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Extracellular overproduction and preliminary crystallographic analysis of a family I.3 lipase

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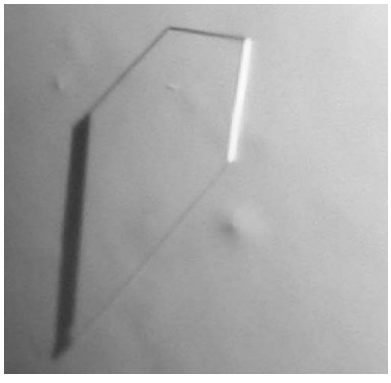

A family I.3 lipase from *Pseudomonas* sp. MIS38 was secreted from *Escherichia coli* cells to the external medium, purified and crystallized and preliminary crystallographic studies were performed. The crystal was grown at 277 K by the hanging-drop vapour-diffusion method. Native X-ray diffraction data were collected to 1.7 Å resolution using synchrotron radiation at station BL38B1, SPring-8. The crystal belongs to space group $P2_1$, with unit-cell parameters a = 48.79, b = 84.06, c = 87.04 Å. Assuming the presence of one molecule per asymmetric unit, the Matthews coefficient V_M was calculated to be 2.73 Å^3 Da^{-1} and the solvent content was 55%.

1. Introduction

Bacterial lipolytic enzymes are classified into eight families (families I–VIII) based on differences in their amino-acid sequences and biological properties (Arpigny & Jaeger, 1999). Family I is further classified into seven subfamilies (families I.1–I.7); of these, families I.1, I.2 and I.3 are true lipases from Gram-negative bacteria (Arpigny & Jaeger, 1999; Jaeger & Eggert, 2002). Family I.1 and I.2 lipases share relatively high amino-acid sequence similarity and are secreted *via* the type II secretion system, in which they are first translocated across the inner membrane into the periplasmic space *via* the Sec pathway and then further translocated across the outer membrane *via* the secreton complex following chaperone-assisted periplasmic folding (Johnson *et al.*, 2006).

Family I.3 lipases show poor amino-acid sequence similarity (<20%) to either family I.1 or family I.2 lipases (Arpigny & Jaeger, 1999; Angkawidjaja & Kanaya, 2006). Furthermore, family I.3 lipases are secreted *via* the type I secretion system (T1SS), which spans the inner and outer membranes of Gram-negative bacteria, forming a continuous channel to export proteins directly to the extracellular milieu in one step (Holland *et al.*, 2005). Passenger proteins of the T1SS vary from toxins to extracellular enzymes, most of which contain a secretion signal located near the C-terminus. Enzymes that are secreted *via* the T1SS have two domains: a catalytic N-domain and a C-domain containing several repeats of a GG*x*G*x*D*xux* sequence motif (where *x* is any amino acid and *u* is a large hydrophobic amino acids) in the upstream region of the secretion signal (Delepelaire, 2004). These repeats, which are also termed RTX (repeat in toxin) motifs, form a β-roll structure in the presence of Ca^{2+} ions, as exemplified by the crystal structures of several proteases (Baumann *et al.*, 1993; Baumann, 1994; Hege & Baumann, 2001; Aghajari *et al.*, 2003) and the NMR structure of the R-module of a mannuronan C-5 epimerase (Aachmann *et al.*, 2006), all of which are secreted *via* the T1SS.

Pseudomonas sp. MIS38 lipase (PML) is a family I.3 lipase and consists of an N-domain (residues 1–370) and C-domain (residues 371–617) (Amada *et al.*, 2000). The C-domain contains 12 repetitive sequences. PML accumulates in *Escherichia coli* cells in an insoluble form upon overproduction using a pET system (Amada *et al.*, 2000). In contrast, it is secreted from the *E. coli* cells to the external medium in a soluble form upon co-expression with the genes encoding a heterologous T1SS (Kwon *et al.*, 2004). The C-domain of PML can be

used as a secretion tag for extracellular production of an alkaline phosphatase (Angkawidjaja *et al.*, 2006).

