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Crystallization and preliminary X-ray studies on *Candida cylindracea* lipase

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Abstract

As part of the programme to understand the mechanism and specificity of lipase enzymes used in biotransformation reactions, the lipase from *Candida cylindracea* has been purified and crystallized. This lipase has been widely used by organic chemists for hydrolysis and esterification reactions. Crystals were obtained using polyethylene glycol 6000 as a precipitant and grew to 0.6 mm in the maximum dimension. The enzyme crystallized in the space group $P2_1$ with unit-cell dimensions $a = 94.3$, $b = 117.0$, and $c = 114.2$ Å with $\beta = 109.2°$. Calculations indicate that there are four molecules in the asymmetric unit. The crystals diffract to at least 2.5 Å resolution and the structure has been solved by molecular replacement using the lipase from *Geotrichum candidum* as a search model.

1. Abbreviations

BAM, benzamidine hydrochloride; PEG, polyethylene glycol; MES, 2-[*N*-morpholino]ethanesulfonic acid; EDTA, ethylene diamine tetracetic acid; DTE, dithioerythritol, FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. Introduction

Lipase enzymes are being exploited increasingly for the industrial synthesis of organic compounds; in particular for the resolution of carboxylic acids or secondary alcohols through enantiospecific biocatalytic hydrolysis or formation of an ester derivative. However, the selection of a lipase for such a purpose, and the manner of its use is largely based on empirical experimental observations. In order to exploit this important group of enzymes more fully more information is required as to their specific enzymic mechanism and substrate specificity. Structural studies have been carried out to date on several fungal lipases, *Rhizomucor miehei* (Derewenda, Derewenda & Dodson, 1992), *Geotrichum candidum* lipase (Schrag, Li, Wu & Cygler, 1991), *Candida rugosa* lipase (Grochulski *et al.*, 1993; Grochulski, Li, Schrag & Cygler, 1994), *Candida antarctica* lipase (Uppenberg, Hansen, Patkar & Jones, 1994), a bacterial lipase from *Pseudomonas glumae* (Noble, Cleasby, Johnson, Egmond & Frenken, 1993) and with human pancreatic lipase (Winkler, D'Arcy & Hunziker, 1990) and its complex with colipase (Tilbeurgh *et al.*, 1993). These have aided our understanding of this group of hydrolase enzymes. All of the lipases have a lid or several loops which cover the active site and which need to move when the substrate binds to the enzyme. The displacement of the lid or loops in response to the absorption of the enzyme onto an oil–water interface is postulated as the structure basis for the phenomenon of interfacial activation. A special advantage of the use of lipase enzymes for biotransformation reactions in chemical synthesis is their stability in organic solvents where they can carry out esterification and *trans*-esterification reactions in a stereoselective manner. This work was pioneered by the group of Klibanov (Cambon & Klibanov, 1984; Kirchner, Schollar & Klibanov, 1985). It has been reported that the enantioselectivity of *Candida* lipase ester hydrolysis can be enhanced by noncovalent enzyme modification (Wu, Guo & Sih, 1990). When the *Candida cylindracea* lipase was treated with deoxycholate and organic solvents the enzyme could unfold and refold to generate a more stable conformer, termed lipase A. The lipase A was found to be more enantioselective than the native enzyme towards a variety of (±)-phenoxypropionic esters. Clearly the mode of lipase preparation can have subtle effects on its structure. The common use of the *Candida cylindracea* lipase for biocatalysis has allowed chemists to create a catalogue of known substrates of the enzyme and to predict an active-site model (Kazlauskas, Weissfloch, Rappaport & Cuccia, 1991). This publication examines some 63 different studies on enantioselectivity of this lipase for acetates of secondary alcohols. The Exeter group has recently described the use of lipases to carry out 'doubly enantioselective' esterifications and inter-esterifications using racemic alcohols and racemic acids as substrates (Macfarlane, Roberts, Steukers & Taylor, 1993).

Reports have been made for the crystallization of *Candida rugosa* lipase from methylpentanediol (Grochulski *et al.*, 1993) with a space group $C222_1$ and more recently from PEG 8000 with a space group C_2 (Grochulski *et al.*, 1994). It is important to study the structure of lipase enzymes from different crystallization media and complexed with substrate and inhibitor molecules in order to gain a greater understanding of the catalytically active conformation. Crystallographic studies of lipases complexed with inhibitor molecules have recently been reported (Brzozowski *et al.*, 1991; Derewenda, Brzozowski, Lawson & Derewenda, 1992). Here we describe the crystallization of the lipase from *Candida cylindracea* using PEG 6000. The crystals are in the space group $P2_1$ with four molecules in the asymmetric unit.

