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Crystallization and preliminary X-ray diffraction analysis of cyclic imide hydrolase (CIH) from *Pseudomonas putida* YZ-26

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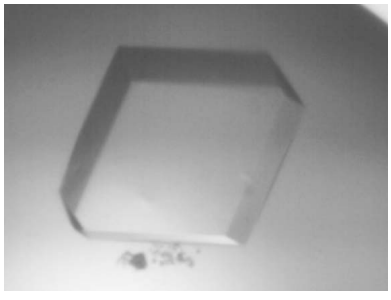
A recombinant form of cyclic imide hydrolase from *Pseudomonas putida* YZ-26 has been crystallized by the hanging-drop method. X-ray diffraction data were collected to 1.9 Å resolution. The crystals belonged to the orthorhombic space group $C222_1$, with unit-cell parameters a = 111.91, b = 176.04, c = 176.06 Å. Assuming the presence of four molecules in the asymmetric unit gives a V_M value of 3.10 Å^3 Da^{-1} and a solvent content of 60.31%.

1. Introduction

Cyclic imide hydrolase (CIH), also named imidase, belongs to the cyclic amidohydrolase superfamily. Cyclic amidohydrolases (cyclic amidases; EC 3.5.2.–) have been shown to participate in pyrimidine metabolism *in vivo* and in the bioconversion of organic acids *in vitro*, which is critical for producing semisynthetic antibiotics, pesticides and food additives (Soong *et al.*, 2001). According to the characteristics of their optimal substrates, the cyclic amidohydrolase superfamily may be divided into two subfamilies: cyclic ureide hydrolases and cyclic imide hydrolases. The former includes hydantoinase, dihydropyrimidinase, allantoinase and dihydroorotase, while the latter only includes cyclic imide hydrolase, which differs from the former enzymes in the molecular mass of the subunit, amino-acid sequence and substrate specificity.

In 1996, Ogawa and coworkers obtained an imidase from *Blasterbacter* sp. A17p-4, which was the first time the enzyme had been purified from a bacterium. The imidase participates in the metabolism of simple imides: it is involved in ring-opening hydrolysis of cyclic imides to half-amides and the resulting half-amide is hydrolyzed by halfamidase to a dicarboxylate, which then undergoes further transformation through tricarboxylic acid (TCA) cyclic reactions (Ogawa *et al.*, 1996, 1997). According to these reports, the imidase is a metal-dependent enzyme and is a homogenous trimer with a subunit molecular weight of 35 kDa (Ogawa *et al.*, 1996). Another CIH from pig liver is a homogenous hexamer with a subunit molecular weight of 51 kDa and is also a metal-dependent enzyme (Yang *et al.*, 1993). We obtained a further CIH from *Pseudomonas putida* YZ-26 (Shi *et al.*, 2007); it is also a zinc-dependent enzyme (Shi *et al.*, 2010).

In order to understand the role of CIH in pyrimidine metabolism and organic acid bioconversion, we cloned the gene for this CIH enzyme and expressed the enzyme in *Escherichia coli*. This is the first report of the crystallization of this CIH and its preliminary analysis by X-ray crystallography.



2. Materials and methods

2.1. Expression and purification

The CIH gene was cloned into the plasmid pET32a vector (Shi *et al.*, 2007). The recombinant plasmid pEI was transformed into *E. coli* strain BL21 (DE3). The cells were grown in LB medium supplemented with 100 µg ml^{-1} ampicillin at 310 K until the OD_{600}

reached 0.6–0.8. Recombinant protein expression was induced by adding 0.25 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for 5 h. Cells were harvested by centrifugation at 4000*g* for 10 min and the cell pellets were resuspended in lysis buffer (500 m*M* NaCl, 20 m*M* Tris–HCl, 5 m*M* imidazole pH 8.0) and lysed by sonication. The cell lysate was centrifuged at 12 000*g* for 45 min at 277 K. The supernatant was loaded onto a nickel-affinity column (Qiagen) pre-equilibrated in lysis buffer and the column containing the His-CIH protein was washed with lysis buffer followed by washing buffer (500 m*M* NaCl, 20 m*M* Tris–HCl, 100 m*M* imidazole pH 8.0). The protein was eluted with elution buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 100 m*M* EDTA). The eluate was immediately ultrafiltrated to remove the EDTA. After concentration, the preparation was loaded onto a HiLoad 16/60 Superdex 200 pg column (Amersham Biosciences, New Jersey, USA) equilibrated with gel-filtration buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl). Fractions containing CIH protein were pooled and concentrated to a concentration of 15 mg ml^{-1} using Amicon Ultra-15 filters with a 10 000 Da cutoff (Millipore, USA).

