Acta Crystallographica Section F
**Structural Biology and Crystallization Communications**
ISSN 1744-3091

# Crystallization and preliminary diffraction analysis of an engineered cephalosporin acylase

**Anandhi Anandan,[a] Corinne Vallet,[a] Travis Coyle,[a] Ibrahim M. Moustafa[b] and Alice Vrielink[a]***

[a]School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia, and [b]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, 201 Althouse Laboratory, University Park, PA 16802, USA

Correspondence e-mail: alice.vrielink@uwa.edu.au

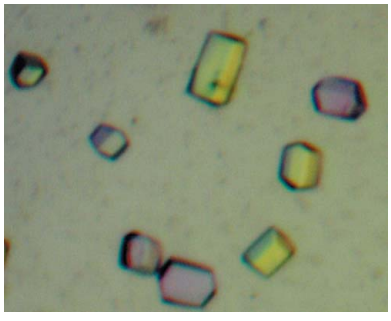
Received 24 March 2010
Accepted 11 May 2010

Crystallization conditions are reported for an engineered cephalosporin acylase based on the sequence of glutaryl-7-aminocephalosporanic acid acylase from *Pseudomonas* strain N176. Initial crystals were grown using polyethylene glycol as a crystallizing agent; however, these crystals diffracted poorly and exhibited high mosaicity. A dehydration procedure in which crystals were transferred to a solution containing a higher concentration of polyethylene glycol as well as glycerol improved the diffraction quality such that a 1.57 Å diffraction data set could be obtained.

## 1. Introduction

The discovery of penicillin as a potent antibacterial agent in the microorganism *Penicillium notatum* in 1928 by Fleming led the way to the use of microbial metabolites as drugs. Cephalosporins are amongst the most widely used classes of antibiotics. They are used to treat both Gram-positive and Gram-negative bacterial infections by inhibiting bacterial cell-wall synthesis. Although they exhibit the same mode of action as other $\beta$-lactam antibiotics such as penicillins, they benefit from being less susceptible to bacterial penicillinases.

Cephalosporin C (CephC) was originally isolated from the microorganism *Cephalosporium* sp. in 1945 as the first $\beta$-lactam fused to a six-membered ring. Thereafter, a number of semi-synthetic analogues were developed from the initial lead compound, with fourth-generation cephalosporins currently being used. Many of the semi-synthetic analogues of CephC are synthesized starting with the conversion of CephC to 7-aminocephalosporanic acid (7-ACA). However, this conversion involves a series of expensive chemical steps that require highly reactive chemicals such as trimethylchlorosilane and phosphorus pentachloride or nitrosyl chloride (Huber *et al.*, 1972), resulting in chemical waste that must be safely disposed of. Hence, altering the production method for semi-synthetic cephalosporins in order to overcome these disadvantages is of great interest to the pharmaceutical industry. An enzymatic method of producing semi-synthetic cephalosporins from 7-ACA using D-amino-acid oxidase and glutaryl-7-aminocephalosporanic acid acylase is also possible (Tischer *et al.*, 1992); although it eliminates the problems associated with toxic waste products, it is expensive and inefficient for industrial production. Therefore, a one-step conversion of CephC to 7-ACA is highly desirable. For this conversion, the utilization of glutaryl-7-aminocephalosporanic acid acylase (gl-7-ACA acylase), altering its substrate specificity and activity for CephC rather than glutaryl-7-aminocephalosporanic acid (gl-7-ACA) as a substrate, offers an ideal solution. Glutaryl acylases have been divided into five classes based on substrate specificity and sequence conservation. The activity of these enzymes towards CephC relative to gl-7-ACA varies from 0 to 4% (Aramori *et al.*, 1992; Li *et al.*, 1999; Matsuda *et al.*, 1987). The crystal structure of a class I acylase (exhibiting 2.3% deacylation activity towards CephC relative to gl-7-ACA) from *P. diminuta* has been determined (Kim *et al.*, 2000). Saturation site-directed mutagenesis of this enzyme resulted in



a 7.9-fold increase in deacylation activity towards CephC relative to the wild-type enzyme (Oh *et al.*, 2003).