3. Experimental

3.1. *Purification of Candida cylindracea lipase*

Candida cylindracea lipase was originally purchased from Meito-Sangyo, Japan. A 50 g amount was purified by water extraction, diafiltration/ultrafiltration and cation-exchange chromatography at Chiroscience Ltd, Cambridge, England. The purification gave 2.1 g of lyophilized enzyme with an activity against olive oil of 680 units mg^{-1}. The enzyme was

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further purified by resuspension of ~30 mg of the lyophilized protein in 5.5 ml of buffer *A* containing 10 m*M* Tris–HCl pH 7.5, 0.2 m*M* EDTA, 2 m*M* dithioerythritol and 1 m*M* benzamidine hydrochloride. The sample was passed over a preparative FPLC Superose 12 gel-filtration column (Pharmacia) to remove aggregated materials and traces of contamination. The lipase fractions which ran as a single band of 71 kDa molecular weight on SDS–PAGE PHAST gel electrophoresis, were pooled. A total of five different lipases have been isolated from *Candida cylindracea* (Lotti *et al.*,1993), which show isoelectric points ranging from p*I* 4.5 to 5.7. The lipase used in this study ran as two closely separated bands with a p*I* of 4.25/4.30, when run on a PHAST Pharmacia isoelectric focussing gel, range p*I* 4–6. The N-terminal sequence of 29 amino acids of this lipase was obtained from gas-phase sequence analysis and was found to agree with that published by Kawaguchi, Honda, Taniguchi-Morimura & Iwasaki, (1989) and lipase I of Lotti *et al.* (1993). The enzyme gave a positive test for glycosylation with the gel blotting assay from the Oxford Glycosystems, GlycoTrack™ kit.

3.2. *Crystallization*

The fractions containing the pure lipase were concentrated an Amicon Centricon 10 microconcentrators to a concentration of protein of 15 mg ml^{-1}. The concentrated samples were 'washed' twice by addition of 2.5 ml of buffer *A* without BAM to the microconcentrators and the volume reduced to 250 µl by centrifugation. The concentrated protein was crystallized by vapour-phase diffusion by the hanging-drop technique against 4% PEG 6000, pH 7.5, 301 K. Crystals were harvested into 10% PEG 6000, 20 m*M* Tris–HCl pH 7.5, 0.2 m*M* EDTA, 2 m*M* DTE.

3.3. *X-ray data collection*

Data was collected at room temperature on a Siemens Xentronics Area Detector system and exposed to a 0.3 mm collimated X-ray beam (Ci $K\alpha$, $\lambda = 1.5418$ Å) supplied by a MAC Science rotating-anode generator operating at 45 kV, 80 mA. A total of 180° of data was collected using the oscillation method (0.25° oscillations, 60 oscillations per frame, 4 min exposure).

The diffraction data was processed using both the *XENGEN* (Howard, 1988) and *XDS* (Kabsch, 1988) software packages independently. Subsequent data processing and structure determination utilized the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994) and the *X-PLOR* 3.1 package (Brünger, 1992).

4. Results and discussion

Crystals appeared after incubation for 1–2 weeks at 301 K at 4% PEG 6000 (Fig. 1). Typical dimensions were 0.6 × 0.5 × 0.3 mm. However, the crystals were very delicate to handle and for some experiments only clusters of small crystals or needle-like bouquets were grown.

Diffraction was observed out to 2.5 Å resolution but low–medium resolution data was collected for use in molecular replacement. One crystal was used to collect an 86% complete data set to 3.5 Å resolution (Table 1). The crystals were of the monoclinic space group $P2_1$ with cell dimensions $a = 94.3$, $b = 117.0$, $c = 114.2$ Å and $\beta = 109.2°$. Calculations indicated four molecules of lipase per asymmetric unit with a specific volume of 2.5 Å^3 Da^{-1}, corresponding to a crystal solvent content of approximately 53% (Matthews, 1968).

