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Expression, purification, crystallization and preliminary crystallographic analysis of a thermostable lipase from *Bacillus stearothermophilus* P1

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The gene encoding a thermostable lipase secreted by *Bacillus stearothermophilus* P1 has been cloned and overexpressed in *Escherichia coli*. The recombinant lipase was purified to homogeneity using ammonium sulfate precipitation, anion-exchange chromatography (Poros 20 HQ) and Sephacryl S-200HR. The molecular mass was shown to be 43 209 Da by mass spectrometry. Crystals suitable for X-ray diffraction analysis were obtained by the hanging-drop method of vapour diffusion with ammonium sulfate as the precipitating agent. Determination of the structure by molecular replacement with existing mesophilic lipase structures has proved unrewarding, as there is less than 20% sequence identity with known lipase structures, but preliminary results with heavy-atom soaking indicate that this strategy will allow the structure to be solved. The availability of this new lipase structure will be of particular significance because it will be the first thermostable lipase to be described.

1. Introduction

Lipases (triacylglycerol acylhydrolases; E.C. 3.1.1.3) are found in various organisms, including animals, plants, fungi and bacteria. They catalyze the hydrolysis and transesterification of triacylglycerols, although some will degrade a fairly broad range of compounds containing an ester linkage. Microbial lipases are of considerable interest for biotechnological applications such as detergents, oleochemistry, cheese production, pharmaceuticals and the industrial synthesis of fine chemicals (reviewed by Jaeger & Reetz, 1998). In particular, thermostable lipases isolated from thermophilic bacteria are playing an increasingly important role in industrial processes because they exhibit relatively high thermodynamic stability both at elevated temperatures and in organic solvents (Omar *et al.*, 1987; Iizumi *et al.*, 1990; Sugihara *et al.*, 1992; Schmidt-Dannert *et al.*, 1996, 1997; Kim *et al.*, 1998).

Numerous lipase structures have been investigated by X-ray crystallography, including mammalian pancreatic lipase (Lombardo *et al.*, 1989; Winkler *et al.*, 1990; Moreau *et al.*, 1992), fungal lipases (Brady *et al.*, 1990; Brzozowski *et al.*, 1992; Schrag & Cygler, 1993; Grochulski *et al.*, 1993; Derewenda *et al.*, 1994; Uppenberg *et al.*, 1994; Lewis *et al.*, 1997) and mesophilic bacterial lipases (see review by Arpigny & Jaeger, 1999). A characteristic feature of these enzymes is the presence of a catalytic triad consisting of Ser-His-Asp/Glu.

We now report the overexpression, purification and preliminary crystallographic analysis of a thermostable lipase from *B. stearothermophilus* P1.

2. Methods and results

2.1. Cloning and expression

Chromosomal DNA from *B. stearothermophilus* P1 (isolated from a hot spring in Chiang Mai, Thailand) was partially digested with *Nco*I and *Hin*dIII, and the fragments were inserted into the same restriction sites of plasmid pQE-60 (Qiagen). After ligation, the recombinant plasmids were transformed into *E. coli* M15[pREP4] (Qiagen) and screening was performed by plating on LB agar containing 100 μg ml^{-1} ampicillin, 25 μg ml^{-1} kanamycin and 1%(*w*/*v*) tricaprylin. Colonies surrounded by a clear zone were chosen and grown in LB medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin.

The recombinant plasmid DNA was isolated and amplified by PCR and shown to contain a 4.5 kbp insert by agarose gel electrophoresis. Both strands of the lipase gene were sequenced automatically. The nucleotide sequence of the gene, submitted to GenBank under accession number AF237623, revealed an open reading frame of 1254 bp encoding a 417 amino-acid polypeptide (Sinchaikul *et al.*, 2001). The sequence was found to be 92% identical to lipases from the thermophilic bacteria *B. thermocatenulatus* and *B. stearothermo-*

philus L1 (Schmidt-Dannert *et al.*, 1996; Kim *et al.*, 1998), but only 15–17% identical to mesophilic bacterial lipases for which crystal structures are available (*Burkholderia glumae*, *Chymobacterium viscosum*, *Burkholderia cepacia* and *Pseudomonas aeruginosa*; Noble *et al.*, 1993; Lang *et al.*, 1996; Kim *et al.*, 1997; Schrag *et al.*, 1997; Nordini *et al.*, 2000).

