Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction studies of the BTL2 lipase from the extremophilic microorganism *Bacillus thermocatenulatus*

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Received 21 July 2008
Accepted 3 October 2008

Bacillus thermocatenulatus lipase 2 (BTL2) is a thermoalkalophilic lipase that has been reported as an enantioselective biocatalyst for diverse reactions and that heads a group of enzymes that share high resistance towards many inactivation agents (heat, organic solvents, pH *etc.*). This makes BTL2 an important research target because of its potential industrial applications. BTL2 was cloned and overexpressed in *Escherichia coli*, purified and concentrated for crystallization using the sitting-drop vapour-diffusion method at 291 K. Crystals grew from a mixture of 13% MPD and 0.2 *M* ammonium acetate in 0.05 *M* sodium citrate pH 5.5–5.6. The crystals, which belonged to the orthorhombic space group *I*222 with unit-cell parameters a = 73.07, b = 129.08, c = 127.49 Å, allowed the collection of an X-ray data set to 2.2 Å resolution.

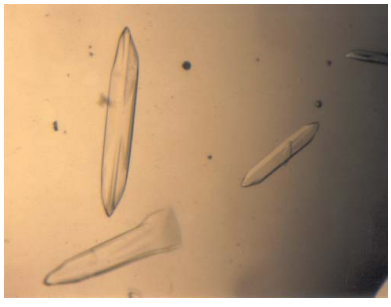
1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are the most common enzymes used in biocatalysis (Ghanem & Aboul-Enein, 2004, 2005). These proteins have an extraordinary catalytic activity and substrate specificity over a wide range of conditions, including high organic solvent concentrations, high temperatures (food chemistry) or both (*e.g.* biodiesel production; Ghanem & Aboul-Enein, 2005; Aravindan *et al.*, 2007).

Molecular stability is a critical requirement for the suitability of an enzyme as a biocatalyst in all biotechnological applications (Burton *et al.*, 2002; Ó'Fágáin, 2003). Enzymes from thermophilic microorganisms are especially resistant towards common inactivation agents (heat, organic solvents, pH *etc.*) owing to their natural structural stability and rigidity (Owusu & Cowan, 1989; Cowan, 1997), which allows them to play an important role in several industrial applications (Schmidt-Dannert *et al.*, 1997). Moreover, their natural properties (specificity, selectivity, activity and stability) can be artificially improved further *via* immobilization techniques, therefore amplifying the range of conditions under which these enzymes can be used (Mateo *et al.*, 2007).

The thermoalkalophilic bacterial lipases show a high amino-acid sequence identity (95%) and share significant homology (30–35%) to lipases that are involved in the pathogenic processes of some infectious Gram-positive organisms such as *Staphylococcus* strains. The fact that thermophilic and *Staphylococcus* lipases (of unknown structure) are grouped into the I.5 family (Rosenstein & Götz, 2000) and share significant homology opens new possible approaches for drug development (enzyme inhibitors) against *Staphylococcus* species based on the structure of BTL2.

The lipase BTL2 (43 kDa) from *Bacillus thermocatenulatus* is an enzyme that has high stability towards both organic solvents and



thermal conditions (Schmidt-Dannert *et al.*, 1994, 1997) and that has been reported (Palomo *et al.*, 2003) as an interesting enantioselective biocatalyst in diverse reactions, but its structure has not yet been determined. Here, we describe the initial results obtained for the crystallization of BTL2 and its X-ray diffraction data to 2.2 Å resolution.

2. Experimental

2.1. Expression and purification of BTL2

Escherichia coli BL21 (DE3) strain cells were transformed with the pT1BTL2 plasmid containing the *B. thermocatenulatus* gene that codes for the mature lipase BTL2, as described previously (Schmidt-Dannert *et al.*, 1996). Cells carrying the recombinant pT1BTL2 plasmid were grown at 303 K. Raising the temperature to 315 K for 20 h induced lipase overexpression.

