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Crystallization and preliminary X-ray diffraction studies of a complex of extracellular lipase from *Streptomyces rimosus* with the inhibitor 3,4-dichloroisocoumarin

Ivana Leščić Ašler,[a] Jasenka Pigac,[b] Dušica Vujaklija,[b] Marija Luić[a] and Zoran Štefanić[a]*

[a]Department of Physical Chemistry, Rudjer Bošković Institute, Bijenička cesta 54, 10002 Zagreb, Croatia, and [b]Division of Molecular Biology, Rudjer Bošković Institute, Bijenička cesta 54, 10002 Zagreb, Croatia

Correspondence e-mail: zoran.stefanic@irb.hr

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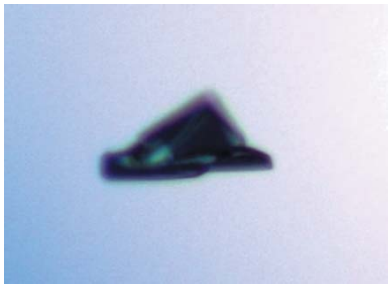
A recombinant lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) from the bacterium *Streptomyces rimosus* was inhibited by the serine protease inhibitor 3,4-dichloroisocoumarin and crystallized by the hanging-drop vapour-diffusion method at 291 K. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 38.1, b = 78.7, c = 56.6 Å, β = 104.5° and probably two molecules in the asymmetric unit. Diffraction data were collected to 1.7 Å resolution using synchrotron radiation on the XRD beamline of the Elettra synchrotron, Trieste, Italy.

1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are enzymes that are involved in the catabolism of fats and oils in living organisms. In their natural environment they hydrolyze the fatty-acid ester bonds of acylglycerols, but many of them are stable in organic solvents and are capable of accepting a wide range of substrates and catalyzing nonphysiological reactions. Their property of high enantioselectivity towards chemically and pharmaceutically important compounds, in addition to their being environmentally friendly, makes them, together with amylases and proteases, the most used enzymes in various branches of industry (Hasan *et al.*, 2006). Lipases derived from microorganisms are especially important for industrial use owing to their interesting catalytic characteristics, high stability and convenient production (Hasan *et al.*, 2006).

Upton & Buckley (1995) recognized a new family of lipolytic enzymes, which they originally named GDS(L) lipolytic enzymes after the conserved motif containing the catalytic serine. A new name, SGNH hydrolases, was proposed after the four amino acids that are conserved in the active site (Mølgaard *et al.*, 2000). A comprehensive review of SGNH hydrolases (Akoh *et al.*, 2004) describes a wide range of hydrolytic functions for these enzymes. Thus, this particular group comprises lipases, proteases, thioesterases, arylesterases, lysophospholipases, carbohydrate esterases and acyltransferases. Some of the SGNH hydrolases display high flexibility in the active site which enables them to accept a wide range of different types of substrates (*e.g.* protease I/thioesterase I/lysophospholipase L1 from *Escherichia coli*; Lee *et al.*, 1997), but it is as yet unclear whether this property of promiscuity is common to all enzymes of this family. Some of the biological functions of SGNH hydrolases include participation in bacterial virulence, plant development and morphogenesis, and they are also involved in plant defence mechanisms (Akoh *et al.*, 2004). As of May 2011, 45 structures of 22 different SGNH hydrolases could be found in the Protein Data Bank and there were 12 644 protein sequences in the InterPro database (mainly hypothetical proteins from genome-sequencing projects), but the family remains poorly investigated, particularly with respect to detailed catalytic characterization.

We have recently discovered that the extracellular lipase from *Streptomyces rimosus* exhibits substrate and catalytic promiscuity (Leščić Ašler *et al.*, 2010), which would make this SGNH hydrolase of great interest for industrial applications. As it is known that



transition-state analogues and inhibitors in general can serve as extremely useful chemical probes to understand the structural and mechanistic basis of enzyme catalysis (Hiratake, 2005), we investigated the interactions of this enzyme with several inhibitors. Of the compounds that we tested, the general mechanism-based serine protease inhibitor 3,4-dichloroisocoumarin (Harper *et al.*, 1985) was shown to be the most effective and covalently bound to the active-site serine (Zehl *et al.*, 2004).