Expression of the lipase enzyme was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture grown at 310 K had attained late log phase (OD_{600} = 0.6). The expression was optimized by testing various IPTG concentrations (0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 5 m*M*) and various induction times (1, 2, 3, 4 and 5 h). Expression levels were measured by SDS–PAGE (total cells lysed by SDS–PAGE sample buffer) and by assay of lipase activity in cell lysates (see below). The best expression was achieved with 0.4 m*M* IPTG for 3 h at 310 K.

2.2. Purification of recombinant lipase

Cells were collected from 4 l of culture by centrifugation at 6500*g* for 30 min and were suspended in 50 ml 20 m*M* Tris–HCl buffer pH 8.5 containing 10 m*M* EDTA, 5 m*M* 3,4-dichloroisocoumarin, 1 m*M* E64 and 100 m*M* 1,10-phenanthroline. After sonication, the cell lysate was centrifuged at 12 000*g* for 30 min and the precipitate was discarded. Streptomycin sulfate was added to the supernatant to a final concentration of 1%(*w*/*v*) and the precipitate was removed by centrifugation at 12 000*g* for 15 min. Protein precipitated between 20% saturated and 40% saturated ammonium sulfate was collected by centrifugation at 12 000*g* for 30 min and suspended in 20 m*M* Tris–HCl buffer pH 8.5. The protein was concentrated, desalted and solvent exchanged into the same Tris buffer with a Vivaspin 20 ml centrifugal concentrator (Vivasciences) with a molecular-weight cutoff of 10 000 Da.

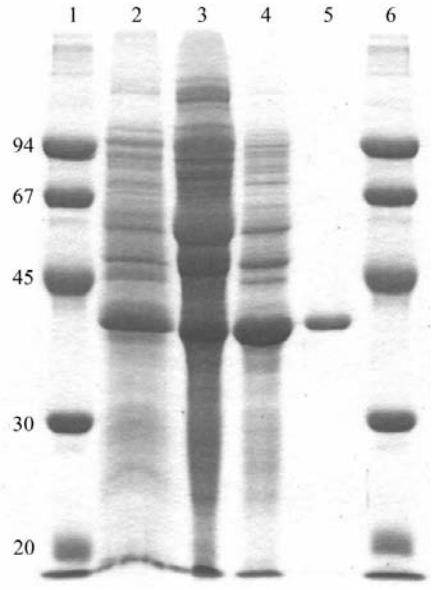

Figure 1
SDS–PAGE analysis of lipase fractions. A 12.5% gel was used and marker proteins (lanes 1 and 6) were from a low-molecular-weight calibration kit (Amersham Pharmacia Biotech) containing phosphorylase b (94 kDa), serum bovine albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). Lane 2, crude cell extract; lane 3, 20–40% saturated ammonium sulfate precipitate; lane 4, Poros HQ pool; lane 5, Sephacryl pool.

Table 1
Purification of thermostable lipase from *B. stearothermophilus* P1.

Step	Volume (ml)	Total activity (10^3 U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	11.8	40.6	611	66.5	1.00	100
$(NH_4)_2SO_4$ precipitation	5.5	30.3	132	230	3.45	74.6
HQ column	14.0	12.2	14.6	836	12.6	30.0
Sephacryl S-200HR	8.7	7.8	3.0	2600	39.1	19.2

The lipase was then purified by anion-exchange perfusion chromatography on a Poros 20 HQ column (4.6 × 100 mm) and gel filtration on Sephacryl S-200 (1.6 × 100 cm). The Poros column was eluted with 20 m*M* Tris–HCl buffer pH 8.5 containing a linear gradient of 0.15–0.6 *M* NaCl at a flow rate of 10 ml min^{-1} using a BioCAD workstation (Applied Biosystems). The largest peak contained high lipase activity and the corresponding fractions were pooled and concentrated with a Vivaspin tube prior to loading onto the Sephacryl column and eluting with the same Tris buffer. The fractions with high lipase activity were pooled and characterized by SDS–PAGE (Fig. 1) and specific activity (Table 1). The purified lipase showed a single band by SDS–PAGE and was purified 39-fold over the crude extract, with a yield of 19%. The molecular mass of the purified lipase was shown to be 43 209 Da by mass spectrometry (LCQTM, Finnigan).