The cells were collected by centrifugation, resuspended (10 ml per litre of culture) in buffer *A* (10 m*M* sodium phosphate pH 7.0, 3 m*M* benzamidine) and disrupted using a French press. The lysate was centrifuged at 5000*g* for 30 min at 277 K using a Sorvall centrifuge and the protein concentration was measured. The extract containing overexpressed BTL2 was diluted in buffer *A* to 5 mg ml^{-1} protein. Octyl-Sepharose was then added [1:10(*w*:*v*) using the batch method] to extract the protein and the mixture was gently stirred for 12 h at 298 K. The lipase adsorbed onto the octyl-Sepharose was eluted with 400 ml buffer *B* [12 m*M* sodium phosphate pH 7 and 0.125%(*v*/*v*) Triton X-100] and the supernatant (S1) was obtained by filtration. The BTL2 recovered in the S1 fraction was mixed with 25 g PEI–agarose and stirrred gently for 2 h at 298 K (in this step, the BTL2 was not adsorbed by the resin). The support (PEI–agarose) with the adsorbed contaminating proteins was then discarded and the supernatant (S2) with the remaining BTL2 activity (near to 100%) was recovered by filtration. 10 g SP-550 Toyopearl was then added to the S2 supernatant using the batch method and the suspension was filtrated; the new supernatant (S3) containing the BTL2 activity was collected. The supernatant (S3) temperature was decreased to 277 K and solid sodium bicarbonate was added to a final concentration of 12.5 m*M*. The pH was readjusted to 10.0 using NaOH. Q-Sepharose (10 g) was then added to this suspension also using the batch method and BTL2 was adsorbed onto this support. After a few minutes, the support (Q-Sepharose) was recovered by filtration and washed in a sintered glass funnel with distilled water containing 10 n*M* Triton X-100. The support was resuspended in 50 ml buffer *C* (500 m*M* sodium phosphate pH 7) to desorb the enzyme. The enzyme was concentrated 15-fold by centrifugation using an Amicon Ultra-15 membrane. Finally, the purified enzyme was dialyzed against double-distilled water.

2.2. Crystallization

High-throughput techniques with a NanoDrop robot (Innovadyne Technologies Inc.) were used to assay crystallization conditions using a few milligrams of pure BTL2 (5 mg ml^{-1} in double-distilled water) with Crystal Screens I, II and Lite, Index Screen and SaltRx from Hampton Research and PACT Suite and JCSG+ Suite from Qiagen. Initial assays were carried out by the sitting-drop vapour-diffusion method at 291 K on Innovaplate SD-2 microplates (Innovadyne Technologies Inc.), mixing 250 nl protein solution with 250 nl precipitant solution and equilibrating against 80 µl well solution. BTL2 microcrystals grew under a condition containing 15% MPD in 0.05 *M* sodium citrate pH 5.6 buffer with 0.1 *M* ammonium acetate as an additive. This initial condition was optimized using sitting drops by mixing 1 µl protein solution with 1 µl precipitant solution and equilibrating against 600 µl well solution. Good-quality crystals with a rice-grain shape were obtained using 0.05 *M* sodium citrate pH 5.6, 13% MPD and 0.2 *M* ammonium acetate. Crystals reached their maximum dimensions of 0.3 × 0.1 × 0.1 mm in 3 d (Fig. 1).

Table 1
Data-collection statistics for BTL2 crystals diffracting to 2.2 Å resolution.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	*I*222
Unit-cell parameters (Å)	
a	73.07
b	129.08
c	127.49
Data processing	
Temperature (K)	100
Wavelength (Å)	0.9792
Resolution (Å)	63.75–2.2 (2.36–2.2)
Unique data	37406
Redundancy	4.1 (4.5)
Data completeness (%)	99.8 (100)
Average $I/\sigma(I)$	8.9 (2.5)
Molecules per ASU	1
Matthews coefficient (Å^3 Da^{-1})	3.27
Solvent content (%)	62
$R_{\rm merge}$†	0.14 (0.48)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the weighted mean of all measurements.

2.3. X-ray data collection and processing

Prior to flash-cooling to 100 K using a cryogenic system, all crystals were soaked for 5 s in a cryoprotectant solution consisting of 20%(*v*/*v*) glycerol in the crystallization solution. A native data set was collected using synchrotron radiation at ESRF (Grenoble) on beamline BM16 using a ADSC reverse-φ detector and a wavelength of 0.9792 Å. Collected images were processed and scaled using *MOSFLM* (Leslie, 1992) and *SCALA*, respectively (Collaborative Computational Project, Number 4, 1994).