DLS measurements were carried out at 298 K using a DynaPro Titan instrument (Wyatt Technology, Santa Barbara, California, USA). Protein concentrations were determined using the BCA protein assay according to the manufacturer's instructions (Pierce).

2.2. Crystallization

Crystallization screening was carried out with Crystal Screen and Crystal Screen 2 (Hampton Research). Crystallization trials for CIH were performed at 289 K using the hanging-drop vapour-diffusion method by mixing 1 µl 5 mg ml^{-1} protein (in buffer consisting of 50 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl) and 1 µl reservoir solution. The reservoir volume was 200 µl. Crystals appeared within 7 d in several different conditions. Diffraction-quality crystals were obtained using the following conditions: 0.1 *M* bicine pH 9.0, 2%(*v*/*v*) 1,4-dioxane, 10%(*w*/*v*) polyethylene glycol 20 000.

2.3. Data collection and processing

The crystals were first transferred into cryoprotectant containing 15% glycerol for about 30 s and were then mounted on nylon loops and flash-cooled in a stream of gaseous nitrogen at 100 K. Diffraction data were collected using an in-house X-ray source (Rigaku MicroMax-007 desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA) and an R-AXIS IV^{++} imaging-plate detector at a wavelength of 1.5418 Å. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using *HKL*-2000 (Minor *et al.*, 2000).

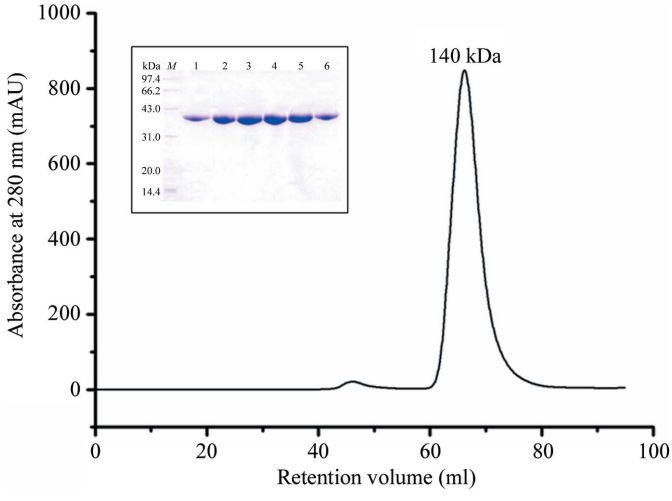


Figure 1
Gel-filtration chromatogram of recombinant CIH using a HiLoad 16/60 Superdex 200 pg column and SDS–PAGE of the CIH eluted from the gel-filtration column (inset).

3. Results and discussion

The gene encoding CIH from *P. putida* YZ-26 (GenBank AAY98498.1) was cloned and the protein was overexpressed and purified. The open reading frame for CIH is 882 bp coding for 293 amino-acid residues. The purified CIH showed a single band of 35 kDa on SDS–PAGE (Fig. 1), which is in agreement with the calculated molecular weight with a 6×His tag and a thrombin site (MHHHHHHSSGLVPRGS) at the N-terminus. During gel filtration on a Superdex 200 pg column CIH eluted at the volume expected for a molecular weight of about 140 kDa (Fig. 1). This result is consistent with the DLS experiments (Fig. 2), suggesting that the protein exists as a tetramer in solution.

Crystals appeared within 7 d (Fig. 3) and an X-ray diffraction image is shown in Fig. 4. The crystals belonged to space group $C222_1$,

