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Crystallization and preliminary diffraction studies of SFC-1, a carbapenemase conferring antibiotic resistance

SFC-1, a class A carbapenemase that confers antibiotic resistance, hydrolyzes the β -lactam rings of β -lactam antibiotics (carbapenems, cephalosporins, penicillins and aztreonam). SFC-1 presents an enormous challenge to infection control, particularly in the eradication of Gram-negative pathogens. As SFC-1 exhibits a remarkably broad substrate range, including β -lactams of all classes, the enzyme is a potential target for the development of antimicrobial agents against pathogens producing carbapenemases. In this study, SFC-1 was cloned, overexpressed, purified and crystallized. The SFC-1 crystal diffracted to 1.6 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 65.8$, $b = 68.3$, $c = 88.8$ Å. Two molecules are present in the asymmetric unit, with a corresponding V_M of 1.99 Å³ Da⁻¹ and a solvent content of 38.1%.

1. Introduction

Severe clinical problems have arisen from the appearance of antibiotic resistance in pathogenic bacteria that cause nosocomial and/or community-acquired infections (Chopra *et al.*, 2008; Lee *et al.*, 2009, 2012). β -Lactamases hydrolyze the β -lactam rings of β -lactam antibiotics and thus provide pathogenic bacteria with resistance to these antibiotics (Schneider *et al.*, 2011). Based on their primary structures, β -lactamases have been grouped into four classes (A–D), with classes A, C and D using a serine-based covalent catalysis mechanism (Bush & Jacoby, 2010; Frère, 1995). Class A β -lactamases possess a very high degree of sequence diversity (more than 600 variants with 11–66.2% sequence identity to mature SFC-1) and are particularly troublesome in the eradication of Gram-negative pathogens (Bush & Jacoby, 2010). To overcome inactivation by these β -lactamases, carbapenems such as imipenem and meropenem have been developed (Higgins *et al.*, 2009). However, class A carbapenemases that can hydrolyze a broad variety of β -lactams (carbapenems, cephalosporins, penicillins and aztreonam) have recently emerged (Papp-Wallace *et al.*, 2011; Queenan & Bush, 2007).

To date, five major families of class A carbapenemases have been identified in Gram-negative bacteria: the IMI/NMC-A (imipenemase/non-metallo-carbapenemase-A), SME (*Serratia marcescens* enzyme), KPC (*Klebsiella pneumonia* carbapenemase), GES (Guiana extended-spectrum) and SFC (*S. fonticola* carbapenemase) families (Fonseca *et al.*, 2007; Henriques *et al.*, 2004; Queenan & Bush, 2007). Increasing numbers of these β -lactamases are being found in the clinic and the environment, the genes of which are primarily located on plasmids or chromosomes (Henriques *et al.*, 2004; Queenan & Bush, 2007). This potential for wide dispersal together with their broad substrate specificity highlights the importance of investigating the structural details of these enzymes. Only four crystal structures of class A carbapenemases have been reported: those of KPC-2 (Ke *et al.*, 2007), SME-1 (Sougakoff *et al.*, 2002), GES-2 (Smith *et al.*, 2007) and NMC-A (Swarén *et al.*, 1998). SFC-1 has 66.2, 63.4, 62.2 and 33.1% sequence identity to mature KPC-2, NMC-A, SME-1 and GES-1, respectively. However, the mechanism responsible for carbapenemase activity in class A enzymes remains relatively obscure and the structure of SFC-1 (the only member of the SFC family) has not thus far been reported. Furthermore, SFC-1 exhibited catalytic efficiencies for imipenem and ceftazidime that were higher than those

of the structurally known carbapenemases (Fonseca *et al.*, 2007). Thus, there is a need to determine the crystal structure of SFC-1 in order to understand the biochemical characteristics of class A carbapenemases and to elucidate the structural basis of the expanded catalytic activity of SFC-1 towards carbapenems. Here, we expressed, purified, crystallized and performed a preliminary X-ray crystallographic analysis of SFC-1.

