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Crystallization and X-ray diffraction analysis of nylon-oligomer hydrolase (NylC) from *Agromyces* sp. KY5R

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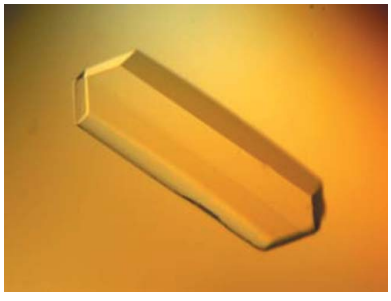

6-Aminohexanoate-oligomer hydrolase (NylC) from *Agromyces* sp. KY5R was expressed in *Escherichia coli* JM109 and purified by ammonium sulfate fractionation, anion-exchange column chromatography and gel-filtration chromatography. NylC was crystallized by the sitting-drop vapour-diffusion method with sodium citrate as a precipitant in 0.1 *M* HEPES buffer pH 7.5 containing 0.2 *M* NaCl. Diffraction data were collected from native and K_2PtCl_4-derivative crystals to resolutions of 2.00 and 2.20 Å, respectively. The obtained crystal was plate-shaped, with an *I*-centred orthorhombic space group and unit-cell parameters a = 155.86, b = 214.45, c = 478.80 Å. The anomalous difference Patterson map of the K_2PtCl_4-derivative crystal suggested that the space group was $I222$ rather than $I2_12_12_1$.

1. Introduction

Biodegradation of xenobiotic compounds that have been released into the natural environment since the development of the chemical industry has been recognized as a useful method for the elimination of environmental pollutants. In addition, it provides a suitable system to investigate how microorganisms have evolved the enzymes that are essential for this degradation. We have been studying the degradation of a byproduct of nylon 6 manufacture, the 6-aminohexanoate (Ahx) oligomer (nylon oligomer), as a model system (Okada *et al.*, 1983; Negoro *et al.*, 1992, 2005, 2007; Kakudo *et al.*, 1993, 1995; Negoro, 2000; Ohki *et al.*, 2006, 2009; Yasuhira, Uedo *et al.*, 2007; Yasuhira *et al.*, 2010; Kawashima *et al.*, 2009).

Three enzymes, Ahx-cyclic dimer hydrolase (NylA), Ahx-dimer hydrolase (NylB) and Ahx-oligomer hydrolase (NylC), are responsible for the degradation of nylon oligomers (Okada *et al.*, 1983; Negoro, 2000; Yasuhira, Uedo *et al.*, 2007). Previous biochemical studies have revealed that NylA specifically hydrolyses one of the two equivalent amide bonds in the 6-aminohexanoate cyclic dimer, generating a 6-aminohexanoate linear dimer, and is thus classified into the amidase signature (AS) hydrolase family (Yasuhira, Uedo *et al.*, 2007). NylB, on the other hand, hydrolyses Ahx oligomers *via* an exo-type mode and is a member of the penicillin-recognizing family of serine-reactive hydrolases (Negoro *et al.*, 2005; Ohki *et al.*, 2006, 2009; Negoro *et al.*, 2007; Kawashima *et al.*, 2009).

NylC degrades cyclic and linear oligomers of Ahx with a degree of polymerization of more than three by an endo-type mode, but is barely active towards the Ahx cyclic dimer (the specific substrate for NylA), the Ahx linear dimer (the specific substrate for NylB) and various tested peptides (Negoro *et al.*, 1992; Kakudo *et al.*, 1993, 1995; Yasuhira, Uedo *et al.*, 2007). NylC has been found in *Arthrobacter* (plasmid pOAD2-encoded NylC; $NylC_{p2}$), *Agromyces* ($NylC_A$) and *Kocuria* ($NylC_K$) (Yasuhira, Tanaka *et al.*, 2007). These enzymes are composed of 355 amino-acid residues (molecular mass of 36 kDa). However, $NylC_A$ and $NylC_K$ have 5–15 amino-acid substitutions compared with the $NylC_{p2}$ sequence and are more thermostable (by 10–20 K; Yasuhira, Tanaka *et al.*, 2007).

Knowledge of the crystal structure of NylC will allow us to study its catalytic mechanism and the molecular basis of the stability of this protein. In this paper, we report the crystallization and preliminary crystallographic analysis of the $NylC_A$ protein.