2.3. Lipase assay

Lipase activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl caprate (Lesuisse *et al.*, 1993). Lipase solution (20 µl) was added to 880 µl reaction buffer containing 20 m*M* Tris–HCl buffer pH 8.5, 0.1%(*w*/*v*) gum arabic and 0.2%(*w*/*v*) sodium deoxycholate. The reaction mixture was prewarmed at 328 K and then mixed with 100 µl 8 m*M* freshly prepared *p*-nitrophenyl caprate (Sigma) solubilized in 2-propanol. The mixture was incubated at 328 K for 2 min and the reaction stopped by addition of 0.5 ml of 3 *M* HCl. After centrifugation, 333 µl of supernatant was mixed with 1 ml of 2 *M* NaOH and the A_{405} was measured against an enzyme-free control. One enzyme unit is defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol per minute. The protein concentration was measured spectrophotometrically at 280 nm or by using a dye-binding assay (Sedmak & Grossberg, 1977) with bovine serum albumin (Sigma) as a standard.

2.4. Crystallization and data collection

The hanging-drop vapour-diffusion method was used to grow crystals in 24-well Linbro plates. Crystals were obtained from preliminary ammonium sulfate screens in a wide range of concentrations over a range of pH values. In optimized conditions, the well solution consisted of 20% saturated ammonium sulfate in 0.1 *M* HEPES buffer pH 6.8–7.0. The hanging drop (4 µl) contained 2 µl of protein solution at 15 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 8.5 plus 2 µl of well solution.

Monoclinic crystals with dimensions up to 0.15 × 0.15 × 0.1 mm grew within 24 h at 289 K (Fig. 2). The space group is *C*2, with unit-cell parameters a = 118.5, b = 81.23, c = 99.78 Å, β = 96.33°. This gives V_M = 2.76 Å^3 Da^{-1} (55% solvent content) for two molecules in the asymmetric unit. Heavy-atom derivatives were prepared by

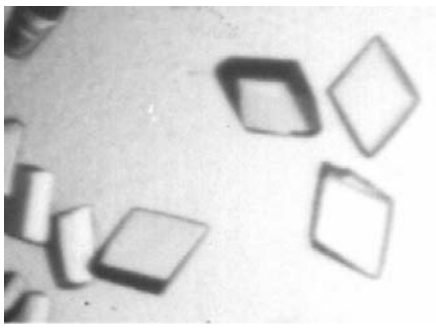

Figure 2
Crystals of *B. stearothermophilus* P1 lipase. See text for details.

soaking crystals in a solution consisting of 10 m*M* heavy-atom compound, 22% ammonium sulfate, HEPES buffer and 25% glycerol. After approximately 30 min, the crystals were flash-frozen in liquid nitrogen. Data were collected at the Daresbury SRS (station 14.1, $\lambda = 1.488$ Å) with an ADSC Q4 CCD detector using a φ scan with a step size of 1.50. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) (Table 2).

Table 2
Data-collection details and statistics.

Values in parentheses are for the highest resolution shell.

Derivative (conditions)	Native	K_2PtCl_4	$HgCl_2$
Resolution (Å)	2.2	2.4	2.5
No. of observations	211130	214389	79129
No. of independent reflections	47208	35676	28038
R_{merge}† (%)	10.6 (29.8)	6.3 (12.7)	7.3 (17.5)
Completeness (%)	99.2 (91.9)	96.8 (95.5)	87.4 (76.7)
$I/\sigma(I)$	11.2 (4.2)	19.2 (13.6)	11.2 (4.6)

† $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$.

3. Discussion

Lipases are a generally well characterized group of enzymes, with crystal structures available for enzymes from mammals, fungi and mesophilic bacteria. However, there is as yet no detailed structural information for a thermostable lipase, although this would be of considerable interest as the basis for tailoring the enzyme for a range of biotechnological applications. We have thus overexpressed, purified and crystallized a thermostable lipase from *B. stearothermophilus* P1.

