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Crystallization and preliminary X-ray crystallographic analyses of CMY-1 and CMY-10, plasmidic class C β-lactamases with extended substrate spectrum

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Plasmid-encoded class C β-lactamases, including CMY-1 and CMY-10, hydrolyze the lactam bonds of β-lactam antibiotics, inducing therapeutic failure and a lack of eradication of clinical isolates by third-generation cephalosporins or cephamycins. Therefore, the enzymes are potential targets for developing agents against pathogens isolated from patients suffering from wound infection, urinary tract infection or pneumonia. CMY-1 and CMY-10 were purified and crystallized at 298 K. X-ray diffraction data from CMY-1 and CMY-10 crystals have been collected to 2.5 and 1.5 Å resolution, respectively, using synchrotron radiation. The crystals of the two proteins are isomorphous and belong to the primitive monoclinic space group $P2_1$.

1. Introduction

β-Lactam antibiotics, including penicillins, cephalosporins, monobactams and carbapenems, induce the death of growing bacteria by inhibition of cell-wall synthesis (Tomasz, 1979). Widespread use of β-lactam antibiotics has led to the emergence of bacterial resistance to these antibiotics. Expression of β-lactamases is a prevalent resistance mechanism of bacteria to β-lactam antibiotics: these enzymes hydrolyze the lactam bonds of the antibiotics. β-Lactamases are grouped into four classes, A, B, C and D, on the basis of sequence homology (Ambler, 1980).

The β-lactamase-mediated resistance of pathogenic bacteria to antibiotics is a continuing threat to public health. Therefore, a third generation of cephalosporins was developed that could escape inactivation by β-lactamases. The new antibiotics, such as cefotaxime and ceftazidime, contain bulky oxyimino groups at the C7 position of the cephalosporin nucleus. After clinical use, however, novel β-lactamases that could inactivate even the oxyimino β-lactams appeared. For example, a chromosomal class C β-lactamase that hydrolyzes oxyimino β-lactams has been isolated from the Gram-negative *Enterobacter cloacae* strain GC1 (Nukaga *et al.*, 1995).

Clinically, class A and C β-lactamases are the most commonly encountered of the four classes (Waley, 1992). However, class C β-lactamases are more problematic than class A enzymes. Class C β-lactamases can confer resistance to cephamycins (cefoxitin and cefotetan), penicillins, cephalosporins and β-lactam/β-lactamase inhibitor combinations and are not significantly inhibited by clinically used β-lactamase inhibitors such as clavulanic acid. In contrast, class A β-lactamases are not able to confer resistance to cephamycins and the enzymes are generally susceptible to inhibition by clavulanic acid.

Class C β-lactamases are typically synthesized by Gram-negative organisms and are mainly chromosomal. Recently, plasmid-encoded class C β-lactamases have been reported in several bacterial species (Lee *et al.*, 2002). Plasmid-encoded class C β-lactamases pose more problems since they are transmissible to other bacterial species and are often expressed in large amounts (Marchese *et al.*, 1998).

CMY-1 was the first plasmidic class C β-lactamase to be identified. CMY-10 is a variant of CMY-1 with a point mutation at position 346 from Asn to Ile. CMY-1 and CMY-10 display the characteristics of extended-spectrum β-lactamases (ESBLs) (Lee *et al.*, 2003; Horii *et al.*, 1993). The enzymes can confer resistance to cefoxitin and cefotetan as well as to penicillins, the third-generation cephalosporins and monobactams (Bauernfeind *et al.*, 1989). The high sequence identity between plasmidic β-lactamases and chromosomal lactamases clearly define the origin of the plasmidic enzymes: MIR-1, a plasmidic β-lactamase, shows over 90% sequence identity to a chromosomal enzyme, AmpC, from *E. cloacae*. In the case of CMY-1 and CMY-10, however, the root is obscure since there is no closely related chromosomal class C enzyme.

Structural information on class C β-lactamase is very restricted. All available structures have been determined using chromosomal β-lactamases (Crichlow *et al.*, 1999; Lobkovsky *et al.*, 1993; Oefner *et al.*, 1990; Usher *et al.*, 1998). Thus, the structures of CMY-1 and

CMY-10 will open new opportunities for structural comparison between chromosomal and plasmidic class C β-lactamases and for the design of new antibiotics that can escape hydrolysis by plasmidic class C ESBLs. Here, we report the overexpression, crystallization and preliminary X-ray crystallographic analyses of CMY-1 and CMY-10 as a first step toward their structure determination.

