oligomeric class C β -lactamase (Est-Y29) from a metagenomic library was expressed, purified and crystallized. The recombinant protein was expressed in *Escherichia coli* with an N-terminal 6 $\times$ His tag and purified to homogeneity. Est-Y29 was crystallized and X-ray intensity data were collected to 1.49 Å resolution using synchrotron radiation.

1. Introduction

The expression of β -lactamases, which catalyze the inactivation of antibiotics such as penicillins and cephalosporins by hydrolysis of the critical β -lactam ring, is a major defence mechanism of pathogenic bacteria (Fisher *et al.*, 2005; Wilke *et al.*, 2005). To date, four classes (A, B, C and D) of β -lactamases, each of which is characterized by a specific reaction mechanism, have been identified (Philippon *et al.*, 1998). In particular, class C β -lactamases are of great interest for therapeutic studies because of the high incidence of chromosomally or plasmid-encoded class C β -lactamases in pathogenic bacteria such as *Enterobacter cloacae* and *Citrobacter freundii* (Nicasio *et al.*, 2008; Philippon *et al.*, 2002). Therefore, there is a great need to develop new antibiotics targeting these enzymes in order to prevent dangerous bacterial infections (Pelto & Pratt, 2008). However, structural information on class C β -lactamases, which is necessary for structure-based antibiotic design, is still very limited (Wilke *et al.*, 2005; Wagner *et al.*, 2002).

In a previous report, we identified a novel oligomeric class C β -lactamase, Est-Y29, from a metagenomic library (Yoon *et al.*, 2007). Interestingly, Est-Y29 can catalyze the kinetic resolution of ketoprofen ethyl ester used in the production of the anti-inflammatory drug (*S*)-ketoprofen (Yoon *et al.*, 2007; Kim *et al.*, 2007). Est-Y29 possesses the Ser-X-Lys motif which is highly conserved among β -lactamases and site-directed mutagenesis of Ser to Ala resulted in complete loss of activity (unpublished results). In addition, Est-Y29 showed a high stability towards pH change, heat and addition of organic solvents, which is promising for the use of this enzyme in the production of biopharmaceuticals. To our knowledge, Est-Y29 is the first multimeric class C β -lactamase with broad hydrolytic activity. The amino-acid sequence of Est-Y29 is only marginally homologous to those of EstB from *Burkholderia gladioli* (26%; PDB code 1ci9; Wagner *et al.*, 2002) and penicillin-binding protein (PBP) from *Pyrococcus abyssi* (25%; PDB code 2qmi; V. Delfosse, E. Girard, L. Moulinier, S. Magnet, P. L. Anelli, C. Birck, P. Schultz & C. Mayer, unpublished work). In this report, we describe the protein preparation, crystallization screening and preliminary crystallographic analysis of Est-Y29. The crystal structure of Est-Y29 will provide an invaluable framework for understanding its unique features such as its oligomeric state, high stability and (*S*)-specific stereoselectivity, as well as its significance for the development of antibiotic drugs against class C β -lactamases.

Figure 1

(a) Est-Y29 crystals obtained using the initial crystallization screening solution. (b) Diffraction-quality crystal of Est-Y29 grown in 0.1 M sodium acetate trihydrate buffer pH 4.6 and 2.0 M sodium formate precipitant. (c) SDS-PAGE analysis of the crystallized Est-Y29 (42 kDa).

2. Experimental procedures

2.1. Protein expression and purification

A novel class C β -lactamase from a metagenomic library, Est-Y29, was overexpressed in *Escherichia coli* and crystallized for structural analysis. In a previous report, a biophysical analysis determined the oligomeric state of Est-Y29 under physiological conditions (Yoon *et al.*, 2007). Molecular cloning of Est-Y29 has been described previously (Streit & Schmitz, 2004; Yoon *et al.*, 2007). For purification of Est-Y29 protein, cDNA was inserted into the T5 promoter-driven expression vector pQE30 (Qiagen, USA). The forward and reverse primers were 5'-GCGGATCCATGCCAGATTGCTGACCAAT-3' (*Bam*HI site in bold) and 5'-CCAAGCTTAGCCAAGCATCGA-CATCGCAG-3' (*Hind*III site in bold), respectively. Overexpression of Est-Y29 was performed in *E. coli* XL1-Blue strain according to the manufacturer's instructions (Qiagen, USA). Briefly, cells were inoculated in 100 ml LB medium containing 100 µg ml⁻¹ ampicillin. When the cell density reached an A_{600} of 0.4–0.5, 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside) was added to induce recombinant protein expression at 310 K. After 3 h induction, cells were harvested by centrifugation, resuspended in lysis buffer (50 mM phosphate pH 8.0, 10 mM imidazole) and disrupted by sonication. After centrifugation at 12 000 rev min⁻¹, the supernatant was applied onto a Ni-NTA column equilibrated with 10 mM imidazole and the His-tagged protein was purified according to the manufacturer's instructions (Qiagen, USA). The purity of the purified Est-Y29 was verified by SDS-PAGE and mass-spectrometric analysis and the protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, USA) with bovine serum albumin (BSA) as a standard protein (Bradford, 1976). The purified protein was concentrated to 10 mg ml⁻¹ in phosphate-buffered saline pH 7.4 using an Amicon Ultracore-15 centrifugal filter (30 kDa MWCO, Millipore, USA). The recombinant Est-Y29 used in crystallization trials had an additional 11 amino acids (MRGSHHHHHHGS) at the N-terminus of the intact enzyme.

