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Cloning, crystallization and preliminary X-ray study of XC1258, a CN-hydrolase superfamily protein from *Xanthomonas campestris*

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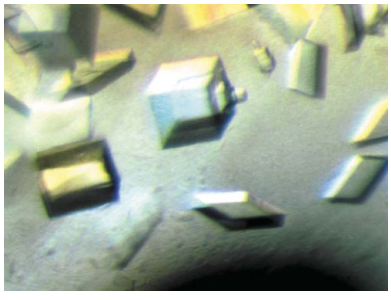

CN-hydrolase superfamily proteins are involved in a wide variety of non-peptide carbon–nitrogen hydrolysis reactions, producing some important natural products such as auxin, biotin, precursors of antibiotics *etc.* These reactions all involve attack on a cyano or carbonyl carbon by a conserved novel catalytic triad Glu-Lys-Cys through a thiol acylenzyme intermediate. However, classification into the CN-hydrolase superfamily based on sequence similarity alone is not straightforward and further structural data are necessary to improve this categorization. Here, the cloning, expression, crystallization and preliminary X-ray analysis of XC1258, a CN-hydrolase superfamily protein from the plant pathogen *Xanthomonas campestris* (Xcc), are reported. The SeMet-substituted XC1258 crystals diffracted to a resolution of 1.73 Å. They are orthorhombic and belong to space group $P2_12_12$, with unit-cell parameters a = 143.8, b = 154.63, c = 51.3 Å, respectively.

1. Introduction

XC1258 (gi|21113352) from the plant pathogen *Xanthomonas campestris* pv. *campestris* strain 17 (Xcc) is classified as belonging to the CN-hydrolase superfamily (previously called the nitrilase superfamily; Pace & Brenner, 2001) in the Pfam database (Bateman *et al.*, 2000). The monomer consists of 266 amino acids and shares sequence identities of 91, 49, 44, 32, 30 and 30% with similar proteins from the *X. campestris* pv. *vesicatoria*, *Pseudomonas putida*, *Erwinia carotovora* subsp. *atroseptica*, *Staphylococcus aureus*, *Bacillus anthracis* and *Lactobacillus salivarius* subsp. *salivarius*, respectively. The CN-hydrolase superfamily is relatively large, containing enzymes such as nitrilases, aliphatic amidases, carbamylase and β-alanine synthase (Pace & Brenner, 2001; Bork & Koonin, 1994). Their reactions usually involve attack on a cyano or carbonyl carbon by a conserved novel catalytic triad Glu-Lys-Cys through a thiol acylenzyme intermediate (Pace & Brenner, 2001).

Currently, only a few tertiary structures are available for proteins belonging to this superfamily, including a NitFhit protein from *Caenorhabditis elegans* (Pace *et al.*, 2000), the *N*-carbamyl-D-amino-acid amidohydrolases (DCases) from *Agrobacterium* sp. strain KNK712 (Nakai *et al.*, 2000) and *A. radiobacter* (Wang *et al.*, 2001) and a putative CN hydrolase from *Sacchromyces cerevisiae* strain S288C (Kumaran *et al.*, 2003), which are either dimeric or tetrameric $\alpha\beta\beta\alpha$ sandwich proteins. Intriguingly, while the structures of *Rhodococcus* nitrilases (nitrile hydratases that convert a nitrile to the corresponding amide; Huang *et al.*, 1997; Nagashima *et al.*, 1998) also comprise $\alpha\beta\beta\alpha$ sandwiches, they are not classified as belonging to the CN-hydrolase superfamily owing to very low structural similarity (Pace & Brenner, 2001). Therefore, more structural studies of proteins from this superfamily are necessary to unambiguously classify this interesting class of proteins. In this manuscript, we describe the cloning, expression, crystallization and preliminary X-ray analysis of XC1258 from *X. campestris*. Good crystals that diffracted to a resolution of 1.73 Å have been obtained. The preliminary structure obtained indicates that the protein adopts a novel quaternary struc-

ture variant of previously determined CN-hydrolase superfamily protein structures.