Using error-prone PCR mutagenesis, molecular modelling and site-directed mutagenesis, mutant variants of a class III gl-7-ACA acylase (the wild-type enzyme exhibits 4% deacylation activity towards CephC relative to gl-7-ACA) were designed to increase the catalytic efficiency towards CephC compared with gl-7-ACA (Pollegioni *et al.*, 2005). This work resulted in a double mutant of gl-7-ACA acylase (H296S/H309S) that exhibits 22-fold enhanced specificity and reactivity for CephC over the natural substrate gl-7-ACA. This double mutant also gives high yields of 7-ACA compared with the other mutants that were produced and tested; hence, it is considered to be the best engineered mutant for this reaction (Pollegioni *et al.*, 2005). In order to fully understand the molecular features of the enzyme that facilitate the binding and deacylation of both CephC and glutaryl-7-aminocephalosporanic acid, we have undertaken structural investigations of both the wild-type gl-7-ACA acylase and the double-mutant variant (H296S/H309S). Here, we describe the initial results obtained for the crystallization and space-group determination, and the diffraction parameters of the double-mutant variant enzyme.

## 2. Materials and methods

### 2.1. Protein overproduction and purification

The pET24 expression vector containing the synthetic gene for either the full-length wild-type glutaryl-7-aminocephalosporanic acid acylase from *Pseudomonas* strain N176 (Aramori *et al.*, 1991) or the double-mutant variant (H296S/H309S) was transformed into *Escherichia coli* BL21 (DE3) pLysS as described previously (Pollegioni *et al.*, 2005). A starter culture was prepared from a single colony that was grown overnight at 310 K in LB medium containing kanamycin (30 µg ml$^{-1}$) and chloramphenicol (34 µg ml$^{-1}$). This starter culture was used to inoculate 600 ml of medium containing 44 g l$^{-1}$ tryptone, 30 g l$^{-1}$ yeast extract and 10 g l$^{-1}$ NaCl as well as kanamycin and chloramphenicol (as above) to give an $OD_{600}$ of 0.1. Growth was continued at 310 K with shaking and expression was induced by the addition of 0.6 m*M* IPTG once the cells reached the exponential phase (typically an $OD_{600}$ of 0.4–0.8). Incubation was continued at 293 K for 20 h, at which point the cells were collected by centrifugation at 12 000 rev min$^{-1}$ for 1 h.

A crude extract was prepared by sonication of the cell pellet (eight cycles of 30 s pulse at output control 4 followed by 30 s rest at 277 K on a Branson Sonifier B12) in 50 m*M* potassium phosphate buffer pH 7.5 containing 1 m*M* PMSF (10 ml per gram of cell pellet). The lysate was centrifuged at 15 000 rev min$^{-1}$ for 1 h at 277 K and the pellet was discarded. The supernatant was filtered using a 0.2 µm Acrodisc syringe filter (Pall Corporation), NaCl was added to a final concentration of 1 *M* and the resulting solution was applied onto a 1 ml HisTrap crude extract chelating affinity column (GE Healthcare) equilibrated with a binding buffer consisting of 50 m*M* sodium pyrophosphate pH 7.5, 1.0 *M* NaCl and 5% glycerol using an ÄKTA Purifier FPLC system (GE Healthcare). The column was washed with the binding buffer until the absorbance at 280 nm reached a steady baseline value. The bound enzyme was eluted using an increasing gradient of elution buffer, which consisted of 20 m*M* sodium pyrophosphate pH 7.5, 500 m*M* imidazole and 10% glycerol. A peak corresponding to the acylase was eluted at 25–30% elution buffer. The protein was dialysed first into 20 m*M* sodium pyrophosphate pH 7.5 and then into 20 m*M* HEPES pH 7.5. The dialysed sample was concentrated to 10–20 mg ml$^{-1}$ as determined by the Bradford assay using an Amicon centrifugal filter unit (30 kDa cutoff) and aliquots were stored at 193 K.

