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Received 4 December 2012

Accepted 20 February 2013

PDB Reference: lipase, 3auk

Crystallization and X-ray structure determination of a thermoalkalophilic lipase from *Geobacillus* SBS-4S

A thermoalkalophilic lipase (LIP_{SBS}) from the newly isolated *Geobacillus* strain SBS-4S which hydrolyzes a wide range of fatty acids has been characterized. In the present study, the crystallization of purified LIP_{SBS} using the sitting-drop vapour-diffusion method and its X-ray diffraction studies are described. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 55.13$, $b = 71.75$, $c = 126.26$ Å. The structure was determined at 1.6 Å resolution by the molecular-replacement method using the lipase from *G. stearothermophilus* L1 as a model.

1. Introduction

The combination of structural studies and protein engineering has resulted in the enhancement of our understanding of the relationship between the structure and activity of enzymes. Crystal structures of lipases originating from mammals, yeast, fungi and bacteria have been determined (Jaeger *et al.*, 1999). Most of these lipases show the presence of an α/β -hydrolase fold and have the active-site serine residue buried in a Gly-X-Ser-X-Gly pentapeptide motif. In the thermoalkalophilic lipases of family I.5, the first Gly is replaced by Ala, resulting in a new Ala-X-Ser-X-Gly motif (Jeong *et al.*, 2002). These lipases show optimal activity at 333–348 K and pH 8–10 and share about 95% amino-acid sequence identity. One of the prominent features of this family is its extraordinary activation mechanism, which allows enzyme activity under extreme conditions. The active conformation of thermoalkalophilic lipases is achieved by a complicated process that has been described for the BTL2 lipase from *Geobacillus thermocatenulatus*. The activation of the enzyme involves large structural rearrangements of around 70 amino acids and the concerted movement of two lids, the $\alpha 6$ and $\alpha 7$ helices, unmasking the active site (Carrasco-López *et al.*, 2009).

We have isolated the thermophilic *Geobacillus* strain SBS-4S and characterized a thermostable lipase (Tayyab *et al.*, 2011) from this microorganism. The lipase (LIP_{SBS}) is stable over a wide range of temperature (288–338 K) and pH (4–12) and has the ability to hydrolyze fatty-acyl esters of acids ranging in length from two to 18 C atoms. The availability of calcium ions enhanced the enzyme activity of LIP_{SBS}. In the present study, we report the crystallization and structure determination of this thermoalkalophilic lipase from *Geobacillus* SBS-4S.

2. Materials and methods

2.1. Protein preparation

The gene for lipase was cloned in pET-21a and expressed in *Escherichia coli* BL21 CodonPlus (DE3) cells. The insoluble form of recombinant LIP_{SBS} was denatured with 8 M urea and then refolded by fractional dialysis in 6, 4, 2 and 0 M urea in 50 mM Tris-HCl pH 8.0.

Purification of the refolded lipase was performed by ion-exchange chromatography (using Resource Q and HiTrap DEAE FF columns) and gel-filtration chromatography (using a Superdex 200 HR10/300 column). In the first case the columns were equilibrated with 20 mM Tris-HCl pH 8 with 0.01% Triton X-100 and were eluted with a

gradient to the same buffer containing 1 M NaCl. In the case of gel filtration the buffer used was 20 mM Tris–HCl pH 8 containing 150 mM NaCl. The fractions containing the monomeric form of the lipase, with a molecular weight of 43 kDa, were collected and the purity of the fractions was analyzed by SDS–PAGE. Freshly purified protein was used in each crystallization screening.

2.2. Crystallization and data collection

Purified monomeric protein was utilized in screening using Crystal Screen (Hampton Research) and Wizard (Emerald BioSystems) crystallization kits. In order to find the optimum conditions for the development of crystals, screening was performed at temperatures in the range 277–293 K using various concentrations of LIP_{SBS} in the range 5–10 mg ml^{−1}. For screening, the protein was mixed with buffers (containing precipitants) in a 1:1 ratio and incubated at various temperatures. The presence of crystals was examined using a Nikon SMZ1500 stereomicroscope (Excel Technologies Inc., USA). The crystals obtained were kept carefully to avoid damage.

The crystals were then mounted on a Cryo-Loop (Hampton Research), protected by soaking in the mother liquor with 18% ethylene glycol and flash-cooled in a stream of nitrogen gas at 100 K. The synchrotron-radiation experiments were performed on beamlines BL38B1 and BL44XU at SPring-8, Hyogo, Japan. Data were collected using a Quantum 210 CCD (ADSC) image-plate detector with an oscillation of 1° per image. The data sets were indexed, integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997).

2.3. Structure determination and refinement

The initial processed data were further analyzed using the *CCP4* package (Winn *et al.*, 2011). The structure of LIP_{SBS} was solved by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) from *CCP4* using the lipase from *G. stearothermophilus* L1 as a model (PDB entry 1ku0; Jeong *et al.*, 2002). An initial electron-density map was generated using *CNS* (Brünger *et al.*, 1998). Several cycles of structure refinement were performed using *REFMAC* (Murshudov *et al.*, 2011) and *Coot* (Emsley & Cowtan, 2004). The progress of the structural refinement was evaluated from the free *R* factor and using *PROCHECK* (Laskowski *et al.*, 1993). An inspection of the stereo-

Figure 1

Crystals of LIP_{SBS}. Protein in Tris–HCl pH 8.0 was incubated with 0.1 M imidazole pH 8.0, 10% polyethylene glycol 8000, 0.2 M calcium acetate in a 1:1 ratio.

Table 1

Data-collection and refinement statistics for LIP_{SBS}.

Values in parentheses are for the highest resolution shell.