The crystals were shown to diffract well and an attempt was made to find a solution through molecular replacement. The structure of a lipase from *Burkholderia cepacia* (PDB code 4lip; Lang *et al.*, 1998) with a sequence identity of 17% was used as a search model. Fig. 3 shows a *CLUSTALW* sequence alignment (Thompson *et al.*, 1994) of the two proteins with the corrresponding secondary structure of the Burkholderia lipase and the predicted secondary structure (PSIPRED; http://insulin.brunel.ac.uk/psipred/) of the enzyme from *B. stearothermophilus*. Of note is the relatively good agreement between the predicted and known secondary structures, despite the low sequence identity. *MOLREP* (Vagin & Teplyakov, 1997; Vaguine *et al.*, 1999) and *AMoRe* (Navaza, 1994) were used unsuccessfully to try to find the molecular-replacement solution, with search models corresponding to the apo enzyme or to a truncated model omitting the obvious differences in loop structures. Preliminary MIR (multiple isomorphous replacement) results using two heavy-atom derivatives suggest that this strategy will allow the structure to be solved.

The sequence of the lipase studied here is very similar indeed to the lipases isolated from the thermophilic bacteria *B. thermocatenulatus* (Schmidt-Dannert *et al.*, 1996) and *B. stearothermophilus* L1 (Kim *et al.*, 1998). The availability of the detailed structure of the *B. stearothermophilus* P1 lipase will thus enable work on another family of thermostable enzymes to be placed in a structural context.

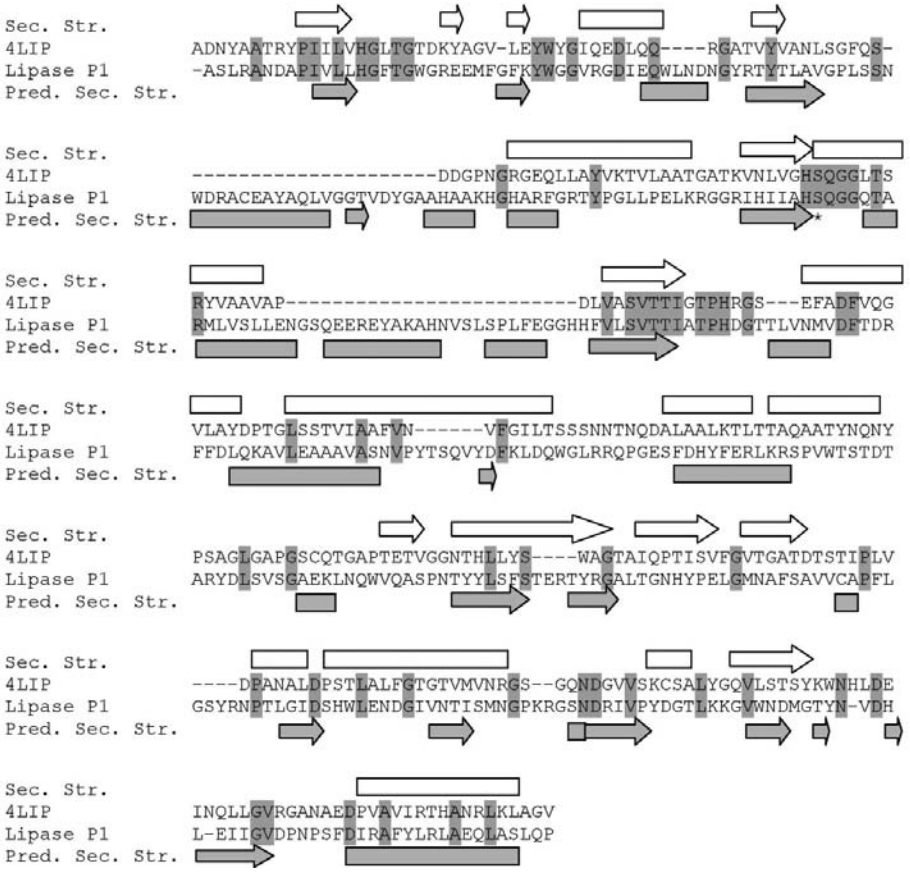

Figure 3
Comparison of lipases from *B. stearothermophilus* P1 and *Burkhardia cepacia*. A *CLUSTALW* alignment of the two sequences (designated Lipase P1 and 4LIP, respectively) is shown flanked by the known secondary structure (Sec. Str.) of the *Burkhardia* enzyme (taken from the PDB entry) and the predicted secondary structure (Pred. Sec. Str.) of the *B. stearothermophilus* enzyme. Arrows indicate β-strands and bars show α-helices. The shaded residues are identical in both sequences. The catalytic serine is denoted by an asterisk.

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