### 2.2. Crystallization, dehydration and X-ray data collection

Crystallization trials were carried out by vapour diffusion using the hanging-drop method at 291 K. Drops containing 1 µl protein solution and 1 µl crystallizing solution were equilibrated against a 1 ml reservoir of the crystallizing solution in a 24-well Linbro tray. Crystals appeared from Crystal Screen Lite condition No. 36 (4% PEG 8000, 100 m*M* Tris–HCl pH 8.5) from Hampton Research. Further optimization was achieved by screening around this initial condition. The final crystallization conditions for the double-mutant variant protein were 10–20% PEG 8000, 10 m*M* Tris–HCl pH 8–8.5.

Crystals were dehydrated to improve diffraction by transferring cover slips containing crystals grown over 10–20% PEG 8000 to wells containing 30% PEG 8000 and 20% glycerol. The crystals were incubated under these dehydration conditions for 2–4 h. Cryoprotection was carried out by briefly transferring the crystals to a drop of Paratone-N (Hampton Research) followed by flash-cooling in liquid nitrogen. All crystal transfers were carried out using CryoLoops (Hampton Research).

A high-resolution diffraction data set was collected on beamline MX1 at the Australian Synchrotron (Melbourne). Diffraction data were processed using the *MOSFLM* software (Leslie, 1992) and data reduction was carried out using the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

## 3. Results and discussion

Preliminary crystals of the double-mutant acylase could be grown from 4% PEG 8000, 100 m*M* Tris–HCl pH 8.5. Optimization of these conditions resulted in large single crystals of the double-mutant variant of the protein. Crystals appeared within approximately four weeks (Fig. 1). Crystals of the double-mutant variant appeared much more readily than those of the wild-type protein. Also, the wild-type protein crystals tended to form as showers of thinner needles. Further optimization of the conditions for the wild-type enzyme is under way.

Cryoprotectant screens were carried out and the results were tested by measuring diffraction images. Initially, the best cryoprotectant condition consisted of briefly soaking the crystals in the final concentration of the well solution containing 20% glycerol followed

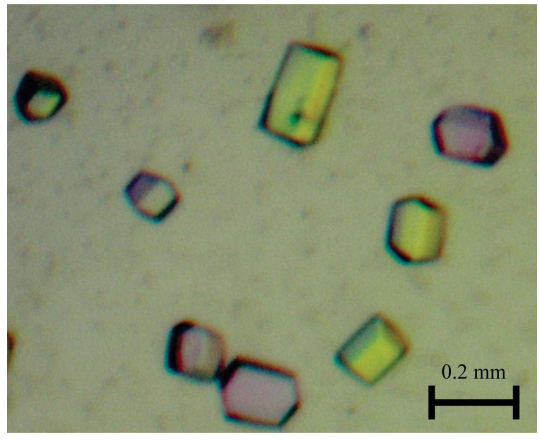



**Figure 1**
Crystals of the H296S/H309S mutant of glutaryl-7-aminocephalosporanic acid acylase obtained by vapour diffusion using the hanging-drop method. The crystals grew to approximately 0.1 × 0.1 × 0.2 mm in size.