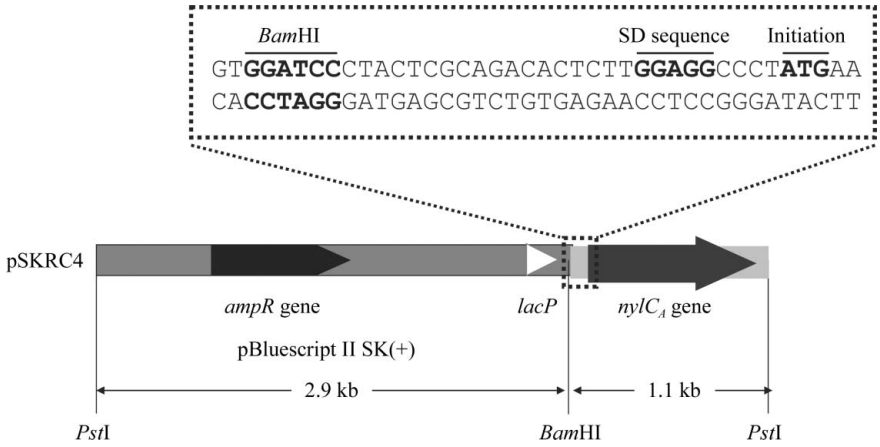

Figure 1
Structure of the pSKRC4 plasmid. The pSKRC4 plasmid is a hybrid plasmid composed of a 1.1 kb *Bam*HI–*Pst*I fragment containing the *nylC*$_A$ gene and a 2.9 kb *Bam*HI–*Pst*I fragment of the pBluescript II SK(+) expression vector (Yasuhira, Tanaka *et al.*, 2007). The vector plasmid contains the ampicillin-resistance (*ampR*) gene as the selection marker for transformation of *E. coli.* The *nylC*$_A$ gene is expressed under the control of the *lac* promoter (*lacP*) of the vector region. The DNA region for the *Bam*HI site, ribosome-binding (Shine–Dalgarno; SD) sequence and initiation codon (ATG) of the *nylC*$_A$ gene is shown in the nucleotide sequence.

2. Materials and methods

2.1. Expression and enzyme purification

To express the *nylC*$_A$ gene in *Escherichia coli*, the pBluescript II SK(+) expression vector (Stratagene, La Jolla, California, USA) was used. Plasmid pSKRC4, in which a 1.1 kb *Bam*HI–*Pst*I fragment containing *nylC*$_A$ was inserted downstream of the *lacP* region in the vector (*Bam*HI/*Pst*I sites), was constructed (Yasuhira, Tanaka *et al.*, 2007; Fig. 1). Competent cells of *E. coli* JM109 were prepared by the $CaCl_2$ method (Sambrook & Russell, 2001) and stocked at 193 K before use. For purification of NylC$_A$, the competent cells (100 µl) were newly transformed with pSKRC4 (10 µg). The cells were incubated at 273 K for 30 min, followed by heat-shock treatment at 315 K for 45 s (Sambrook & Russell, 2001). The cell suspension was transferred to 900 µl SOC medium (Sambrook & Russell, 2001) and incubated at 310 K for 1 h. Since prolonged cultivation at 303 K or higher temperatures resulted in insoluble NylC in the cells, *E. coli* cells harbouring the hybrid plasmid were grown in 400 ml TB medium (Sambrook & Russell, 2001) containing 100 mg l^{-1} ampicillin and 1 m*M* isopropyl *β*-D-1-thiopyranogalactoside at 298 K for 60 h. The cells were harvested by centrifugation at 10 000*g* for 10 min, washed with buffer *A* (20 m*M* phosphate buffer pH 7.3 containing 10% glycerol) and suspended in 20 ml buffer *A*. After disrupting the cells by ultrasonication (20 kHz, 5 min, four times), the cell lysate obtained by centrifugation at 30 000*g* for 30 min was used as the crude enzyme solution.

To precipitate the NylC fraction, ammonium sulfate (40% saturation) was added to the crude enzyme solution (22 mg ml^{-1}, 20 ml) and kept for 30 min on ice. The NylC precipitate was obtained by centrifugation at 30 000*g* for 10 min and dissolved in 20 ml buffer *A*. To decrease the ammonium sulfate concentration in the sample, the enzyme solution was diluted to 140 ml with buffer *A* and the NylC was purified by HiTrap Q Sepharose HP (two sequentially joined 5 ml columns, GE Healthcare) column chromatography with a 0.16–0.4 *M* NaCl linear gradient in a total volume of 160 ml. NylC fractions were concentrated by centrifugation at 4000*g* for 40 min using an Amicon