The active-site residues of PML, which presumably form a catalytic triad, are located in the N-domain, indicating that the N-domain represents the catalytic domain (Kwon *et al.*, 2000). PML requires Ca^{2+} for enzymatic activity (Amada *et al.*, 2001). The Ca^{2+} ion is expected to bind to or in the vicinity of the active site of PML, like the Zn^{2+} ion of the metalloproteases from *Serratia marcescens* (Baumann, 1994) and *Pseudomonas aeruginosa* (Baumann *et al.*, 1993). PML also requires Ca^{2+} for folding of the C-domain, which is probably initiated by the formation of a β-roll structure (Amada *et al.*, 2001). It has been proposed that folding of the C-domain is required not only to make the conformation of the N-catalytic domain functional, but also to protect PML from intracellular proteolytic degradation (Kwon *et al.*, 2004; Angkawidjaja *et al.*, 2005). However, the following questions remain to be answered. Does the catalytically essential Ca^{2+} ion bind to the catalytic N-domain? Do the repetitive sequences form a β-roll structure upon Ca^{2+} binding? How many Ca^{2+} ions bind to the protein in total? Does folding of the C-domain directly or indirectly affect the structure of the catalytic N-domain? To answer these questions, it is necessary to determine the crystal structure of PML.

Here, we report the extracellular production, crystallization and preliminary X-ray crystallographic studies of PML.

2. Experimental procedures

2.1. Extracellular overproduction and purification

Plasmid pUC-PML for the secretion of PML was constructed previously (Kwon *et al.*, 2004). Plasmid pYBCD20 harbouring the *lipBCD* genes from *S. marcescens* SM8000 (Kawai *et al.*, 1998) was kindly donated by K. Omori. The *lipBCD* genes encode T1SS for *S. marcescens* lipase (Lip system). *E. coli* DH5 [F^-, *hsdR17*(r_K^-, m_K^+), *recA1*, *endA1*, *deoR*, *thi*$^{-1}$, *supE44*, *gyrA96*, *relA1*] was transformed with plasmids pUC-PML and pYBCD20 and the recombinant *E. coli* cells were grown in LB medium containing 50 mg l^{-1} ampicillin and 30 mg l^{-1} chloramphenicol at 303 K for 24 h with constant shaking. The culture was centrifuged at 10 000*g* for 30 min at 277 K. The supernatant was collected and its pH was adjusted to 8.0 by adding 1/20(*v*/*v*) of 2 *M* Tris–HCl pH 8.0 (final concentration 0.1 *M*). PML was collected from the culture supernatant by 80% ammonium sulfate precipitation. The protein pellet was redissolved in 50 m*M* Tris–HCl pH 8.0 containing 5% glycerol and 10 m*M* $CaCl_2$ and was dialyzed overnight against the same buffer. The dialysate was centrifugated at 25 000*g* for 30 min to remove insoluble materials and was then applied onto a HiTrapQ HP anion-exchange column (GE Healthcare) equilibrated with the same buffer. The flowthrough fraction was collected, concentrated using a Centriplus YM-50 filter device (Millipore) and applied onto a HiLoad 16/60 Superdex 200 gel-filtration column (Amersham Biosciences) equilibrated with 5 m*M* Tris–HCl pH 8.0 containing 5 m*M* $CaCl_2$. The protein was eluted from the column using the AKTA Prime system (GE Healthcare). The fractions containing PML were collected, concentrated to a final concentration of 10 mg ml^{-1} using Centricon YM-50, filtered through a 0.22 µm filter and used directly for crystallization. The purity of the protein was analyzed by 15% SDS–PAGE (Laemmli, 1970) followed by staining with Coomassie Brilliant Blue. Protein concentration was determined from UV absorption using a cell with an optical length of 1 cm and an A_{280} value of 0.91 for 0.1% protein solution (1.0 mg ml^{-1}), which was calculated based on the amino-acid sequence of PML (Gill & von Hippel, 1989).

