Takamitsu Furuyama,^a Yoshikazu Ishii,^b Norimasa Ohya,^c Kazuhiro Tateda,^b Nancy D. Hanson^d and Akiko Shimizu-Ibuka^{c*}

^aDepartment of Material and Biological Chemistry, Graduate School of Science and Engineering, Yamagata University, 1-4-12 Kojirakawa, Yamagata 990-8560, Japan,

^bDepartment of Microbiology and Infectious Diseases, Toho University School of Medicine, 5-21-16 Omori-nishi, Ohta-ku, Tokyo 143-8540, Japan, ^cDepartment of Material and Biological Chemistry, Faculty of Science, Yamagata University, 1-4-12 Kojirakawa, Yamagata 990-8560, Japan, and ^dDepartment of Medical Microbiology and Immunology, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA

Correspondence e-mail:
ibuka@sci.kj.yamagata-u.ac.jp

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Purification, crystallization and preliminary X-ray analysis of IMP-18, a class B carbapenemase from *Pseudomonas aeruginosa*

Class B β -lactamases are known as metallo- β -lactamases (MBLs) and they hydrolyze most β -lactams, including carbapenems. IMP-18, an MBL cloned from *Pseudomonas aeruginosa*, was overexpressed, purified and crystallized by vapour diffusion for X-ray crystallographic analysis. Preliminary X-ray analysis showed that the crystal diffracted to 2.4 Å resolution and belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 120.77$, $c = 96.54$ Å, $\alpha = \beta = \gamma = 90^\circ$, suggesting the presence of two molecules in the asymmetric unit.

1. Introduction

Bacterial resistance to common and potent antibiotics such as β -lactams, aminoglycosides and quinolones continues to emerge. Bacteria have developed several strategies against β -lactam compounds to escape these lethal molecules: synthesis of β -lactamases to hydrolyze β -lactam antibiotics, decreased target sensitivity and/or development of efflux systems (Walsh, 2000; Nikaido & Pagès, 2012; Alekshun & Levy, 2007). The production of β -lactamases is especially important in Gram-negative bacteria, as they constitute the major defence mechanism against β -lactam-based drugs (Wilke *et al.*, 2005).

Class B or metallo- β -lactamases (MBLs) require one or two zinc ions to catalyze the hydrolysis of β -lactams. They have no sequence or structural homology to the serine β -lactamases of classes A, C or D (Queenan & Bush, 2007). They are generally not impeded by commercially available β -lactamase inhibitors such as clavulanic acid or tazobactam and exhibit a broad-spectrum substrate profile for a wide range of β -lactam antibiotics, including penicillins, cephalosporins, cephamycins and carbapenems (Frère, 1995). Therefore, it is difficult to treat patients infected by bacteria which produce these enzymes at high levels (Suh *et al.*, 2010; Picão *et al.*, 2009).

MBLs are further divided into three subclasses (B1, B2 and B3) based on sequence similarity and structural features (Galleni *et al.*, 2001; Garau *et al.*, 2004). Subclass B1 includes enzymes such as IMPs, VIMs, NDMs, SPM, GIM, SIM, DIM, TMB-1 and KHM-1 (Patel & Bonomo, 2013). In the late 1980s, the first IMP-type enzyme, IMP-1, was discovered in *Serratia marcescens* in Japan, followed by the identification of other IMP-type variants in Europe (Osano *et al.*, 1994; Cornaglia *et al.*, 2011). Although there are many other types of subclass B1 MBLs, IMP-type enzymes remain some of the most widespread MBLs (Borgianni *et al.*, 2011).

IMP-18 was first identified in the United States and then in Mexico and, more recently, Puerto Rico (Garza-Ramos *et al.*, 2008; Hanson *et al.*, 2006). This enzyme efficiently hydrolyzes most β -lactam compounds, with the exceptions of aztreonam and temocillin. Ten of the 11 amino-acid residues that constitute the active site of IMP-18 are identical to those of IMP-1, an IMP-type enzyme whose three-dimensional structure has already been solved. However, the substrate specificity of IMP-18 was significantly different from that of IMP-1: the k_{cat}/K_m value of IMP-1 for meropenem was 100 times higher than that of IMP-18 and the k_{cat}/K_m value of IMP-18 for ticarcillin was 100 times higher than that of IMP-1 (Borgianni *et al.*, 2011). Therefore, X-ray analysis of IMP-18 is crucial and will provide a structural basis for understanding the substrate specificity of IMP-type enzymes. In this study, we report the expression, purification,

crystallization and preliminary X-ray crystallographic analysis of IMP-18.

