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# Crystallization and preliminary X-ray analysis of a thermoalkalophilic lipase from *Bacillus stearothermophilus* L1

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A thermoalkalophilic lipase from *Bacillus stearothermophilus* L1 (L1 lipase) was crystallized in two different crystal forms using a low concentration of the enzyme and a calcium-exchange process. The first, needle-like, crystal form, which diffracts to about 3.5 Å, belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 67.84, $b$ = 72.96, $c$ = 104.41 Å. The second, monoclinic, crystal form, which behaves better than the first form for crystallographic analyses, belongs to the monoclinic space group $C2$ and has unit-cell parameters $a$ = 119.62, $b$ = 85.05, $c$ = 98.36 Å, $\beta$ = 99.73°. From the monoclinic crystals, a native data set and a samarium-derivative data set were collected to 2.0 and 2.3 Å resolution, respectively. The difference Patterson map between the two data sets shows strong heavy-atom peaks, indicating that the crystals are suitable for a high-resolution structure determination.

## 1. Introduction

Microbial lipases from fungi and bacteria have significant potential for various industrial applications (Jaeger *et al.*, 1999; Schmidt-Dannert *et al.*, 1998). Of the microbial lipases, the thermoalkalophilic lipases of bacterial origin exhibit unique thermoactivity and substrate specificities that allow their application in the enzymatic processing of lipids at high temperatures and in an organic solvent phase (Rúa *et al.*, 1998). These thermoalkalophilic lipases, which are produced mainly by the thermophilic *Bacillus* strains, have about 90–95% amino-acid sequence identity and show a significant homology (30–35%) with lipases from pathogenic *Staphylococcus* strains (Rosenstein & Götz, 2000; Eggert *et al.*, 2000; Schmidt-Dannert, 1999). However, they show no apparent sequence homology (less than 15%) with other microbial lipases, including those from other *Bacillus* strains (Eggert *et al.*, 2000). Thus, the thermoalkalophilic and *Staphylococcus* lipases constitute a distinctive subfamily of microbial lipases that was named the lipase subfamily I.5 (Jaeger *et al.*, 1999) or the *Staphylococcus* family (Schmidt-Dannert, 1999).

The *B. stearotheromphilus* L1 lipase (MW 43 kDa) is one of the well characterized thermoalkalophilic lipases (Kim *et al.*, 1998; Kim, Kim, Lee *et al.*, 2000; Kim, Kim, Oh *et al.*, 2000). In addition to its thermostability (its unfolding temperature is 347 K), the L1 lipase exhibits a unique thermoactivity and is most active at 341 K (Kim, Kim, Lee *et al.*, 2000). Calcium ions are required for the enzyme's optimal activity at high temperatures (Kim, Kim, Lee *et al.*, 2000). In contrast to the L1 lipase's thermoactivity, other thermophilic lipases from mesophiles usually show only low activity at temperatures above 333 K (Schmidt-Dannert *et al.*, 1998). The thermoactivity of the L1 lipase can be critically utilized in many industrial applications, as the melting points of some lipids are as high as 323 K (Sigurgísladóttir *et al.*, 1993). The enzyme has a high preference for saturated fatty acids ($C_3$–$C_{16}$) and natural solid lipids such as beef tallow and palm oil that are known to be poor substrates for other lipases (Kim *et al.*, 1998). Other useful characteristics of the enzyme include its high enantiospecificity for producing (*S*)-naproxen from racemic mixtures of (*R*,*S*)-naproxen esters, where only the (*S*)-form is active as an anti-inflammatory drug (Kim, Kim, Oh *et al.*, 2000 and unpublished data).

The three-dimensional structures of the thermoalkalophilic lipases are essential in understanding their unique thermoactivity and substrate specificity as well as in tailoring the lipases to specific applications. However, no structural information on this important subfamily of lipases has been available to date. Although the crystallization of the *Staphylococcus hyicus* lipase has been reported previously (Ransac *et al.*, 1995), the quality of the crystals was not good enough for structural analysis (Rosenstein & Götz, 2000). Here, we report a crystallization of the thermoalkalophilic L1 lipase that yields strongly diffracting crystals. In a preliminary X-ray

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analysis of the crystals, we collected a 2.0 Å native data set and also found a promising samarium derivative.

## 2. Materials and methods

### 2.1. Protein preparation

The structural gene corresponding to the mature L1 lipase was overexpressed in *Escherichia coli* using the pET22b(+) expression vector and the protein was purified using CM-Sepharose and hydroxyapatite chromatography as described previously (Kim, Kim, Oh *et al.*, 2000). The purified protein was stored precipitated with 70%(*w*/*v*) ammonium sulfate, as the purified protein tended to aggregate when stored in solution. For optimal activity and stability of the protein, the precipitated L1 lipase pellet was dissolved in an appropriate volume of calcium-charging buffer (20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 5 m*M* $CaCl_2$) and incubated for 30 min at room temperature. The excess calcium ions in the protein solution were removed by subjecting the protein solution to gel-filtration chromatography (Superose-12, Pharmacia) with calcium-stripping buffer (20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl). The eluent was pooled and concentrated using a Centricon (Amicon) to about 7.5 mg $ml^{-1}$ concentration. The concentrate was frozen in liquid nitrogen and stored until use in crystallization trials.