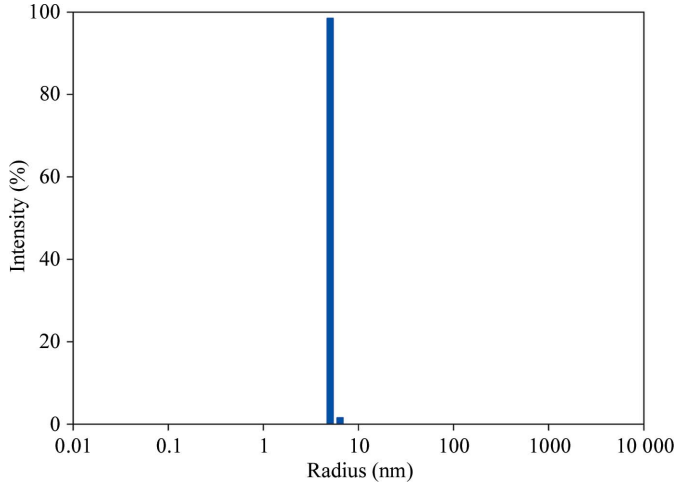


Figure 2
DLS experiment result, showing a single peak of about 140 kDa; the molecular radius is 5.0 nm and the polydispersity is 3.9%.

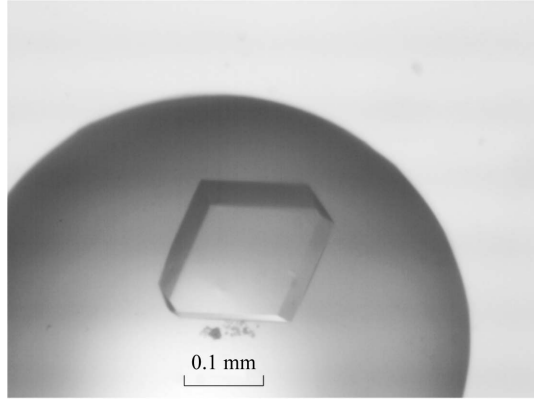


Figure 3
Representative crystal of CIH from *P. putida* YZ-26.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Unit-cell parameters (Å, °)	a = 111.91, b = 176.04, c = 176.06, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	50–1.9 (1.97–1.90)
No. of unique reflections	135717
No. of observed reflections	861080
Completeness (%)	99.7 (100.0)
Multiplicity	36.3 (6.3)
$\langle I/\sigma(I)\rangle$	18.3 (9.1)
$R_{\rm merge}$† (%)	0.09 (0.23)

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

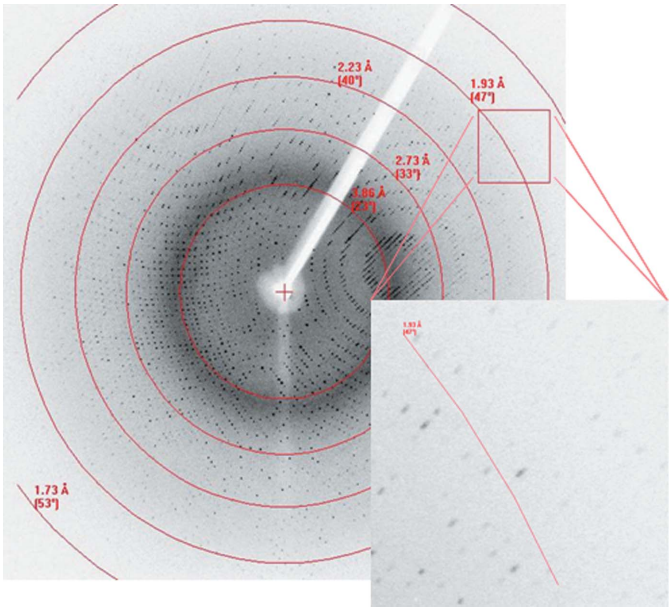


Figure 4
X-ray diffraction image of CIH from *P. putida* YZ-26. The frame edge is at 1.9 Å resolution.

with unit-cell parameters a = 111.91, b = 176.04, c = 176.06 Å, $\alpha = \beta = \gamma = 90°$. Using the molecular weight of the monomer (35 kDa), a Matthews coefficient (Matthews, 1968) of 3.10 Å^3 Da^{-1}, corresponding to a solvent content of 60.31%, was obtained assuming the presence of four monomers in the asymmetric unit. Data-collection and processing statistics are given in Table 1. As CIH has 29% sequence identity to the PuuE allantoinase (PDB entry 3cl6) from *P. fluorescens* (Ramazzina *et al.*, 2008), we attempted molecular-replacement methods for phase determination. However, this approach did not provide sufficient phases for structure determination. As there are 12 Met residues in the sequence, we are currently preparing selenomethionine-substituted crystals for phase determination using the multiwavelength anomalous diffraction (MAD) technique.

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