3. Results

An X-ray data set was collected to 2.2 Å resolution from a single BTL2 crystal and displayed apparently weak but good-quality diffraction patterns (Fig. 2). Despite some pattern weakness, preliminary X-ray data processing showed good processing statistics

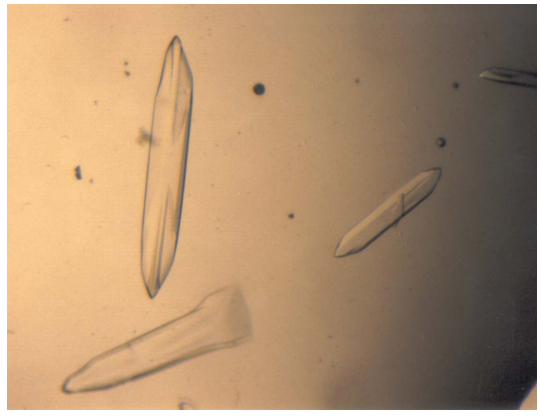


Figure 1
BTL2 crystals obtained using 0.05 *M* sodium citrate pH 5.6, 13% MPD and 0.2 *M* ammonium acetate. The approximate dimensions of the crystals are 0.3 × 0.1 × 0.1 mm.

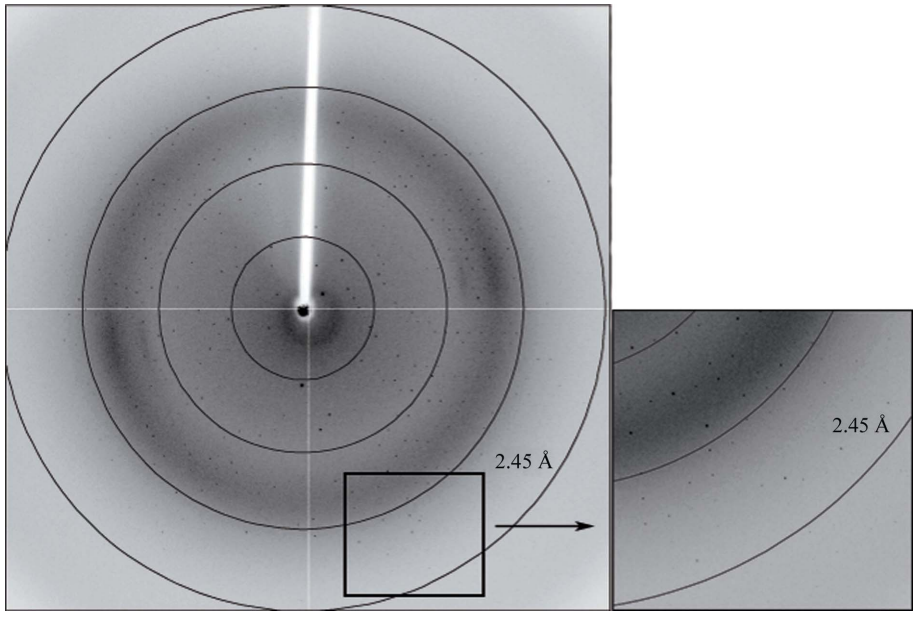


Figure 2
(*a*) X-ray diffraction pattern of BTL2 crystals; (*b*) enlargement showing resolution rings.

(Table 1). The crystal belonged to the orthorhombic space group *I*222, with unit-cell parameters a = 73.07, b = 129.08, c = 127.49 Å. Specific volume calculations based on the molecular weight of BTL2 and the unit-cell parameters indicated the presence of one monomer molecule in the asymmetric unit, with 63% solvent content (V_M = 3.27 Å^3 Da^{-1}; Matthews, 1968).

Structure determination was initiated using the lipase from *B. stearothermophilus* (PDB code 1ji3), which shows 95% sequence identity, as a structural model. Molecular replacement was performed with the *MOLREP* program (Vagin & Teplyakov, 1997) using reflections to 3.5 Å resolution. A single and unambiguous solution for the rotation and translation functions was obtained, which yielded a final correlation coefficient of 0.45 and an R factor of 0.46. The space group was confirmed to be *I*222, with one protein monomer in the asymmetric unit. Structural refinement of the BTL2 model is currently in progress.

CC-L is a fellow of the Fundayacucho Fundation (Venezuela). This work was supported by grant BFU2005-01645 from Dirección General de Investigación. This is a product of the Project 'Factoría Española de Cristalización' Ingenio/Consolider 2010.

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