### 2.2. Crystallization

The frozen L1 lipase was thawed in an ice bath and centrifuged to remove insoluble aggregates. Crystallization of the lipase was conducted by the hanging-drop vapour-diffusion method. Crystal Screens I, II and Lite (Hampton Research) were used for the screening of initial conditions. Typically, 2 µl of the protein solution (7.5 mg $ml^{-1}$) was mixed with a reservoir solution in a 1:1 ratio and the resulting drop was equilibrated against the reservoir solution at 296 K. The initial crystallization conditions were optimized by adjusting protein and precipitant concentrations and by adding different additives.

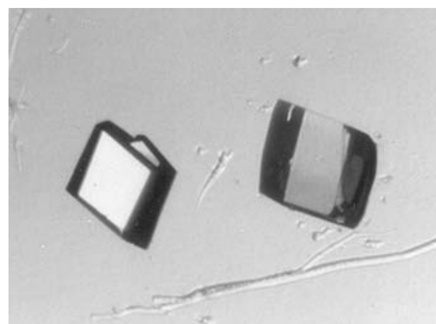

**Figure 1**
Refined monoclinic crystals of the L1 lipase.

### 2.3. Crystallographic data collection

X-ray data were collected at room temperature using Cu $K\alpha$ X-ray radiation from a Rigaku RU-300 rotating-anode generator operating at 40 kV and 100 mA. The diffraction data were collected in 0.5° rotation frames over a total range of 150° using a Rigaku R-AXIS $IV^{++}$ image-plate detector. All diffraction data were indexed and integrated using the program *CrystalClear* 1.2 (Rigaku Corp.).

### 2.4. Heavy-atom derivative search

A preliminary search for heavy-atom derivatives was performed by transferring the L1 lipase crystals to the stabilizing solution (the reservoir solution) containing heavy-atom compounds. Heavy-atom concentrations of 5 m*M* and soaking times of 5–7 d were used for the initial screening. Patterson maps of the potential derivatives were interpreted using the program *XtalView* 3.1 (McRee, 1992) and programs from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

## 3. Results and discussion

The purified L1 lipase exhibits aggregation during concentration, as is observed for other lipases. This tendency towards aggregation has been regarded as one of the major difficulties in crystallizing the lipases from *Staphylococcus* and *Bacillus* strains (Simons *et al.*, 1998). For the crystallization of the L1 lipase, we used a protein concentration of 7.5 mg $ml^{-1}$, which was the maximum concentration we could attain without significant aggregation. Small amounts of aggregates that formed during the concentration were removed by centrifugation (12 000 rev $min^{-1}$, 2 h) before setting up the crystallization trials. Owing to the dependency of the enzyme on calcium for activity and stability (Kim, Kim, Lee *et al.*, 2000), our initial crystallization trials were carried out in the presence of calcium ions. However, the initial trials yielded only salt crystals. We then decided to remove the calcium ions from the protein solution and exchanged the buffer by gel filtration with the calcium-stripping buffer (see §2). The buffer-exchanged protein exhibited full lipase activity comparable to the protein in the buffer containing calcium ions (data not shown). The wild-type activity of the L1 lipase after the calcium-stripping step indicates that the gel filtration seems to remove only excess calcium ions from the solution without affecting the protein-bound calcium ions.

**Table 1**
Data-collection statistics.

Values in parentheses represent statistics for the highest resolution shell.

| Crystal | Native | Sm derivative |
|---|---|---|
| Resolution (Å) | 99.0–2.0 (2.07–2.00) | 99.0–2.3 (2.38–2.30) |
| No. of observations | 202657 (18954) | 106333 (9825) |
| No. of unique reflections | 63443 (6537) | 43260 (4296) |
| $I/\sigma(I)$ | 20.2 (6.1) | 17.0 (6.3) |
| Completeness (%) | 96.9 (95.2) | 93.9 (96.5) |
| $R_{sym}$ (%) | 5.9 (22.2) | 7.2 (21.8) |