2. Materials and methods

2.1. Expression and purification of IMP-18

The gene encoding the entire IMP-18 sequence (including the signal sequence) was inserted into the pET-28a expression vector (Novagen) using *NdeI* and *BamHI* sites. *Escherichia coli* BL21(DE3) cells (Novagen) harbouring this plasmid were grown overnight at 310 K in 200 ml 2× Yeast extract and Tryptone (2× YT) medium with 20 µg ml⁻¹ kanamycin. When the absorbance of the culture at 600 nm reached approximately 0.5, IPTG was added to a final concentration of 0.1 mM for the induction of protein expression. After centrifugation to remove the cells, ammonium sulfate was added to the cell-culture supernatant to 80% saturation. The protein precipitate was collected by centrifugation at 27 000g for 15 min and dissolved in 2 ml buffer consisting of 20 mM HEPES pH 7.2, 50 µM ZnSO₄. The protein suspension was applied onto a PD-10 desalting column (GE Healthcare) followed by a Toyopearl CM-650S cation-exchange column (TOSOH) pre-equilibrated with the same buffer. The bound protein was eluted with a linear gradient of 0–500 mM NaCl. Eluted fractions were applied onto a HiTrap Desalting column (GE Healthcare) equilibrated with a buffer consisting of 20 mM HEPES pH 7.2, 5 mM ZnSO₄, 100 mM NaCl. Size-exclusion chromatography was performed with Superdex 75 10/300 GL (GE Healthcare). At each step, the fractions were analyzed by SDS–PAGE. The protein concentrations were estimated using the calculated molar absorption coefficient 1 mg ml⁻¹ A_{280 nm} = 1.724 OD.

2.2. Crystallization

For crystallization, the protein solution was concentrated to 7–10 mg ml⁻¹ in 20 mM HEPES pH 7.2 containing 5 mM ZnSO₄ by ultrafiltration (Amicon Ultra-4, Millipore). Initial screening of crystallization conditions was performed by the hanging-drop vapour-diffusion method at 283 and 289 K using commercial screening kits from Hampton Research (Crystal Screen, Crystal Screen 2 and Additive Screen). Crystallization drops were prepared by mixing 1 µl protein sample with an equal volume of reservoir solution and were equilibrated against 500 µl reservoir solution. Crystallization

conditions in which crystals or precipitates appeared were further optimized.

2.3. Data collection

Single crystals were transferred to reservoir solution containing 13% ethylene glycol, mounted in cryoloops and immediately cooled in a stream of cold nitrogen gas. X-ray data were collected on beamlines BL5A, NW12A and NE3A of the Photon Factory, Tsukuba, Japan. Diffraction patterns were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997) or *iMosflm* (Battye *et al.*, 2011) followed by programs from the *CCP4* suite (Winn *et al.*, 2011).

2.4. Preliminary structure analysis

Initial molecular-replacement trials were performed with *MOLREP* (Vagin & Teplyakov, 2010) in *CCP4*. The search model was generated by *SWISS-MODEL* (Arnold *et al.*, 2006) based on the amino-acid sequence of IMP-18 and the IMP-1 mutant structure (Yamaguchi *et al.*, 2005). This IMP-1 structure was used as a model since it showed the highest sequence identity to IMP-18 among the structure-solved MBLs and because the resolution of this X-ray structure was one of the highest available at the time.

3. Results and discussion

In the purification of IMP-18, salting out with ammonium sulfate was followed by cation-exchange chromatography. Concentration of the IMP-18 solution after purification by cation-exchange chromatography caused precipitation of the protein. To overcome this problem, we raised the ZnSO₄ concentration in the buffer from 50 µM to 5 mM. The increase to 5 mM ZnSO₄ resulted in increased protein solubility and IMP-18 remained soluble at concentrations up to 10 mg ml⁻¹. Generally, a class B enzyme molecule binds one or two Zn ion(s). Our result indicated that a Zn ion concentration sufficiently higher than that of the protein is required to maintain this protein in a soluble form and possibly adds to its stability.

