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Crystallization and preliminary X-ray analysis of cephalosporin C acylase from *Pseudomonas* sp. strain N176

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Cephalosporin C acylase from *Pseudomonas* sp. strain N176, a heterodimer of 25 and 58 kDa, has been crystallized using polyethylene glycol 6000 as precipitant. The crystals are orthorhombic and have unit-cell parameters a = 141.41, b = 192.10, c = 80.75 Å. They belong to space group $P2_12_12_1$ and diffract to at least 2.7 Å resolution. Calculations indicate that there are two heterodimers in the asymmetric unit. The structure is being solved by molecular replacement using penicillin G acylase from *Escherichia coli* as a search model and by multiple isomorphous replacement.

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1. Introduction

Cephalosporin C acylase catalyses the hydrolysis of cephalosporin C (CC) to give 7-aminocephalosporanic acid (7ACA; Aramori *et al.*, 1991). The CC acylase from a strain of *Pseudomonas* sp. N176 (N176 CC acylase), an 83 600 Da heterodimer of *A* and *B* chains (238 and 535 amino acids, respectively), is the most efficient in terms of production of 7ACA and the recombinant enzyme has been produced by *E. coli* (Ishii *et al.*, 1994). Although the correct catalytic pathway of N176 CC acylase is still uncertain, N176 CC acylase has been confirmed to be a member of the serine-protease family by several experiments (Nobbs *et al.*, 1994).

The homology of N176 CC acylase and penicillin G acylase, an 80 kDa heterodimer of *A* and *B* chains (209 and 566 amino acids, respectively), is approximately 20% including homologous mutations. However, several clusters of high homology are observed in both the *A* and *B* chains. It is suggested that these homologous regions play an important role in the recognition of the β-lactam backbone of the substrates. In particular, noticeable conservation is present at the amino-terminal region of the *B* chain (Ishii *et al.*, 1994). Furthermore, from chemical modification experiments, the amino-terminal serine residue of the *B* chain from both enzymes is thought to act as a nucleophile in catalysis (Nobbs *et al.*, 1994).

7ACA is a key intermediate in the production of semi-synthetic cephem antibiotics. It is usually prepared from CC by chemical methods (Fechtig *et al.*, 1968; Morin *et al.*, 1969). However, the chemical reaction procedure requires highly reactive chemicals, such as trimethylchlorosilane and phosphorus pentachloride, which can only be handled at low temperature and require expensive safety and energy requirements as well as strict requirements for environmental control. In addition, the use of this method in the lowering of the production costs has almost reached its limit. Therefore, an alternative method using cephalosporin acylase, which catalyses the hydrolysis of cephalosporin compounds to 7ACA, is preferred because it is simple mild and ecologically clean. Furthermore, production costs may be lowered still further than with the chemical method (Saito *et al.*, 1996).

In order to establish efficient enzymatic production of 7ACA, CC acylases have been explored during the last two decades and some effective acylases have been found (Shibuya *et al.*, 1981; Ichikawa *et al.*, 1981; Matsuda & Komatsu, 1985). Several approaches aimed at improving the productivity of 7ACA have been also investigated by modification of N176 CC acylase with chemical reagents and by site-directed point-mutation study of the acylases (Nobbs *et al.*, 1994). However, the acylases have not been generally applied in industrial processes owing to their low enzymatic activity as CC acylases. Therefore, we intend to improve the capability of N176 CC acylase by protein engineering to establish enzymatic production of 7ACA. Detailed information on the three-dimensional structure of the enzyme can potentially lead to the design of new acylases that have higher enzymatic activity.

In this paper, the crystallization and preliminary X-ray analysis of N176 CC acylase are described.

2. Methods

Cephalosporin C acylase from *Pseudomonas* sp. N176 has been overexpressed and purified

Figure 1
Crystals of cephalosporin C acylase from *Pseudomonas* sp. strain N176.

from an *E. coli* tryptophan promoter (Ishii *et al.*, 1994). The protein used for crystallization was at 6 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 7.5. All crystallization trials were carried out using the hanging-drop vapour-diffusion method by mixing 2 µl of protein solution with 2 µl of reservoir solution and equilibrating the drops over the reservoir at room temperature. The initial trials were performed using the commercially available sparse-matrix screening kits Crystal Screen I, Crystal Screen II and Crystal Screen Lite from Hampton Research (Jancarik & Kim, 1991). The crystallization conditions were refined using grid screening based on the results from the first screening.

The X-ray diffraction data were collected at room temperature on a Rigaku R-AXIS IIc imaging-plate system using Cu $K\alpha$ radiation from a Rigaku RU-200 rotating-anode generator operated at 40 kV and 100 mA. The crystal-to-detector distance was 150 mm and 82 images were recorded at 1.5° intervals. The exposure time was 30 min per image. Data were reduced using the Rigaku *PROCESS* crystallographic data-reduction package.

Subsequent calculations utilized the *X-PLOR* 3.1 package (Brünger, 1992) and the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Small crystals were obtained from several drops from the Crystal Screen Lite kit. These revealed conditions consisting of 15% polyethylene glycol with 0.2 *M* salts to be promising. As a result of the refinement of the conditions, the crystals used for data collection were grown against reservoirs containing 12–15%(*w*/*v*) polyethylene glycol 6000, 0.1 *M* magnesium chloride, 50 m*M* imidazole buffer pH 6.0. The crystals grew to maximum dimensions of 0.2 × 0.2 × 0.1 mm in two months (Fig. 1).

The crystals diffracted to at least 2.7 Å resolution. A total of 115 226 observed reflections were scaled and reduced to yield a data set containing 42 046 unique reflections with an $R_{\rm merge}$ of 10.9%. The data set was 96.2% complete to 3.5 Å resolution and 94.0% complete to 3.0 Å resolution, with the data in the shell 3.5–3.0 Å resolution being 90.2% complete. The average $I/\sigma(I)$ value in this shell was 4.4.

The crystal system was determined to be orthorhombic, space group $P2_12_12_1$; the unit-cell parameters were a = 141.41, b = 192.10, c = 80.75 Å. The V_m value (Matthews, 1968) was calculated to be 3.6 Å^3 Da^{-1}, assuming two heterodimers per asymmetric unit. Reflections where $h = 2n + 1$ had weaker intensities than those where $h = 2n$. The Patterson function calculated with the observed native data indicated a prominent peak 63% of the origin peak at $u = 0.5$, $v = 0.0$, $w = 0.0$. No significant signal was observed in the self-rotation function calculated with 15–4 Å reflections. These facts suggest that the two independent molecules are located in a similar direction, separated by a half of the unit-cell edge along the a axis.

No significant peak was observed on the cross-rotation function calculated with 15–4 Å reflections. The calculation was performed using the program *AMoRe* (Navaza, 1994) and used penicillin G acylase from *E. coli* (Protein Data Bank code 1pnk; Duggleby *et al.*, 1995) as a starting model. Further calculations are being performed using several models modified from penicillin G acylase, for example, a polyalanine model.

In parallel with these trials, preparation of heavy-atom derivatives and data collection for a detailed X-ray analysis are currently in progress.

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