2. Materials and methods

2.1. Cloning

The class A carbapenemase SFC-1 was produced by *S. fonticola* UTAD54 (Henriques *et al.*, 2004). To express the *SFC-1* gene (*bla*_{SFC-1}) as a soluble form in *Escherichia coli*, the *bla*_{SFC-1} gene with codon optimization was chemically synthesized using GenScript technology (GenScript Corp., Piscataway, New Jersey, USA) and amplified by polymerase chain reaction (PCR). The sequences of the forward and reverse oligonucleotide primers designed from the *bla*_{SFC-1} gene for PCR were as follows: 5'-ATA **CAT ATG CAC CAT** CAT CAT CAT CAT GAC GAC GAC GAC AAG AAT ATG GCC

GAA GCT GCC T-3' (*Nde*I restriction site in bold) and 5'-GAG **CTC GAG** TCA GAA ACC GAT TGA TTT ACC C-3' (*Xho*I restriction site in bold), respectively. The underlined bases indicate the hexahistidine-tag site and the italic bases indicate the enterokinase recognition site. The amplified gene was double-digested with *Nde*I and *Xho*I and inserted into the expression vector pET-30a (Novagen, Madison, Wisconsin, USA) digested with the same DNA-restriction enzymes to produce the pET-30a/His₆-*bla*_{SFC-1} plasmid. After verifying the DNA sequence, the plasmid DNA was transformed into *E. coli* strain BL21 (DE3) for overexpression of His₆-SFC-1 (Fig. 1).

2.2. Overexpression and purification

The transformed cells were grown in Luria-Bertani medium (Difco, Detroit, Michigan, USA) containing 50 µg ml⁻¹ kanamycin to an OD₆₀₀ of 0.5 at 310 K. The expression of His₆-SFC-1 was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at 289 K. The cells were harvested by centrifugation at 3000g (Supra 30K A1000S-4 rotor; Hanil, Seoul, Republic of Korea) for 30 min at 277 K. The resulting cell pellet was resuspended in ice-cold lysis buffer (10 mM Tris-HCl pH 7.0) and homogenized with a sonicator

Figure 1

Schematic diagram of the recombinant protein His₆-SFC-1 including the first residue (methionine, M), the His₆ tag, the enterokinase recognition site (DDDDK) and mature SFC-1 (residues 22–309) without the signal peptide (residues 1–21; MSRTGRLSVFFSAIFPLLTLT). The fused peptide (M, His₆ tag and enterokinase recognition site) was removed from the recombinant protein during the purification process and the purified mature SFC-1 (residues 22–309) was crystallized. 1, 26, 27 and 309 are residue numbers of immature SFC-1 (residues 1–309).

Figure 2

Purified SFC-1 and its crystals. (a) Purified SFC-1 shown on 12% SDS-PAGE. The left lane contains molecular-mass markers (labelled in kDa). (b) Crystals of SFC-1 with approximate dimensions of 0.2 × 0.1 × 0.05 mm.

(Sibata, Saitama, Japan). The crude lysate was centrifuged at 19 960g (Hanil) for 30 min at 277 K and the clarified supernatant was loaded onto a His-Bind column (Novagen, Wisconsin, USA) equilibrated with binding buffer (20 mM Tris-HCl, 10 mM imidazole, 500 mM NaCl pH 7.9). His₆-SFC-1 was eluted with the same buffer containing 1 M imidazole. Eluted fractions containing His₆-SFC-1 were pooled and concentrated to a volume of approximately 1 ml using Vivaspin

20 concentrators (Sartorius, Göttingen, Germany). For further purification, the His₆ tag was removed from His₆-SFC-1 using enterokinase according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture was desalted and concentrated using a Fast Desalting column (GE Healthcare Biosciences AB, Uppsala, Sweden) and then loaded onto a Superdex 200 prep-grade column (GE Healthcare) previously