In this report, we describe the crystallization and preliminary crystallographic data of *S. rimosus* lipase complexed with 3,4-dichloroisocoumarin (DCI).

2. Experimental

2.1. Expression and purification

The overexpression of *S. rimosus* lipase was rather problematic. In heterologous overexpression (in *S. lividans*) the protein was not processed properly, while homologous overexpression resulted in small yields of the protein. Moreover, lipases are very hydrophobic and tend to stick to plastic surfaces, leading to great losses of enzyme during concentration. Therefore, only limited amounts of protein were available. Protein concentration was probed extensively in the initial crystallization trials. When concentrated above 4 mg ml^{-1} the protein tended to aggregate and precipitate, even in the presence of stabilizers such as detergents (*e.g.* β-octyl glucoside). Overexpressed lipase was purified from the culture filtrate of an *S. rimosus* R6-ZGL3(pDJ7) strain that contained a high-copy-number plasmid harbouring the *S. rimosus* lipase gene (Vujaklija *et al.*, 2002). The strain was cultivated in Gr_2d medium (40 g dextrin, 16 g corn extract, 7.0 g $CaCO_3$, 2.0 g ammonium sulfate and 1.4 ml lactic acid per litre) at 303 K in 2 l Erlenmeyer flasks containing 400 ml medium on a rotary shaker at 250 rev min^{-1}. After 3 d of growth the biomass was removed by centrifugation for 30 min at 5800*g*.

Purification started with ion-exchange chromatography, applying 800 ml culture filtrate (550 mg total protein content) to carboxymethyl cellulose (Amersham Biosciences) equilibrated in 10 m*M* sodium phosphate buffer pH 6.0 with 10 m*M* EDTA (buffer 1). The lipase was eluted with 1 *M* NaCl in buffer 1 and active fractions were concentrated in an Amicon ultrafiltration cell through a YM-10 membrane and then transferred to buffer 1 by gel filtration on a Sephadex G-25 column (Amersham Biosciences). Subsequent re-chromatography on the carboxymethyl cellulose column under the same conditions with a shallow NaCl gradient (0–0.35 *M*) elution was the next purification step. Fractions containing lipase activity were desalted using a Sephadex G-25 column into 20 m*M* sodium phosphate buffer pH 7.0 (buffer 2) and were concentrated in an Amicon ultrafiltration cell. Next, the lipase sample was applied onto a Mono Q column for fast protein liquid chromatography (FPLC; Amersham Biosciences). Elution was performed with an NaCl gradient (1 m*M*–0.5 *M* in buffer 2). Active fractions were concentrated in an Amicon ultrafiltration cell and desalted by gel filtration on a PD-10 column into 20 m*M* sodium phosphate buffer pH 6.0 (buffer 3). The final purification step was FPLC on a Mono S column (Amersham Biosciences), from which the lipase was eluted in an NaCl gradient (1 m*M*–1 *M* in buffer 3) at approximately 0.08 *M* NaCl. The purification was monitored by an activity assay with *p*NPP as a lipase substrate (Abramić *et al.*, 1999) and a protein concentration assay following the method of Bradford (1976). The purification yield was 22% and the purification factor was 3.5. The enzyme specific activity after the purification procedure was determined to be 400 µmol min^{-1} mg^{-1} and its purity was verified by SDS–PAGE.

2.2. Crystallization

1 ml purified lipase sample (in buffer 3 with 0.08 m*M* NaCl) was diluted to 0.1 mg ml^{-1} with 2.8 ml buffer 3 and incubated for 45 min at room temperature with 0.2 ml 50 m*M* 3,4-dichloroisocoumarin (DCI) solution in DMSO (the final DMSO concentration was 5% and the molar excess of the inhibitor over the enzyme was 600×). The sample was centrifuged for 5 min at 15 000*g* and was subsequently concentrated to 2.7 mg ml^{-1} in a Vivaspin2 (molecular-weight cutoff 3000) concentrator.