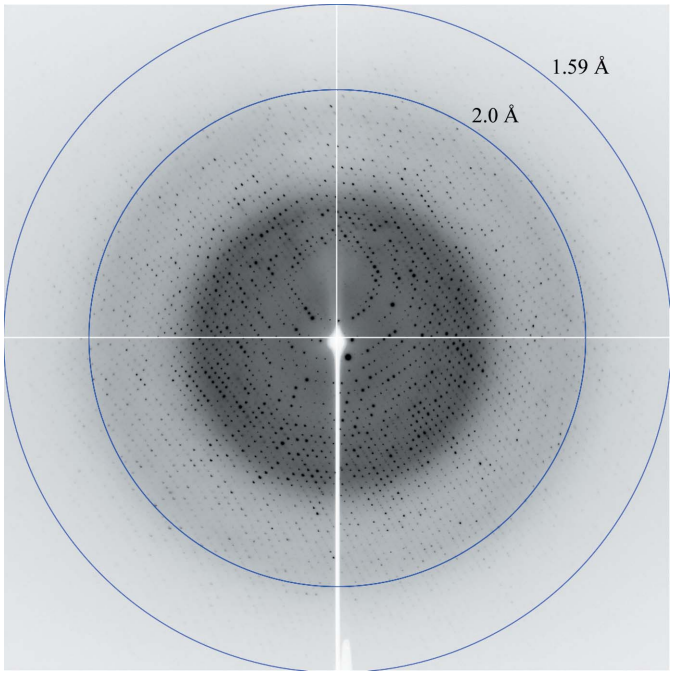



**Figure 2**
Diffraction image of the H296S/H309S mutant of glutaryl-7-aminocephalosporanic acid acylase. The image was recorded using an ADSC Quantum 210r detector on beamline MX1 at the Australian Synchrotron. The wavelength used was 0.9566 Å, the crystal-to-detector distance was 149.9 mm and the crystal was rotated 0.5° while the image was recorded. Resolution rings are shown at 2.0 and 1.59 Å.

by flash-cooling in liquid nitrogen. However, diffraction was limited to 4 Å resolution using an in-house rotating-anode X-ray generator. In addition to the low resolution, the crystals appeared to be highly mosaic.

Dehydration was a critical step in optimizing the diffraction quality of the crystals. The dehydration time and the concentrations of PEG and glycerol were optimized in order to maximize the gain in diffraction quality. After dehydration, the crystals were found to be best cryoprotected with Paratone-N, as described above. The diffraction pattern could be indexed in a primitive orthorhombic lattice of space group $P2_12_12_1$, with unit-cell parameters $a$ = 68.4, $b$ = 77.8, $c$ = 192.0 Å.

**Table 1**
Summary of X-ray data statistics for the H296S/H309S mutant of glutaryl-7-aminocephalosporanic acid acylase.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å) | $a$ = 68.4, $b$ = 77.8, $c$ = 192.0 |
| Wavelength (Å) | 0.95663 |
| Resolution range (Å) | 28.1–1.57 (1.65–1.57) |
| Unique reflections | 133584 (17581) |
| Completeness (%) | 93.5 (85.3) |
| Redundancy | 9.6 (9.7) |
| $R_{\rm merge}$ (%) | 0.088 (0.338) |
| $I/\sigma(I)$ | 15.2 (5.6) |

A 1.57 Å data set was collected from the mutant crystals at the Australian Synchrotron (Fig. 2). The processed data statistics are shown in Table 1. MAD phasing is currently under way using a selenomethionine mutant form of the protein.

## References

Aramori, I., Fukagawa, M., Tsumura, M., Iwami, M., Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Kohsaka, M., Ueda, Y. & Imanaka, H. (1991). *J. Ferment. Bioeng.* **72**, 232–243.
Aramori, I., Fukagawa, M., Tsumura, M., Iwani, M., Ono, H., Ishitani, Y., Kojo, H., Kohsaka, M., Ueda, Y. & Imanaka, H. (1992). *J. Ferment. Bioeng.* **73**, 185–192.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Huber, F., Chauvette, R. & Jackson, B. (1972). *Cephalosporins and Penicillins: Chemistry and Biology*, edited by E. Flynn, pp. 27–48. New York: Academic Press.
Kim, K., Yoon, K.-H., Khang, Y., Turley, S. & Hol, W. G. J. (2000). *Structure*, **8**, 1059–1068.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Li, Y., Chen, J., Jiang, W., Mao, X., Zhao, G. & Wang, E. (1999). *Eur. J. Biochem.* **262**, 713–719.
Matsuda, A., Matsuyama, K., Yamamoto, K., Ichikawa, S. & Komatsu, K. (1987). *J. Bacteriol.* **169**, 5815–5820.
Oh, B., Kim, M., Yoon, J., Chung, K., Shin, Y., Lee, D. & Kim, K. (2003). *Biochem. Biophys. Res. Commun.* **310**, 19–27.
Pollegioni, L., Lorenzi, S., Rosini, E., Marcone, G. L., Molla, G., Verga, R., Cabri, W. & Pilone, M. S. (2005). *Protein Sci.* **14**, 3064–3076.
Tischer, W., Giesecke, U., Lang, G., Roder, A. & Wedekind, F. (1992). *Ann. NY Acad. Sci.* **672**, 502–509.