2.2. Crystallization

Crystallization was performed using the microbatch crystallization method (Chayen *et al.*, 1990) under a thin layer of Al's oil, using commercially available screening kits from Hampton Research (Hampton Research, California, USA) at 295 K. A drop consisting of 1 µl screening solution and 1 µl protein solution (10 mg ml⁻¹) was

placed in each well of a Nunc 96-well Mini Tray (Nalgen Nunc International, USA) and the whole plate was then overlaid with 4 ml Al's oil. Initial crystals were observed under various screening conditions, but only the hexagonal rod-shaped crystals grown in condition No. 34 of Crystal Screen I (0.1 M sodium acetate trihydrate buffer pH 4.6 and 2.0 M sodium formate; Hampton Research, USA) showed diffraction (Fig. 1a). However, its diffraction pattern did not appear to be homogeneous, probably owing to crystal cracks. We optimized the crystallization condition by changing the pH of the protein solution and the temperature. When the temperature was lowered from 295 to 287 K and the protein solution buffer was replaced by 0.05 M sodium phosphate pH 7.0, the shape of the crystal altered to a tetragonal prism and the cracks disappeared (Fig. 1b). Finally, diffraction-quality crystals were obtained using the same hardware and volumes as used for the initial crystals with 0.1 M

Figure 2

Typical X-ray diffraction pattern of the Est-Y29 crystal. The diffraction spots at 1.49 Å resolution are magnified at the top right.

Table 1

X-ray data-collection and processing statistics for Est-Y29.

Values in parentheses are for the highest resolution shell.

Space group	<i>I</i> 4
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 121.95, <i>c</i> = 154.46
Wavelength (Å)	0.983
Resolution (Å)	50.00–1.49 (1.54–1.49)
Unique reflections	179641
Total observations	894503
Completeness (%)	98.1 (99.5)
Redundancy	5.0 (4.8)
<i>R</i> _{merge} † (%)	4.7 (41.4)
Mean <i>I</i> /σ(<i>I</i>)	18.5 (5.4)

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

sodium acetate trihydrate buffer pH 4.6 and 2.0 *M* sodium formate at 287 K. The crystals grew to final dimensions of 0.1 × 0.3 × 0.3 mm within a day (Fig. 1*b*). The Est-Y29 crystals were dissolved and analyzed using 15% SDS–PAGE to confirm the molecular size and purity of the protein (Fig. 1*c*).

2.3. X-ray data collection and data processing

Crystals were serially transferred to cryosolvents which contained increasing amounts of the precipitant sodium formate. Finally, crystals were equilibrated in a cryosolvent containing 6 *M* sodium formate before being flash-frozen in a cold nitrogen stream. X-ray diffraction data were collected to 1.49 Å resolution using a wavelength of 0.983 Å at 100 K with an ADSC Quantum 210 detector on beamline NW12 at the Photon Factory (PF), Japan (Fig. 2) and were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The data-collection and processing statistics for Est-Y29 are summarized in Table 1. Given the absence of reflections violating $h + k + l = 2n$, the Est-Y29 crystals belonged to the tetragonal space group *I*4, with unit-cell parameters $a = b = 121.95$, $c = 155.46$ Å. Est-Y29 is known to form tetramers in solution. Therefore, it is expected that each monomer in a tetrameric complex is packed by crystallographic fourfold symmetry.

3. Results and discussion

Assuming a molecular weight of 42 700 Da for the Est-Y29 monomer with the N-terminal His tag and the presence of two molecules in a crystallographic asymmetric unit, the crystal volume per protein mass (*V*_M) and the solvent content of the crystal were estimated to be 3.34 Å³ Da^{−1} and 63.2%, respectively (Matthews, 1968). Of the similar structures in the PDB, DFP-inhibited esterase EstB (PDB

code 1ci9) showed the highest sequence identity of 26% (Wagner *et al.*, 2002). Although we attempted to solve the structure of Est-Y29 by molecular replacement using EstB as a template model with the programs *MOLREP* (Vagin & Teplyakov, 1997), *AMoRe* (Navaza, 1994) and *Phaser* (McCoy, 2007), we failed to obtain the phase information. Therefore, multiple-wavelength anomalous dispersion methods as well as further molecular-replacement experiments using other template structures will be performed. The crystal structure of Est-Y29 will provide a structural explanation of the (*S*)-stereospecific substrate selectivity of Est-Y29.

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