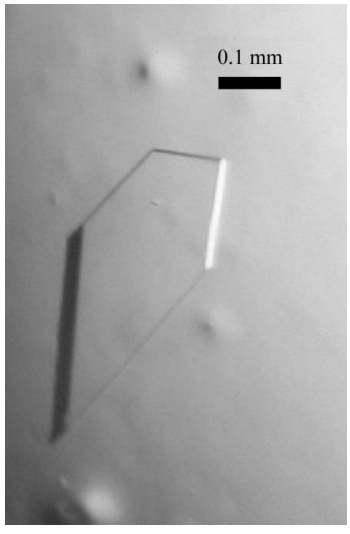

Figure 1
Crystal of PML grown by the hanging-drop vapour-diffusion method. The approximate dimensions of the crystal are 0.3 × 0.2 × 0.05 mm.

2.2. Crystallization and data collection

Preliminary screening of the crystallization conditions was performed using the sitting-drop vapour-diffusion method at 277, 283 and 293 K. Wizards I and II (Emerald Biostructures) and Crystal Screens I and II (Hampton Research) were initially used. Drops were prepared by mixing 1 µl each of protein and reservoir solutions and were vapour-equilibrated against 100 µl reservoir solution. Small needle-shaped crystals were obtained after one week using Wizard I solution No. 7 (0.1 *M* MES pH 6.0, 10% PEG 8K, 0.2 *M* zinc acetate) at 277 K. Further optimization was performed at this temperature by changing the pH and the type and concentration of the precipitant and the metal ion. Diffraction-quality crystals grew by incubating 2 µl protein solution with 1 µl of an optimized crystallant solution (0.1 *M* MES pH 6.0, 10% PEG 20K, 0.2 *M* calcium acetate, 5 m*M* zinc acetate) using the hanging-drop vapour-diffusion method. The drops were vapour-equilibrated against 300 µl of a modified crystallant solution that contains 5% PEG 20K instead of 10%. All crystallization trials were performed at atmospheric pressure.

A PML crystal was mounted on a CryoLoop (Hampton Research) following separation from surrounding crystals using Micro-Tools (Hampton Research) and was soaked in artificial mother liquor plus 20% ethylene glycol as cryoprotectant. The mounted crystal was flash-frozen in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected at a wavelength of 0.9 Å on beamline BL38B1 at SPring-8, Japan. A total of 352 images were recorded with an exposure time of 10 s per image and an oscillation angle of 1.0°. Diffraction images were indexed, integrated and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Extracellular overproduction of PML was achieved by employing the T1SS for *S. marcescens* lipase with a secretion level of 25 mg per litre of culture. Following ammonium sulfate precipitation and dialysis, the protein solution was passed through an anion-exchange column. PML did not bind to this column, while most of the medium components did. Gel-filtration column chromatography of the resultant flow-through fraction yielded PML with a purity of more than 95% as

Table 1
Statistics of data collection.

Values in parentheses are for the highest resolution shell (1.76–1.70 Å).

X-ray wavelength (Å)	0.9
Temperature (K)	100
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 48.79, b = 84.06, c = 87.04
Resolution range (Å)	50.00–1.70
No. of measured reflections	553967
No. of unique reflections	76888
Average redundancy	7.3 (6.3)
$R_{\rm merge}$† (%)	7.0 (35.1)
Data completeness (%)	99.2 (92.3)
Average $I/\sigma(I)$	10.7 (3.3)

† $R_{\rm merge} = \sum|I_{hkl} - \langle I_{hkl}\rangle|/\sum I_{hkl}$, where I_{hkl} is the intensity measurement for the reflection with indices hkl and $\langle I_{hkl}\rangle$ is the mean intensity for multiply recorded reflections.

estimated by SDS–PAGE (data not shown). The amount of protein purified from 1 l culture was approximately 5 mg. The molecular weight of PML was estimated to be 65 kDa using either SDS–PAGE or gel-filtration column chromatography, which was comparable to that calculated from its amino-acid sequence (64 510 Da), suggesting that secreted PML exists as a monomer.