Refinement statistics	
Unit-cell parameters (Å, °)	$a = 55.13$, $b = 71.75$, $c = 126.26$, $\alpha = \beta = \gamma = 90.0$
Space group	$P2_12_12_1$
Resolution limits (Å)	27.15–1.66 (1.70–1.66)
R_{work}	0.16 (0.25)
R_{free}	0.19 (0.27)
R.m.s.d., bond lengths (Å)	0.01
R.m.s.d., bond angles (°)	0.98
Data-collection statistics	
Resolution limits (Å)	50.00–1.66 (1.69–1.66)
No. of reflections	59613
R_{merge}	0.06 (0.47)
Completeness (%)	99.30
Multiplicity	4.90 (4.70)
$\langle I/\sigma(I) \rangle$	5.00

chemical parameters was performed using *RAMPAGE* (Lovell *et al.*, 2003). The refinement statistics are reported in Table 1 and the figures were prepared with the help of *PyMOL* (<http://www.pymol.org>).

3. Results and discussion

3.1. Crystallization of LIP_{SBS}

LIP_{SBS} was overexpressed in *E. coli* and purified according to a previously reported method (Tayyab *et al.*, 2011). Crystals were prepared using the sitting-drop vapour-diffusion method. LIP_{SBS} crystals with a good diffraction pattern were obtained when equal volumes of 7 mg ml^{−1} LIP_{SBS} and 0.1 M imidazole pH 8.0, 10% polyethylene glycol 8000, 0.2 M calcium acetate were mixed and incubated for 72 h at 293 K (Fig. 1). The dimensions of the crystal were 0.65 × 0.3 mm.

3.2. Structure determination and refinement

The structure was determined at 1.6 Å resolution. The LIP_{SBS} crystal belonged to the orthorhombic space group $P2_12_12_1$ (unit-cell parameters $a = 55.13$, $b = 71.75$, $c = 126.26$ Å). One Zn²⁺ ion, one Ca²⁺ ion and two Cl[−] ions were found in the structure of LIP_{SBS} (Fig. 2). The structure contained the α/β -hydrolase fold characteristic of family I.5 lipases. Each monomeric form of LIP_{SBS} contained a Zn²⁺ ion as part of the crystal structure even when Zn²⁺ ions were not present in the purification and crystallization buffers. Investigations indicated that the Trizma base (Sigma–Aldrich Life Sciences, USA) used in the preparation of the Tris–HCl buffer contained traces of Zn²⁺. This suggests that LIP_{SBS} has a high affinity for its cofactor.

The active-site residues of LIP_{SBS} were Ser114, Asp318 and His359, similar to those of the BTL2 lipase from *G. thermocatenulatus* (Carrasco-López *et al.*, 2009). The active-site serine was found in a conserved Ala-X-Ser-X-Gly pentapeptide motif, as reported previously (Jeong *et al.*, 2002). The amino-acid residues responsible for the stabilization of Zn²⁺ are Asp62, His82, His88 and Asp239 (Fig. 3), while Gly287, Glu361, Asp366 and Pro367 are involved in the binding of Ca²⁺ similar to as observed in BTL2 lipase from *G. thermocatenulatus* (Carrasco-López *et al.*, 2009). We did not observe any enzyme activity-enhancing effect of Zn²⁺ ions. The crystal structure also showed that the Zn²⁺ ion was distant from the catalytic serine residue. These results suggest that the Zn²⁺ ion does not participate in catalysis. Instead, it is a structural Zn²⁺ ion and plays a role in the enhanced thermal stability of LIP_{SBS}. In addition to the Zn²⁺ and Ca²⁺ ions, two Cl[−] ions could be observed in the crystals of LIP_{SBS} which may contribute to increased surface-charge

Figure 2

Cartoon diagram showing the crystal structure of LIP_{SBS} obtained by X-ray crystallography. Helices are shown as green spirals, β -sheets are shown as red arrows, loops are represented by smooth lines and Ca^{2+} , Zn^{2+} and Cl^- ions are shown as green, yellow and red spheres, respectively. Active-site residues are shown in stick representation. The amino- and carboxyl-termini are labelled N and C, respectively. The inset shows the active-site residues.

Figure 3

A cartoon representation of the metal-binding motif, showing the amino acids involved in the binding of Zn^{2+} (yellow sphere). The diagram was generated using PyMOL (<http://www.pymol.org>).

networks, thereby enhancing the thermal stability. These results pave the way for site-directed mutagenesis experiments to examine the role of the amino acids involved in interaction with these metal ions in the thermostability of the enzyme.

A large lid consisting of two consecutive helices together with a long loop (Fig. 2) covered the active-site residues (Ser114, Asp318 and His359). Helix I consisted of 16 amino acids (Phe177–Ala192), helix II consisted of ten amino acids (Phe222–Pro231) and the long loop consisted of 29 amino acids (Ala193–Ser221). The structure obtained from X-ray crystallographic data was found to be in a closed configuration, *i.e.* the active site was covered by the lid.

The addition of various concentrations of Triton X-100 (0–5%) and Ca^{2+} (0–1 mM) to the crystallization buffer was unsuccessful in producing crystals of LIP_{SBS} in the open conformation. Triton X-100

and Ca^{2+} were used because Triton X-100 was involved in the development of hydrophobic interactions with the lid and Ca^{2+} ions were involved in enhancement of the enzymatic activity of LIP_{SBS} (Tayyab *et al.*, 2011). Efforts are being made to obtain crystals of the active configuration.

The synchrotron-radiation experiments were performed on beamlines BL44XU and BL38B1 at SPring-8 with the approval of the Japan Synchrotron Radiation Research Institute (JASRI; Proposal Nos. 2009B6915 and 2010A1158).

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