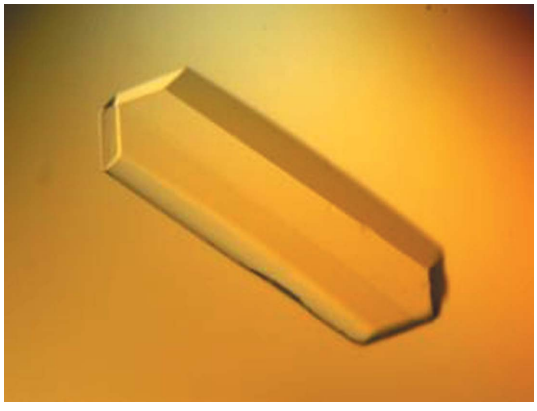

Figure 2
Crystals of NylC$_A$. The crystal (0.8 × 0.4 × 0.3 mm) of NylC$_A$ was obtained using a reservoir solution consisting of 1.0 *M* sodium citrate, 0.1 *M* HEPES buffer pH 7.5 and 0.2 *M* NaCl.

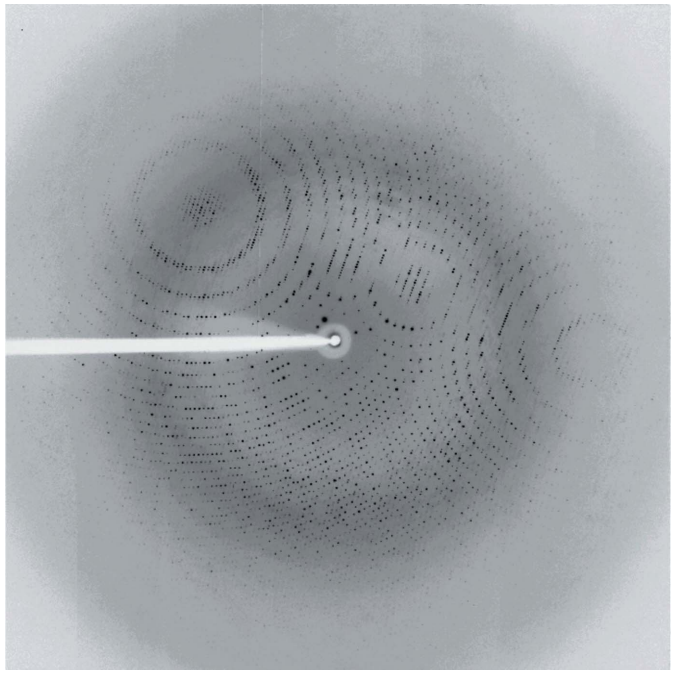

Figure 3
X-ray diffraction image of the native NylC$_A$ crystal.

Ultra microconcentrator (cutoff size of membrane 10 kDa; Millipore Co.) and purified by Sephacryl S-200 HR column (16 × 650 mm; GE Healthcare) chromatography. All purification procedures were carried out at 277 K and 25 mg purified NylC was obtained.

2.2. Crystallization

Initial crystallization tests were performed by the sitting-drop vapour-diffusion method in 96-well plates using Wizard I and Wizard II screen kits (Emerald BioSystems). Droplets were prepared by mixing 2 µl purified $NylC_A$ solution (10 mg ml^{-1} protein in 20 m*M* phosphate buffer containing 10% glycerol pH 7.3; buffer *A*) and 2 µl reservoir solution and were equilibrated against 100 µl reservoir solution at 283 K. To obtain crystals suitable for X-ray diffraction, a survey for optimum conditions was conducted by varying the concentration of sodium citrate (0.8, 0.9 and 1.0 *M*) and the acidity of the buffers (0.1 *M* sodium acetate pH 4.0–5.5 in 0.5 pH-unit intervals, 0.1 *M* MES pH 5.5–7.0 in 0.5 pH-unit intervals and 0.1 *M* HEPES pH 7.0–8.5 in 0.5 pH-unit intervals] in the presence or absence of salt solutions (0.2 *M* NaCl, 0.2 *M* Li_2SO_4).

2.3. Data collection and crystallographic analysis

For data collection, native crystals were soaked for 24 h in cryoprotectant solution (1.0 *M* sodium citrate, 0.1 *M* HEPES pH 7.5, 0.2 *M* NaCl, 25% glycerol) prior to flash-cooling in a nitrogen cold stream. For preparation of heavy-atom derivatives, the crystals were soaked in cryoprotectant solution containing 5 m*M* K_2PtCl_4 for 72 h and then back-soaked in cryoprotectant solution for 1 h. Cryocooling was performed by placing the crystal in a cold nitrogen stream at 100 K.

