Crystallization and preliminary X-ray diffraction studies of the BTL2 lipase from the extremophilic microorganism *Bacillus thermocatenulatus*César Carrasco-López,^a Cesar Godoy,^b Blanca de las Rivas,^b Gloria Fernández-Lorente,^c José M. Palomo,^b José M. Guisán,^b Roberto Fernández-Lafuente,^b Martín Martínez-Ripoll^a and Juan A. Hermoso^{a*}^aGrupo de Cristalografía Macromolecular y Biología Estructural, Instituto de Química-Física Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain, ^bDepartamento de Biocatálisis, Instituto de Catálisis, CSIC Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain, and ^cDepartamento de Microbiología, Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain

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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

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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 mM sodium phosphate pH 7.0, 3 mM benzimidazole) and disrupted using a French press. The lysate was centrifuged at 5000g 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 mM 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 stirred 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 filtered; 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 mM. 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 mM Triton X-100. The support was resuspended in 50 ml buffer C (500 mM 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

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>I</i> 222
Unit-cell parameters (Å)	
<i>a</i>	73.07
<i>b</i>	129.08
<i>c</i>	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>I</i> /σ(<i>I</i>)	8.9 (2.5)
Molecules per ASU	1
Matthews coefficient (Å ³ Da ⁻¹)	3.27
Solvent content (%)	62
<i>R</i> _{merge} [†]	0.14 (0.48)

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted mean of all measurements.

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).

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 ($V_M = 3.27$ Å³ 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.

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References

- Aravindan, R., Anbumathi, P. & Viruthagiri, T. (2007). *Ind. J. Biotechnol.* **6**, 141–158.
- Burton, S. G., Cowan, D. A. & Woodley, J. M. (2002). *Nature Biotechnol.* **20**, 37–45.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
- Cowan, D. A. (1997). *Comp. Biochem. Physiol.* **118**, 429–438.
- Ghanem, A. & Aboul-Enein, H. Y. (2004). *Tetrahedron Asymmetry*, **15**, 3331–3351.
- Ghanem, A. & Aboul-Enein, H. Y. (2005). *Chirality*, **17**, 1–15.
- Leslie, A. G. W. (1992). *Crystallographic Computing 5: From Chemistry to Biology*, edited by D. Moras, A. D. Podjarny & J. C. Thierry, pp. 39–50. Oxford University Press.
- Mateo, C., Palomo, J. M., Fernández-Lorente, G., Guisan, J. M. & Fernández-Lafuente, R. (2007). *Enzyme Microb. Technol.* **40**, 1451–1463.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Ó'Fágáin, C. (2003). *Enzyme Microb. Technol.* **33**, 137–149.
- Owusu, R. K. & Cowan, D. A. (1989). *Enzyme Microb. Technol.* **11**, 568–574.
- Palomo, J. M., Fernández-Lorente, G., Rúa, M. L., Guisán, J. M. & Fernández-Lafuente, R. (2003). *Tetrahedron Asymmetry*, **14**, 3679–3687.
- Rosenstein, R. & Götz, F. (2000). *Biochimie*, **82**, 1005–1014.
- Schmidt-Dannert, C., Rúa, M. L., Atomi, H. & Schmid, R. D. (1996). *Biochim. Biophys. Acta*, **1301**, 105–114.
- Schmidt-Dannert, C., Rúa, M. L. & Schmid, R. D. (1997). *Methods Enzymol.* **284**, 194–220.
- Schmidt-Dannert, C., Sztajer, H., Stocklein, W., Menge, U. & Schmid, R. D. (1994). *Biochim. Biophys. Acta*, **1214**, 43–53.
- Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.