SDS–PAGE of the purified enzyme showed a single band with a molecular weight of 25.2 kDa, which corresponds to the molecular weight of IMP-18 (Fig. 1, lane 3). However, concentration of this purified IMP-18 caused smearing during electrophoresis and a broad band was observed in the range from 25.2 to 50 kDa (Fig. 1, lane 4). In order to determine the exact size, we carried out size-exclusion

Figure 1
SDS–PAGE of IMP-18. Lane M, protein marker (labelled in kDa); lane 1, cell-culture supernatant; lane 2, fraction purified by ammonium sulfate precipitation; lane 3, fractions eluted from cation-exchange column; lane 4, concentrated IMP-18 after cation-exchange chromatography.

Figure 2
Gel-filtration chromatogram for the final stage of purification of IMP-18. The elution peak indicated with an asterisk was collected for crystallization.

chromatography, which showed a single main peak at the position equivalent to 25 kDa (Fig. 2). Thus, these two pieces of data show that IMP-18 exists as a monomer with no aggregation. In addition, SDS-PAGE analysis of the main peak fraction from the size-exclusion

(a)

(b)

(c)

Figure 3

Crystals of IMP-18 obtained with reservoir solutions consisting of (a) 0.1 M sodium citrate pH 5.6, 20% 2-propanol, 20% polyethylene glycol, (b) 0.1 M Tris-HCl pH 8.5, 2.0 M ammonium sulfate and (c) 0.1 M sodium citrate pH 5.2, 3% ethylene glycol, 20% polyethylene glycol. The scale bar is 0.1 mm in length.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Wavelength (Å)	1.0
Resolution range (Å)	54.02–2.40 (2.53–2.40)
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 120.77$, $c = 96.54$
Completeness (%)	95.0 (92.6)
Mosaicity (°)	1.57
Total observations	500584 (58960)
Unique reflections	51579 (7324)
$\langle I/\sigma(I) \rangle$	8.5 (2.2)
$R_{\text{merge}}^\dagger$	0.151 (0.604)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observed intensity of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity for all i observations of reflection hkl .

chromatography (marked with an asterisk in Fig. 2) revealed that this purification step significantly increased the purity of the protein, removing most of the minor bands observed in lane 4 of Fig. 1. This fraction was concentrated and was used in the subsequent crystallization. Approximately 2 mg pure IMP-18 was obtained from 200 ml bacterial culture.

In the initial screening of crystallization conditions, crystals of IMP-18 were observed in two conditions. One was with a reservoir solution consisting of 0.1 M sodium citrate pH 5.6, 20% (v/v) 2-propanol, 20% (w/v) polyethylene glycol 6000 at a temperature of 283 K (Fig. 3a). The other was in 0.1 M Tris-HCl pH 8.5, 2.0 M ammonium sulfate at a temperature of 289 K (Fig. 3b). Crystals grown in the former condition gave diffraction to high resolution, but the diffraction quality was not good enough for subsequent analysis. We searched for crystallization conditions that improved the crystal quality and obtained crystals suitable for data collection under the following conditions: 0.1 M sodium citrate pH 5.2, 3% (v/v) ethylene glycol, 20% (w/v) polyethylene glycol 4000 at 283 K (Fig. 3c). The crystals appeared in 12 h and reached maximum dimensions of $0.3 \times 0.15 \times 0.15$ mm in approximately 72 h.

Preliminary characterization of the IMP-18 crystals indicated that they belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 120.77$, $c = 96.54$ Å. Data-collection statistics are summarized in Table 1. The high R_{merge} value and low $I/\sigma(I)$ in the outer resolution shell may be a consequence of high mosaicity of the crystals. It is expected that higher resolution data may be obtainable when the crystallization conditions are further optimized and/or the flash-cooling technique is improved. The Matthews coefficient calculated using the cell-content analysis program in CCP4 showed that there are two or three molecules in the asymmetric unit; two molecules give a V_M value of $3.19 \text{ Å}^3 \text{ Da}^{-1}$ with 61.5% solvent content, and three molecules give a V_M of $2.13 \text{ Å}^3 \text{ Da}^{-1}$ with 42.2% solvent content (Matthews, 1968).

An initial molecular-replacement trial confirmed the presence of two monomers in the asymmetric unit. The molecules were subjected to rigid-body refinement with REFMAC (Murshudov *et al.*, 2011) in CCP4. A total of 5% of the reflections were used for the calculation of R_{free} . The R and R_{free} values of the refined structure were 41.1 and 41.8%, respectively. We are in the process of optimizing the crystallization conditions to obtain higher resolution data, improving the flash-cooling techniques for data collection and preparing for crystallographic analysis of IMP-18-inhibitor complexes.

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