Initial crystallization trials were carried out with Crystal Screen and Crystal Screen 2 (Hampton Research) using vapour diffusion in a hanging-drop format in VDX plates (Hampton Research) at a constant temperature of 291 K. The drops consisted of 2 µl protein solution mixed with 2 µl well solution; the well volume was 700 µl. Tiny needle-shaped crystals appeared in many drops containing various conditions (different pH, salts and precipitants; the protein concentration was 1–8 mg ml^{-1}). The best-looking needles were grown from 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6 and 25%(*w*/*v*) polyethylene glycol 4K. To optimize the crystallization conditions, the concentrations of the components of the solution were varied and this procedure yielded a few diffraction-quality crystals. The best crystals were grown in 0.16 *M* ammonium sulfate, 0.08 *M* sodium acetate pH 4.6, 20%(*w*/*v*) polyethylene glycol 4K, 20%(*v*/*v*) glycerol using a protein concentration of 3 mg ml^{-1}. These crystals diffracted to approximately 3.5 Å resolution on a rotating-anode instrument.

Further screening was performed using a Phoenix dispenser (Art Robbins Instruments). 100 nl protein droplets were mixed with 100 nl reservoir solution and set up as sitting drops against 200 µl reservoir solution using the following 96-well plate screens: Classics, pH I, pH II, PEGs, PEGs II, $AmSO_4$ and JCSG (Qiagen). One crystal appeared and grew to approximate dimensions of 0.05 × 0.1 × 0.1 mm in condition No. 12 of the PEGs Suite (Qiagen) consisting of 0.1 *M* MES pH 6.5, 25% PEG 2000 MME (Fig. 1). This crystal was used for data collection on the XRD beamline of the Elettra synchrotron, Trieste, Italy.

2.3. Data collection and processing

The crystal was flash-cooled prior to data collection. A 98.4% complete data set was collected to a resolution of 1.7 Å at 100 K using

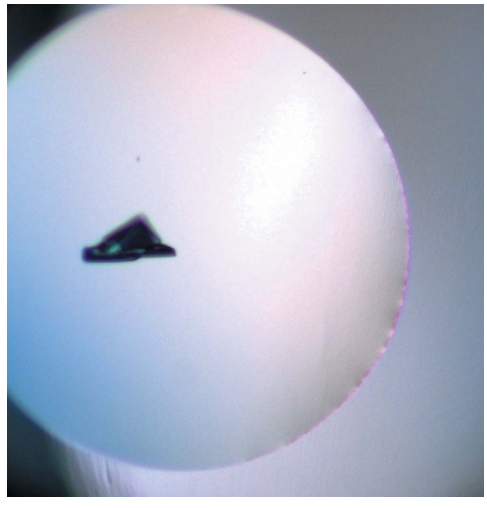


Figure 1
Crystal of the *S. rimosus* lipase complexed with the inhibitor DCI grown in a 96-well crystallization plate. The drop is approximately 1 mm in diameter.

synchrotron radiation (0.97 Å wavelength) and a 165 mm MAR CCD detector on Elettra XRD1 beamline 5.2 (Trieste, Italy). A total of 190 images of 1° rotation were collected (Fig. 2). The protein crystallized in the monoclinic space group $P2_1$, with unit-cell parameters $a = 38.1$, $b = 78.7$, $c = 56.6$ Å, $\beta = 104.5°$. The reflections were indexed using the *iMOSFLM* program (Battye *et al.*, 2011) and scaled with *SCALA* (Evans, 2006). The *CTRUNCATE* program (Padilla & Yeates, 2003) was used to convert intensities to structure factors. The diffraction data statistics are presented in Table 1.

3. Results and discussion

The starting model for the molecular-replacement procedure was built using the *Phyre* server (http://www.imperial.ac.uk/phyre/; Kelley & Sternberg, 2009). The *Phyre* server constructs a profile and a secondary-structure prediction for the query sequence and scans them against the fold library (which contains profiles and secondary structures of proteins with experimentally solved three-dimensional structures) using a profile–profile alignment algorithm. Full three-dimensional models of the query are constructed from the ten highest scoring alignments. For the *S. rimosus* lipase, the best alignment was obtained with an esterase from *Streptomyces scabies* (PDB entry 1esc; Wei *et al.*, 1995), a 306-amino-acid protein which shares only 51/234 (22%) identical amino acids.