Diffraction data sets were collected on SPring-8 (Hyogo, Japan) beamline BL38B1 equipped with a Rigaku Jupiter CCD detector system. The following parameters were chosen for data collection for both the native crystal and the Pt derivative: wavelength, 1.0000 Å; crystal-to-detector distance, 180 mm; oscillation range per image, 0.5°. Indexing, integration and scaling of reflections were performed using the *HKL*-2000 program package (Otwinowski & Minor, 1997). Diffraction data were collected from the native $NylC_A$ crystal and the K_2PtCl_4 derivative to resolutions of 2.00 and 2.20 Å, respectively (Table 1). Substructure determination for the platinum derivative was performed using *BnP* (Weeks *et al.*, 2002). Anomalous difference Patterson and self-rotation functions ware calculated using *CCP*4 (Winn *et al.*, 2011) and *MOLREP* (Vagin & Teplyakov, 2010), respectively.

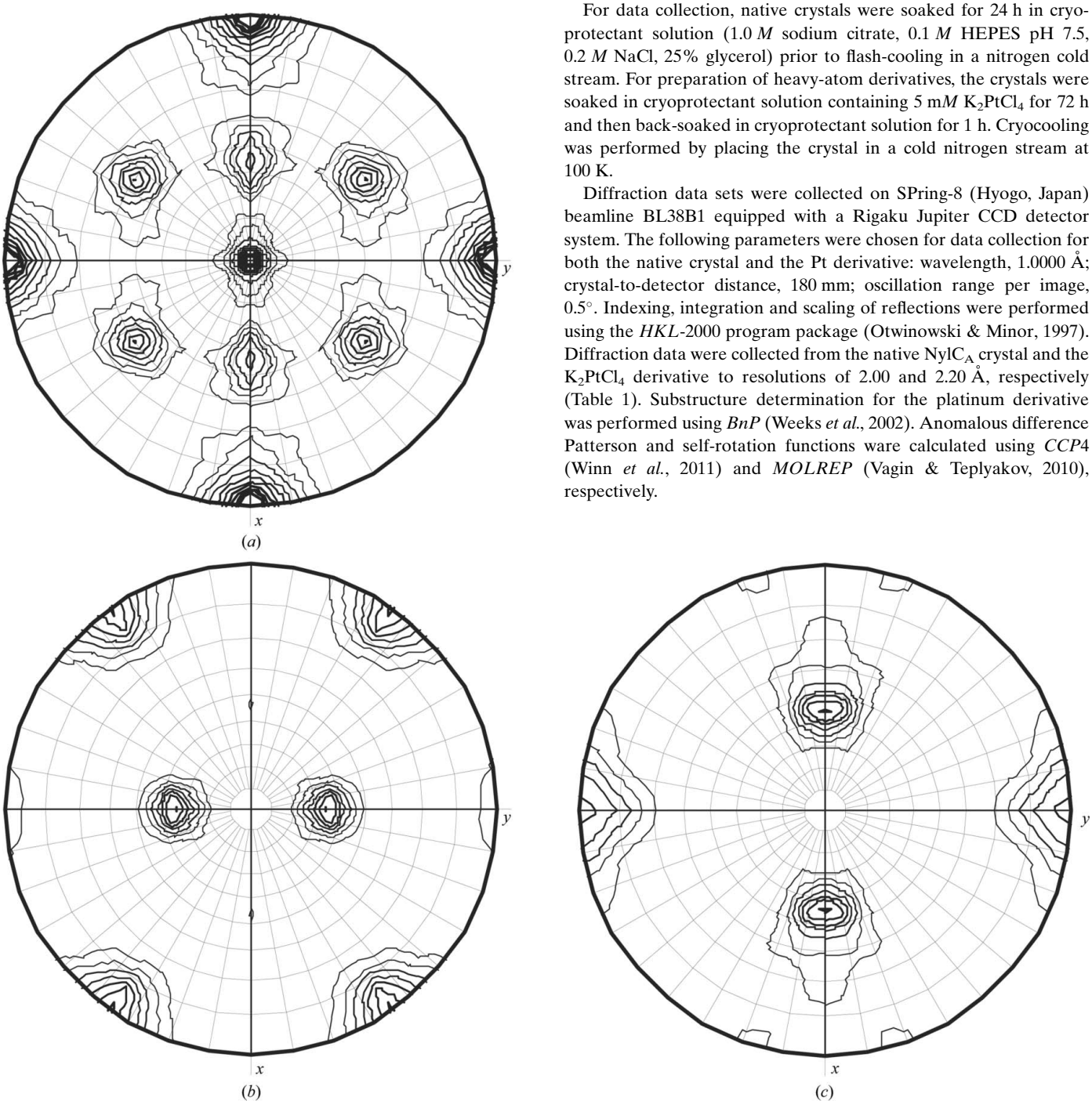

Figure 4
Self-rotation function of the native $NylC_A$ crystal in the (*a*) $\chi = 180°$, (*b*) $\chi = 120°$ and (*c*) $\chi = 90°$ sections.