Figure 3 Self-rotation function. (a) $\chi = 180^\circ$, (b) $\chi = 120^\circ$, (c) $\chi = 90^\circ$ and (d) $\chi = 60^\circ$ sections of the self-rotation function calculated with MOLREP (Vagin & Teplyakov, 2010) using data from 50 to 3 Å resolution.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	5C SB II, PAL
Wavelength (Å)	0.97951
Resolution range (Å)	50.00–1.60 (1.63–1.60)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 65.8, b = 68.3, c = 88.8$
Total No. of reflections	863384
No. of unique reflections	65381
Completeness (%)	96.8 (73.4)
Molecules per asymmetric unit	2
Solvent content (%)	38.1
Average $I/\sigma(I)$	52.5 (4.2)
$R_{\text{merge}}^{\dagger}$ (%)	7.1 (27.1)
Multiplicity	13.2 (7.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

equilibrated with 10 mM MES pH 6.8, 5 mM dithiothreitol for further purification by gel filtration. The homogeneity of the purified protein was analyzed *via* SDS–PAGE (Fig. 2a). The purified SFC-1 without the His₆ tag was dialyzed against 10 mM MES pH 6.8 and subsequently concentrated to 7 mg ml^{−1} for crystallization trials.

2.3. Crystallization and X-ray data collection

Crystallization conditions were screened by the sitting-drop vapour-diffusion method using a Hydra II eDrop automated pipetting system (Matrix) at 287 K. Drops consisted of 0.5 µl protein solution (7 mg ml^{−1}) and 0.5 µl reservoir solution and were equilibrated against 70 µl reservoir solution at 287 K. The initial crystallization conditions tested were from the Index kit (Hampton Research). After two weeks, crystals were observed in Index condition No. 44 [0.1 M HEPES pH 7.5, 25% (w/v) PEG 3350]. Crystals were cryoprotected in the reservoir solution supplemented with 20% (v/v) PEG 400. The crystal was mounted in a loop and transferred into the cryoprotectant solution for 1 min prior to cooling in liquid nitrogen. The cryoprotected crystal was then mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected using an ADSC Q315r detector on beamline 5C SB II at Pohang Light Source (PLS), Republic of Korea. X-ray diffraction data to 1.6 Å resolution were collected from the SFC-1 crystal. The crystal was oscillated by 1.0° per frame over a range of 360° to obtain maximum redundancy. All data were integrated and scaled using the *DENZO* and *SCALEPACK* crystallographic data-reduction routines (Otwinowski & Minor, 1997).

3. Results and discussion

Crystals were obtained by mixing 0.5 µl protein solution with 0.5 µl reservoir solution consisting of 0.1 M HEPES pH 7.5, 25% (w/v) PEG 3350. After three weeks, crystals grew to maximum dimensions of approximately 0.2 × 0.1 × 0.05 mm (Fig. 2b). X-ray diffraction data were collected on beamline 5C SB II at PLS. Data-collection and processing statistics are given in Table 1. Auto-indexing was performed with *DENZO* and the results indicated that the crystal

belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 65.8, b = 68.3, c = 88.8$ Å. For space group $P2_12_12_1$ two protomers exist in the asymmetric unit, with a corresponding V_M of 1.99 Å³ Da^{−1} and a solvent content of 38.1%. Self-rotation functions were calculated at $\chi = 180, 120, 90$ and 60° to detect twofold, threefold, fourfold and sixfold rotation axes, respectively (Fig. 3). Only twofold rotation axes were observed, as expected for this space group. The *MOLREP* program was used for molecular replacement using the class A carbapenemase KPC-2 from *K. oxytoca* (PDB entry 2ov5; 66.2% sequence identity; Ke *et al.*, 2007) as a search model. The molecular-replacement solution provided informative $2F_o - F_c$ and $F_o - F_c$ electron-density maps for model improvement. The two protomers in the asymmetric unit were related by twofold noncrystallographic symmetry (NCS) and the twofold NCS axis was parallel to the a axis. The structural details will be described in a separate paper.

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