2. Materials and methods

2.1. Cloning, expression, and purification

The XC1258 gene fragment was PCR amplified directly from a local Xcc genome (*X. campestris* pv. *campestris* strain 17) with forward 5′-TACTTCCAATCCAATGCTATGCACGACCTGCGC-ATTTCCCTC and reverse 5′-TTATCCACTTCCAATGCTACTCG-CCCAGCACAAAGCTGTCCC primers. A ligation-independent cloning (LIC) approach from to a previously published protocol (Wu *et al.*, 2005) was carried out to obtain the desired construct. The final construct codes for an N-terminal His_6 tag, a 17-amino-acid linker and an XC1258 target protein (266 amino acids) under the control of a T7 promoter. Overexpression of the His_6-tagged target protein was induced by the addition of 1 m*M* IPTG at 293 K for 20 h. The target protein was purified by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma). The His_6 tag and linker were cleaved from XC1258 by TEV (tobacco etch virus) protease at 288 K for 24 h. For crystallization, XC1258 was further purified on an anion-exchange column (AKTA, Pharmacia Inc.). The final target protein (266 amino acids) has greater than 99% purity (Fig. 1) and contains only an extra tripeptide (SNA) at the N-terminal end. The over-expression and purification of XC1258 was monitored by SDS–PAGE as shown in Fig. 1.

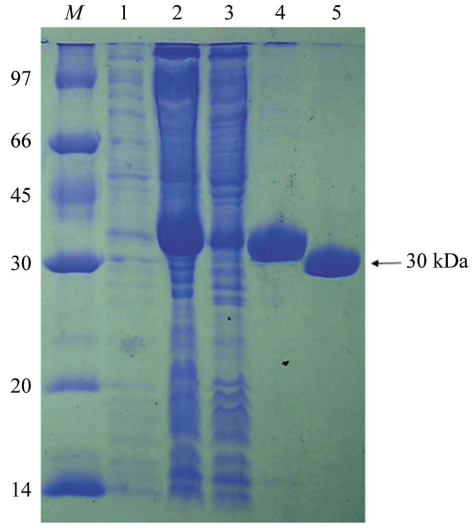

Figure 1
SDS–PAGE monitoring of the overexpression and purification of XC1258. Lane *M*, molecular-weight markers in kDa; lane 1, whole cell lysate before IPTG induction; lane 2, whole cell lysate after IPTG induction; lane 3, soluble fraction after IPTG induction; lane 4, purified XC1258 before TEV cleavage; lane 5, purified XC1258 after TEV cleavage.

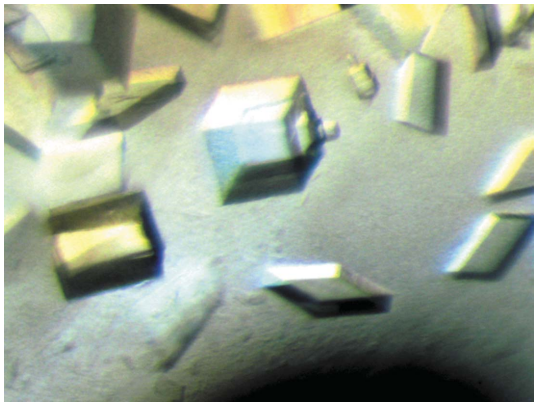

Figure 2
Cube-shaped crystals of XC2981 from *X. campestris* grown by the hanging-drop vapour-diffusion method. The crystallization condition used was 0.1 *M* cacodylate pH 6.5 and 1 *M* sodium citrate. The average dimensions of these crystals were around 0.15 × 0.15 × 0.1 mm.