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

Data collection	Native ($NylC_A$)	K_2PtCl_4 derivative ($NylC_A$)
Space group	$I222$ or $I2_12_12_1$	$I222$ or $I2_12_12_1$
Unit-cell parameters (Å)		
a	155.86	155.99
b	214.45	214.72
c	478.80	477.82
Wavelength (Å)	1.0000	1.0000
Resolution (Å)	30–2.00 (2.07–2.00)	30–2.20 (2.28–2.20)
Total reflections	1789588	2786883
Unique reflections	501977 (38621)	398011 (36644)
Completeness (%)	94.3 (73.0)	99.0 (91.7)
R_{merge}† (%)	11.1 (25.4)	6.4 (46.2)
$\langle I/\sigma(I)\rangle$	10.6 (2.32)	26.1 (2.87)
Multiplicity	3.6 (2.0)	7.0 (4.8)
Mosaicity (°)	0.16	0.22

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

3. Results

The *nylC*$_A$ gene, which encodes a polypeptide of 355 amino acids, was cloned and expressed in *E. coli* and the $NylC_A$ protein was purified to homogeneity. The purity of the protein sample used for crystallization was confirmed by SDS–PAGE, native PAGE and light-scattering experiments. Of the 100 conditions (Wizard I and II) tested in crystallization screening, microcrystals were obtained after one week using Wizard I condition No. 14 (cacodylate buffer pH 6.5 containing 1.0 *M* sodium citrate) and Wizard II condition No. 16 (CHES buffer pH 9.5 containing 1.0 *M* sodium citrate). Crystals with the highest quality were obtained after one week using 1.0 *M* sodium citrate as a precipitant in 0.1 *M* HEPES buffer pH 7.5 containing 0.2 *M* NaCl. Plate-like crystals grew to typical dimensions of 0.8 × 0.4 × 0.3 mm (Fig. 2).

Diffraction data for the native crystal (Fig. 3) and the K_2PtCl_4 derivative were collected to 2.00 and 2.20 Å resolution, respectively. Neither a dose-dependent increase in R_{merge} nor a decrease in the signal-to-noise ratio [*i.e.* $\langle I/\sigma(I)\rangle$] per image were detected, indicating that there was no significant radiation damage during data collection. Autoindexing with *HKL*-2000 clearly indicated that the crystal belonged to the *I*-centred orthorhombic space group $I222$ or $I2_12_12_1$. The unit-cell parameters were $a = 155.86$, $b = 214.45$, $c = 478.80$ Å for the native crystal and $a = 155.99$, $b = 214.72$, $c = 477.82$ Å for the K_2PtCl_4-derivative crystal. Crystal parameters and diffraction data statistics are summarized in Table 1. The anomalous difference Patterson function showed 15 major peaks other than that at the origin, all of which were on the $h = 0$, $k = 0$ and $l = 0$ planes, suggesting that the crystal belonged to space group $I222$ rather than $I2_12_12_1$.

There could be 15–30 molecules in the asymmetric unit, as estimated from the Matthews coefficient (V_M) of 1.8–3.6 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of 32–66%. A platinum-substructure determination from the anomalous differences yielded 15 significant peaks, suggesting that 15 molecules per asymmetric unit was most probable. The oligomeric structure of NylC in aqueous solution has been suggested previously based on the estimation of the molecular weight of native $NylC_{p2}$ by gel-filtration chromatography as 93 000, while that of the polypeptide as deduced from the nucleotide sequence was calculated to be 36 902 (Kakudo *et al.*, 1995). The self-rotation function calculated using the *MOLREP* program (Vagin & Teplyakov, 2010) shows peaks corresponding to noncrystallographic twofold, threefold and fourfold axes (Fig. 4). *MOLREP* also detected pseudo-translation of $x = 0.000$, $y = 0.000$ and $z = 0.333$. These biochemical and crystallographic analyses indicate that the enzyme is likely to exist as a dimer or trimer. However, the exact oligomeric state needs to be elucidated by structural analysis.

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