PML was also overproduced in *E. coli* cells in an insoluble form, solubilized in the presence of 8 *M* urea, purified in a denatured state, refolded by removing the urea and used for biochemical characterization; its enzymatic activity was comparable to that of secreted PML (Kwon *et al.*, 2004). However, crystallization trials using refolded PML have so far been unsuccessful, probably because the protein molecules of refolded PML do not always assume the native structure. Therefore, secreted PML was used to screen for the crystallization conditions.

PML crystals appeared after 7–10 d and grew to maximum dimensions of 0.3 × 0.2 × 0.05 mm after several weeks to one month (Fig. 1). The crystal diffracted X-rays to beyond 1.7 Å resolution (Fig. 2). A total of 553 967 measured reflections were merged into 76 888 unique reflections with an $R_{\rm merge}$ of 7.0%. The crystal belongs to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 48.79, b = 84.06, c = 87.04 Å. Table 1 summarizes the data-collection statistics. Based on the molecular weight and the space group, it was assumed that the crystal contained one protein molecule per asymmetric unit, giving a Matthews coefficient ($V_{\rm M}$) value of 2.73 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 55%. We are currently searching for suitable heavy-atom derivatives. The structure of PML is of particular interest because no structural data are available for family I.3 lipases. This structure should provide valuable information on the catalytic mechanism and role of the C-terminal domain of these lipases.

We have previously shown that *E. coli* alkaline phosphatase is secreted to the external medium in an amount sufficient for biochemical characterization using TISS (Angkawidjaja *et al.*, 2006). Therefore, the success in crystallization of secreted PML may suggest the use of T1SS for the extracellular overproduction of proteins that normally form inclusion bodies in *E. coli*.

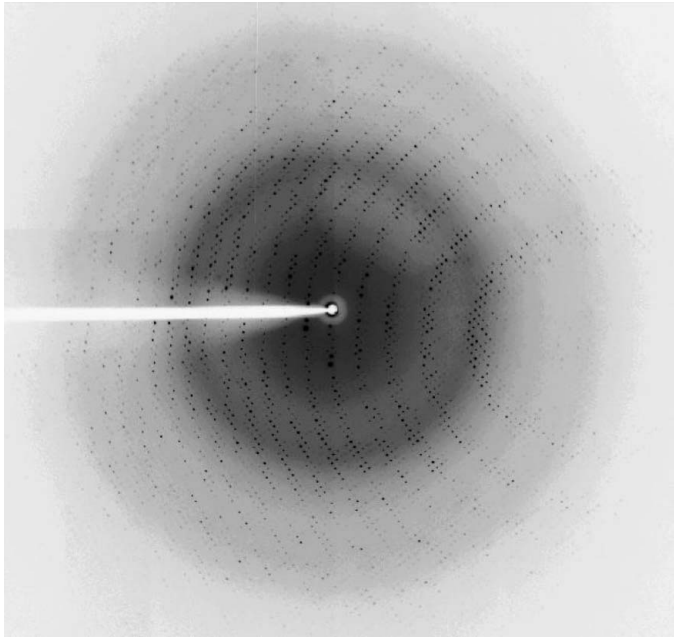

Figure 2
X-ray diffraction pattern of the native PML crystal. The crystal was exposed at 100 K after soaking in artificial mother liquor containing 20% ethylene glycol for cryoprotection. The photograph shows a single frame of 1.0° oscillation with an exposure time of 10 s per image and a crystal-to-detector distance of 170 mm. The crystal diffracted to 1.7 Å (the edge of the plate).

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