Table 1
Data-collection statistics for XC1258.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12$
Unit-cell parameters (Å)	a = 143.8, b = 154.63, c = 51.3
Temperature (K)	100
Wavelength (Å)	0.96108
Resolution range (Å)	30.0–1.73 (1.79–1.73)
Mosaicity (°)	0.4
Unique reflections	731068 (112277)
Redundancy	6.5 (5.9)
Completeness (%)	98.4 (95.3)
R_{merge} (%)	4.3 (13.5)
Mean $I/\sigma(I)$	33.1 (13.1)
Solvent content (%)	48.5

2.2. Crystallization

For crystallization, the protein was concentrated to 28 mg ml^{-1} in 20 m*M* Tris pH 8.0 and 80 m*M* NaCl using an Amicon Ultra-10 (Millipore). Screening for crystallization conditions was performed using sitting-drop vapour diffusion in 96-well plates (Hampton Research) at 295 K by mixing 0.5 µl protein solution with 0.5 µl reagent solution. Initial screens, including the Hampton Research sparse-matrix Crystal Screens 1 and 2, a systematic PEG–pH screen and a PEG/Ion Screen were performed using a Gilson C240 crystallization workstation. Cube-shaped crystals appeared in 3 d from a reservoir solution comprising 0.1 *M* sodium cacodylate pH 6.5 and

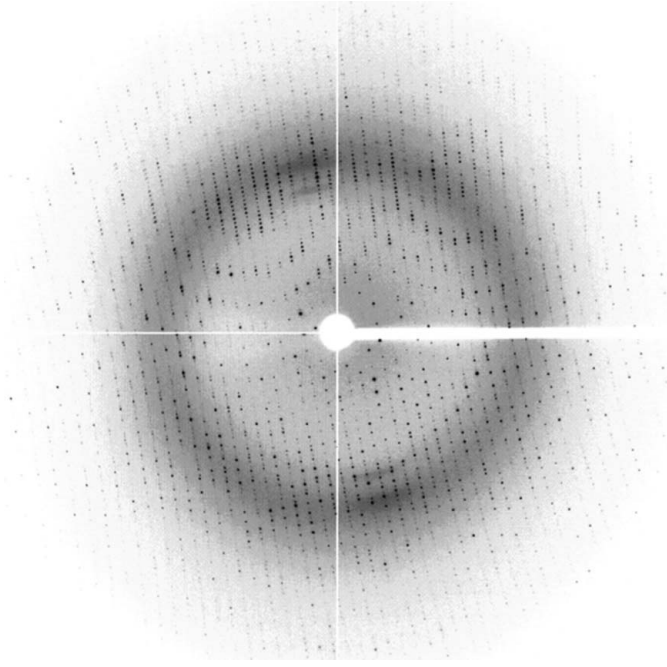

Figure 3
Diffraction pattern of Se-XC1258 collected at NSRRC beamline 13B1 from a crystal flash-frozen in reservoir solution. The exposure time was 3 s, with an oscillation range of 1° and a crystal-to-film distance of 150 mm.