The best model obtained using the *Phyre* server was used as a model for molecular-replacement trials. Matthews coefficient analysis (Kantardjieff & Rupp, 2003) gave ambiguous results, predicting 63.8 or 27.7% solvent content with probabilities of 68 or 32% (at a resolution of 1.7 Å) depending on whether one or two protomers, respectively, were found in the asymmetric unit. The Patterson map showed a large off-origin peak at (0.5, 0.0, 0.5) with an intensity of 57% of the origin peak, indicating high pseudo-translational symmetry in the structure along the diagonal of the *ac* plane. In our opinion, the only way that this can occur for a monomeric protein is in the case of two molecules in the asymmetric unit connected by an approximate $\frac{1}{2}a + \frac{1}{2}c$ translational vector. Therefore, we searched for two molecules in the asymmetric unit despite the very dense packing and very low solvent content. Molecular replacement was attempted with the following programs: *MOLREP* (Vagin & Teplyakov, 2010), *Phaser* (McCoy *et al.*, 2007), *EPMR* (Kissinger *et al.*, 2001) and *BALBES* (Long *et al.*, 2008). The solutions given by *EPMR* using various parameters were the most consistent and led to plausible packing in the unit cell. Also, the calculated structure factors from the *EPMR* solutions reproduced the correct pseudo-symmetry very well, giving the correct off-origin peak in the Patterson map. Despite our best efforts, however, the solutions obtained did not produce maps of interpretable quality that could lead to a traceable model that could subsequently be refined.

Our results are in accordance with the hypothesis of Rost (1999) that at sequence identities in the range 20–35% models are in the so-called 'twilight zone' in which the standard molecular-replacement protocols often fail.

Table 1
Crystallographic data for *S. rimosus* lipase.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 38.09$, $b = 78.69$, $c = 56.56$, $\beta = 104.49$
Resolution (Å)	24.3–1.71 (1.80–1.71)
Rotation per exposure (°)	1.0
Temperature (K)	100
Measured reflections	131164
Independent reflections	34875
Mosaicity (°)	1.46
Completeness (%)	98.4 (89.5)
Multiplicity	3.7 (3.0)
Mean $I/\sigma(I)$	9.9 (2.9)
R_{meas} (%)	7.1 (39.1)

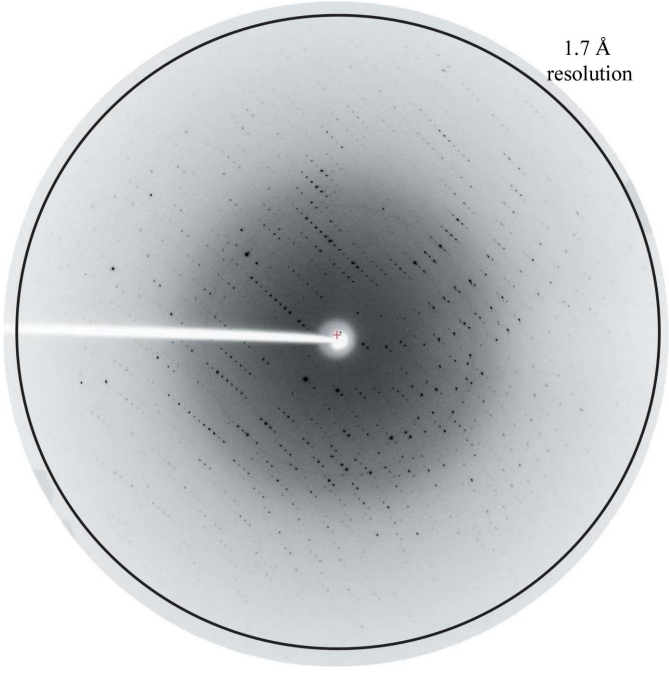


Figure 2
A diffraction photograph of the crystal obtained using a 165 mm MAR CCD detector on Elettra XRD1 beamline 5.2 (Trieste, Italy).

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