2. Experimental

2.1. Subcloning and purification of CMY-1 and CMY-10

The subcloning and purification of CMY-1 are exactly identical to those of CMY-10. For brevity, we only describe the preparation processes of CMY-1.

To express CMY-1 as a histidine-tagged fusion form, the $bla_{\text{CMY-1}}$ gene encoding the β-lactamase, which was produced by *Klebsiella pneumoniae* CHO isolated from a wound infection of a patient in Seoul, South Korea, was amplified by the polymerase chain reaction (PCR) from previously constructed plasmids (Lee *et al.*, 2002). The amplified gene was digested with *Bgl*II and *Xho*I and inserted downstream of the T7 promoter of the expression plasmid pET-26b (Novagen, Wisconsin, WI, USA) to produce the pET-26b/His_{11}-$bla_{\text{CMY-1}}$ plasmid. After verifying the DNA sequence, plasmid DNA was transformed into *Escherichia coli* strain BL21 (DE3) for overexpression of His_{11}-CMY-1. The transformed cells were grown in Luria–Bertani medium (Difco) containing 50 µg ml^{-1} kanamycin to an OD_{600} of 0.6 at 303 K and expression of CMY-1 was induced with 0.5 m*M* IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 16 h at 301 K. Cells were harvested by centrifugation at 5000*g* for 10 min at 277 K and resuspended in ice-cold 20 m*M* sodium phosphate buffer pH 7.0. DNase I (100 µg ml^{-1}) and 1 m*M* PMSF (phenylmethylsulfonyl fluoride) were added to the suspension and cells were disrupted by sonication. The crude lysate was centrifuged at 20 000*g* for 30 min at 277 K and the clarified supernatant was loaded onto a His-Bind column (Novagen, Wisconsin, WI, USA) equilibrated with binding buffer (20 m*M* sodium phosphate, 10 m*M* imidazole and 500 m*M* NaCl pH 7.9). His_{11}-CMY-1 was eluted with the same buffer containing 500 m*M* imidazole. For further purification, the His_{11} tag was removed from His_{11}-CMY-1 by enterokinase according to the manufacturer's instructions (Novagen). The reaction mixture was desalted and concentrated using a Fast Desalting column (Amersham Biosciences, UK) and was then loaded onto a Mono S column (Amersham Biosciences) pre-equilibrated with 10 m*M* sodium phosphate buffer pH 7.0. CMY-1 was eluted with a linear gradient of NaCl (0–0.5 *M*) in phosphate buffer. The soluble form of CMY-1 without the His_{11} tag was obtained with a yield of 9.2 mg of homogeneous protein per litre of culture. The purified CMY-1 and CMY-10 were dialyzed against 10 m*M* phosphate buffer and subsequently concentrated to 17 mg ml^{-1} for crystallization trials. Like other class C β-lactamases, the apparent molecular weight of the purified CMY-1 and CMY-10 was estimated to be 38 kDa by SDS–PAGE.

2.2. Microbatch crystallization and X-ray data collection

Crystallization screening was only performed with CMY-1 owing to the restricted quantity of CMY-10 sample. Crystals of CMY-1 were obtained by the batch-crystallization method at 298 K set up by an automatic crystallization machine, (IMPAX 1-5 system; Douglas Instruments Ltd, UK). Small drops composed of 1 µl protein solution and an equal volume of crystallization reagent were pipetted under a layer of a 1:1 mixture of silicon oil and paraffin oil in 72-well HLA plates (Nunc). The unique oil formulation allows vapour diffusion from the drop through the oil layer and thus this kind of batch-crystallization method mimics the conventional vapour-diffusion method. Initial crystallization conditions were tested by using all the available screening kits from Hampton Research and Emerald BioStructures Inc. Diffraction data were collected from flash-cooled crystals at 100 K with a MacScience 2030b area detector at beamline 6B of Pohang Light Source (6B, PLS), South Korea. The wavelength of the synchrotron radiation was 1.12714 Å. A total of 90 frames of 2° oscillation were measured with the crystal-to-detector distance set to 300 mm. Prior to data collection, crystals were soaked briefly in a cryoprotectant solution consisting of the precipitant solution containing 15%(*v*/*v*) glycerol. Diffraction data were processed and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