After removing the excess calcium ions from the protein solution, the Lite condition 18 (10% PEG 8K, 0.1 *M* sodium cacodylate pH 6.5 and 0.2 *M* magnesium acetate) produced a large number of needle-like crystals within one week. In addition, the Lite condition 34 (1.0 *M* sodium formate, 0.1 *M* sodium acetate trihydrate pH 4.6) produced small monoclinic crystals about three months after the crystallization setup. Although the PEG-grown needle-like crystals did not improve very much after refinement, we were able to determine the space group ($P2_12_12_1$) and unit-cell parameters ($a$ = 67.84, $b$ = 72.96, $c$ = 104.41 Å) of the crystal form by using a preliminary native data set to 3.5 Å resolution. It is most probable that one molecule of L1 lipase is present in the asymmetric unit of these crystals, with a solvent content of 58.7% ($V_M$ = 3.00 $Å^3$ $Da^{-1}$). Two molecules in the asymmetric unit would have a solvent content of 17.5% ($V_M$ = 1.50 $Å^3$ $Da^{-1}$), which is unlikely in protein crystals (Matthews, 1968). In contrast to the difficulties in the refinement of the PEG-grown crystals, the formate-grown monoclinic crystals could be highly refined by varying the precipitant and protein concentrations; the refinement yielded reproducible crystals with typical dimensions 0.2 × 0.2 × 0.2 mm. The best monoclinic crystals formed when a crystallization drop with a protein concentration of 2.5 mg $ml^{-1}$ was equilibrated at 296 K with reservoir solution containing 0.1 *M* sodium acetate pH 4.6 and 0.9 *M* sodium formate (Fig. 1). Under these conditions, the crystals reached their maximum sizes within a week. The crystals diffract beyond 2.0 Å resolution with X-rays from the home source and are relatively insensitive to radiation. From the monoclinic

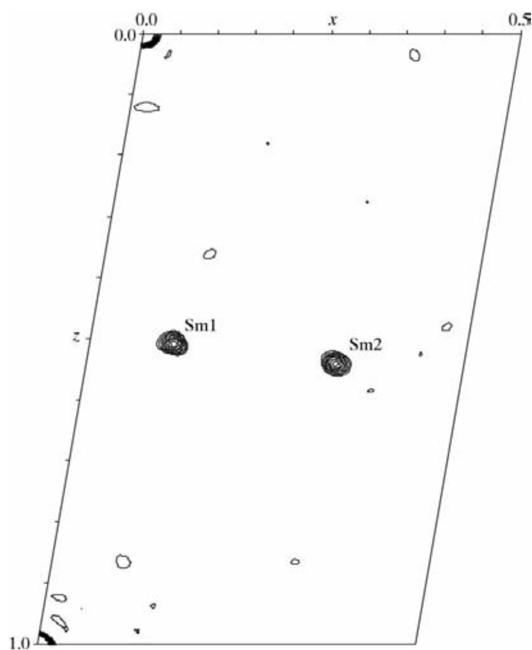


**Figure 2**
Heavy-atom difference Patterson map of the samarium derivative at 3.5 Å resolution. The asymmetric unit of the Harker section ($y$ = 0) is shown. The two strong Harker peaks are labelled Sm1 and Sm2. The map is contoured with an interval of $2\sigma$ from a start level of $3\sigma$. The figure was drawn using the program *XtalView* (McRee, 1992).

crystal, a native data set was collected to 2.0 Å resolution (Table 1). The data set could be indexed in the monoclinic space group $C2$, with unit-cell parameters $a$ = 119.62, $b$ = 85.05, $c$ = 98.36 Å, $\beta$ = 99.73°. Two or three lipase molecules in the asymmetric unit correspond to $V_M$ values of 2.87 or 1.91 $Å^3$ $Da^{-1}$ and solvent contents of 56.8 or 35.1%, respectively. The $V_M$ and solvent-content values are in the range usually found in protein crystals (Matthews, 1968). Based on the good quality and behaviour of the monoclinic crystals, we chose this crystal form for the structure determination of the L1 lipase.

Our initial efforts to determine the structure by the molecular-replacement method using other lipase structures were unsuccessful owing to the low sequence homology of the L1 lipase (about 15%) to other lipases for which three-dimensional structures are known. Instead, in an attempt to search for heavy-atom derivatives, we found a good derivative using a samarium compound (5 m*M* samarium acetate, 5 d soaking). The Sm-derivative crystals diffracted X-rays as well as the native crystals and were highly isomorphous. A 2.3 Å data set from the Sm-derivatized crystal was collected (Table 1). An isomorphous difference Patterson map calculated at 3.5 Å resolution shows two strong Harker peaks ($\sim 17\sigma$) (Fig. 2). The self-Patterson map calculated from the native data does not show such peaks, giving support to the two Harker peaks arising from heavy-atom incorporations in the crystals. Self-rotation searches using the program *AMoRe* (Navaza, 1994) revealed a clear peak ($\sim 11\sigma$) at ($\omega$, $\varphi$, $\kappa$) = (0.0, 0.0, 108.4). The next highest peak has an intensity of $\sim 6\sigma$ and is followed by background peaks with continuously decreasing intensities. The almost identical peak heights of the two Harker peaks in the asymmetric unit and the clear solution in the self-rotation function indicate that the asymmetric unit contains two L1 lipase molecules (with a solvent content of 56.8%). In contrast to the strong heavy-atom peaks in the isomorphous difference Patterson map, the anomalous difference Patterson map of the Sm derivative does not show clear peaks, indicating that the anomalous signal is not strong enough for use in the phase calculation (data not shown). Also, the magnitude of the anomalous differences for non-centric reflections is not significantly higher than that for centric reflections. We are searching for more heavy-atom derivatives to determine the three-dimensional structure of the L1 lipase by the multiple isomorphous replacement method.

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