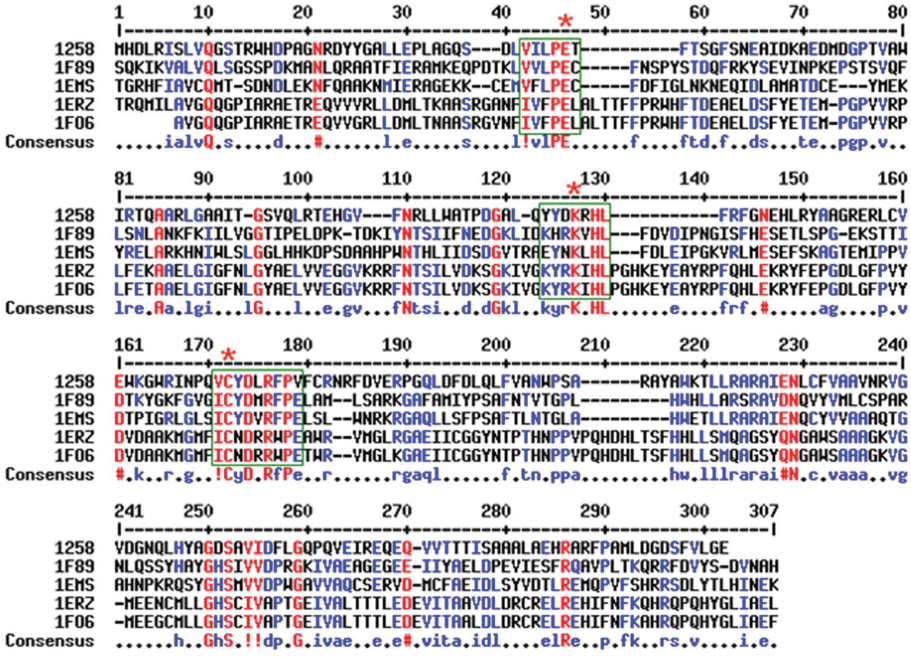

Figure 4
Sequence alignment of XC1258, yeast CN hydrolase (1F89), NitFhit from *C. elegans* (1EMS), DCase from *Agrobacterium* sp. strain KNK712 (1ERZ) and DCase from *A. radiobacter* (1FO6) by *MultiAlign* (Corpet, 1988). Strictly conserved residues are in red and highly conserved residues are in blue. The CN-hydrolase superfamily catalytic triad motifs are boxed in green, with the triad Glu, Lys and Cys residues marked with purple stars.

1 *M* sodium citrate. Crystals suitable for diffraction experiments were grown by mixing 1.5 µl protein solution with 1.5 µl reagent solution at 277 K and reached maximum dimensions of 0.15 × 0.15 × 0.1 mm after one week (Fig. 2).

2.3. Data collection

Crystals were flash-cooled at 100 K without cryoprotectant in a stream of cold nitrogen to prevent crystal cracking. X-ray diffraction data were collected using the National Synchrotron Radiation Research Center (NSRRC) beamline 13B1 in Taiwan. Only a faint ice ring was observed under such circumstances and a data set to 1.73 Å resolution could be obtained for SeMet-substituted XC1258. The data were indexed and integrated using the *HKL*-2000 software (Otwinowski & Minor, 1997), giving a data set that was 98.4% complete with an overall $R_{\rm merge}$ of 4.3%. The crystals belong to the orthorhombic space group $P2_12_12$. The data-collection statistics are summarized in Table 1; an X-ray diffraction image collected at the NSRRC facility is shown in Fig. 3.

3. Results and discussion

The XC1258 gene consists of 801 bp coding for 266 amino-acid residues. The isoelectric point was calculated to be 5.83 using the *Compute pI/MW* tool at the ExPASy website (http://us.expasy.org/tools/pi_tool.html). Purified XC1258 protein shows a single band at the 30 kDa position on SDS–PAGE (Fig. 1), close to its monomer MW.

The high-resolution diffraction data obtained from the SeMet-substituted XC1258 crystals established their suitability for X-ray structural analysis (Fig. 3). A three-wavelength data set has been collected at the remote, peak height and inflection points of Se absorption using beamline 13B1 at the NSRRC, Taiwan. A good preliminary structure of XC1258 was obtained using the MAD approach with the *SOLVE* and *RESOLVE* programs (Hendrickson & Ogata, 1997; Terwilliger & Berendzen, 1999); the protein was found to adopt a novel quaternary structure variant of previously determined CN-hydrolase superfamily protein structures. Detailed structural refinement of XC1258 is currently under way.

A multiple sequence alignment of XC1258 with other CN-hydrolase superfamily proteins for which tertiary structures have been obtained (PDB codes 1f89, 1ems, 1erz and 1fo6) revealed very low overall sequence identities (23, 17, 14.2 and 16.1%, respectively) despite the presence of conserved residues in the catalytic triad motif regions (Fig. 4; Pace & Brenner, 2001). Of the three histidine residues (129, 144 and 215) identified to be important for catalytic activity in DCase (Wang *et al.*, 2001), only His129 was found to be conserved in XC1258; the other two residues His144 and His215 are replaced by Phe and Ala, respectively. Five gap-deletion regions were also observed in the XC1258 primary sequence compared with those of DCases (1fo6 and 1erz; Fig. 4). These sequence-alignment studies indicate that XC1258 possibly does not belong to the DCase subfamily of the CN superfamily. Further structural and functional studies are necessary to decipher its exact function.

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