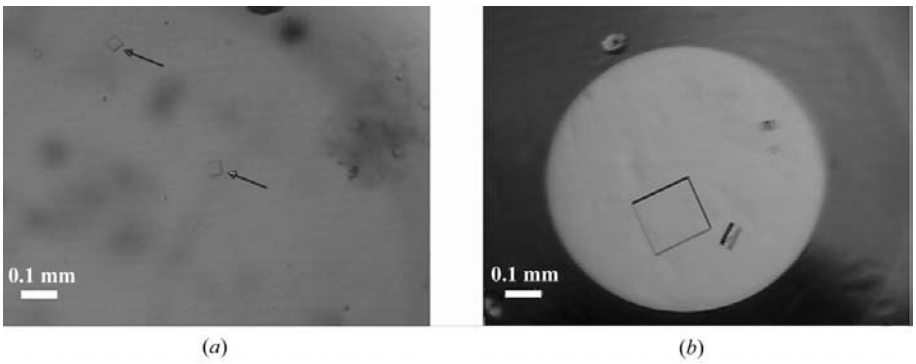

Figure 1
Crystals of CMY-1 (*a*) and CMY-10 (*b*).

3. Results and discussion

CMY-1 crystallized as thin plates with a square shape using a precipitant solution containing 18%(*w*/*v*) polyethylene glycol 8000, 0.1 *M* sodium cacodylate pH 6.5 and 0.2 *M* zinc acetate dehydrate (condition No. 45 of Crystal Screen from Hampton Research). Although the initial condition was varied widely to try to obtain large crystals, we were able to grow only microcrystals with a maximum size of 30 µm (Fig. 1*a*). To obtain larger crystals and to easily handle crystals, we tried the hanging-drop vapour-diffusion method in 24-well tissue-culture plates (Supercon, South Korea). However, we could not obtain any CMY-1 crystals with the hanging-drop method. In the case of CMY-10, fortunately, crystals grew to a size of 0.3 mm in the initial condition for CMY-1 (Fig. 1*b*).

The microcrystals of CMY-1 diffracted to 2.5 Å resolution despite their small size. We collected 1.5 Å resolution data using the large crystals of CMY-10. The crystals of the two proteins were revealed to be isomorphous, belonging to the monoclinic space group $P2_1$, with unit-cell parameters $a = 49.7$, $b = 59.5$, $c = 63.7$ Å, $\beta = 102.5°$. We calculated the crystal volume per unit molecular weight (V_M) to be 2.25 Å^3 Da^{-1} with a solvent content of 44.84% by volume (Matthews, 1968) when the unit cell is assumed to contain two molecules. This corresponds to one molecule per asymmetric unit. The statistics of data collection are summarized in Table 1.

Table 1
Crystal information and data-collection statistics.

Values in parentheses refer to the highest resolution shell, 2.50–2.55 Å for CMY-1 and 1.50–1.55 Å for CMY-10.

	CMY-1	CMY-10
Wavelength (Å)	1.12714	1.12714
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 49.73, b = 59.54, c = 63.78, β = 102.52	a = 49.70, b = 59.51, c = 63.75, β = 102.57
Resolution range (Å)	20.0–2.5	20.0–1.55
Completeness (>0σ) (%)	97.5 (94.6)	96.6 (99.6)
Total/unique reflections	37717/12252	267900/50744
R_{sym}† (%)	8.7 (22.5)	5.8 (18.1)
$I/\sigma(I)$	11.30	30.77

† $R_{sym} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$.

The extended substrate specificity of CMY-1 and CMY-10 toward third-generation antibiotics is highly likely to have arisen by a mechanism different from that of the chromosomal β-lactamase from *E. cloacae* GC1, the only class C ESBL whose structure is available. A peptide insertion consisting of an unusual tandem repeat of three residues in the Ω-loop is responsible for the extended activity of the GC1 β-lactamase (Nukaga *et al.*, 1995), which widens the active site enough to accommodate the oxyimino group of the third-generation cephalosporins (Crichlow *et al.*, 1999). However, CMY-1 and CMY-10 do not have such an insertion mutation; in fact, the two enzymes are two amino acids shorter than general class C β-lactamases with a narrow substrate spectrum. It is therefore an attractive challenge to discover the structural basis for the extended substrate spectrum of CMY-1 and CMY-10. In addition, the structural details affecting the enzymatic activities of CMY-1 and CMY-10 will be of interest because only a single amino-acid difference between them induces a difference in the hydrolysis of ceftazidime. Isolates producing CMY-10 displayed an eightfold higher minimum inhibitory concentration (MIC) of ceftazidime compared with bacteria containing CMY-1 (Lee *et al.*, 2003). The structures should provide an invaluable framework for understanding the biochemical characteristics of CMY-1 and CMY-10. Our trials to determine structures by molecular replacement by use of *AMoRe* with available structures of the four class C β-lactamase as search models resulted in failure. Various efforts such as molecular replacement with other programs (or modified models) and growth of selenium-incorporated crystals for MAD are in progress.

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