Table 1. *Data-collection statistics*

Total No. of observations	50002
No. of independent reflections	25278
Resolution (Å)	3.5
Completeness (%)	86
R_{merge} (%)*	12.6
No. of reflections in outer shell (%)	64
Average $I/\sigma(I)$*	13.7

* $R_{merge} = \sum(|I_j - \langle I_j \rangle|)/(\sum\langle I_j \rangle)$ where I_j is the intensity of an observation of reflection j and $\langle I_j \rangle$ is the average intensity of reflection j. I = average intensity for all scaled observations of a reflection.

Zero-level precession photograph analogues were calculated using *HKLVIEW* in the *CCP*4 suite of programs and showed weak reflections for h and l odd at the highest orders and absent at the lower. These spots were examined individually to determine whether they may have been noise but their intensities were all well defined as their σ's were comparable to other intensities. This is a contributing factor to the high R_{merge}.

A self Patterson map was calculated which revealed a large peak, approximately 67% of the origin peak height, at approximately 0.5 *U*, 0 *V*, 0.5 *W* (Fig. 2). This was interpreted as being a result of having two molecules lying in similar orientations and separated by approximately half a unit-cell edge along *a* and *c*. The distribution of the 0*kl* and *hk*0 intensities, noted above, also reflected this relationship.

The structure was solved by the molecular replacement method using the *X-PLOR* package (Brünger, 1992) and later using *AMoRe* (Navaza, 1994). A real-space cross-rotation search was performed in *X-PLOR* using an intergration radius of 15–4 Å. The search model used was that of triacylglycerol lipase from *Geotrichum candidum* with which the lipase from *Candida cylindracea* has 42% identity. The search yielded two significant peaks each of approximately ten times the average peak height (Fig. 3). Initially two translation searches

Fig. 1. Crystals of *Candida cylindracea* lipase grown at 301 K from PEG 6000.

were made to find the molecules with different orientations within the unit cell termed *A* and *B*. In order that all the search models and the collected data had the same origin in the translation search, the data was first combined into one file using *CAD* (from the *CCP*4 suite). These two translation searches yielded two distinct peaks (Table 2*a* and 2*b*). The relative distance between molecules *A* and *B* were then determined using the non-crystallographic option within *TFFC* (*CCP*4 suite) by fixing molecule *A* and searching for molecule *B* in three dimensions. The second maxima of this search gave the *Y* position of molecule *B* relative to molecule *A* (Table 2*c*).

Molecules *A* and *B* were then fixed in the unit cell and a search was made for the third molecule in the asymmetric unit which was in the same orientation as molecule *A*. Two peaks were obtained again using *TFFC* in non-crystallographic mode (Table 2*d*). The first corresponds to molecule *C* mapping onto molecule *A*. It is the second peak which gives the correct position of the third molecule. Finally, all three molecules were fixed and a search performed for the final molecule, *D*, in the same orientation as molecule *B*. The results are shown in Table 2(*e*).

It can be seen that molecules *A* and *C* and *B* and *D* are related by approximately (0.5*X*, 0, 0.5*Z*). This confirms, independently, the conclusion made from the native self Patterson map. The *Y* positions of each pair of molecules is also equivalent. The arrangement of the four molecules is therefore planar. The whole unit cell was constructed and displayed using *FRODO* (Jones, 1985). The crystal packing showed no bad contacts between neighbouring molecules. An initial rigid-body refinement *X-PLOR*, using non-crystallographic restraints, reduced the *R* factor from 52 to 40% with a corresponding drop in R_{free} (falling from 52 to 45% for a 5% excluded data set).

The structure was also solved by molecular replacement using *AMoRe* (Navaza, 1994). The rotation function (Navaza, 1994) was calculated using diffraction data in the resolution range 10–4 Å and a Patterson radius of 30 Å. The rotation peaks which corresponded to the solutions used had a height of 8.6σ and 8.5σ, respectively, with the next peak at a height of 6.9σ.

The translation search was performed in the resolution range 10–4 Å using the Crowther & Blow (1967) translation function. The translation search gave solutions with the correlation coefficients of 0.144 and 0.181 and the *R* factors of 0.625 and 0.601, respectively. The other solutions had correlation coefficients not higher than 0.082. These solutions corresponded to two molecules in the asymmetric unit. Two further solutions were generated by translating each of these two molecules by (0.5, 0, 0.5) and gave a correlation of 0.439 and an *R* factor of 0.534 in each case. These solutions corresponded to the four molecules in the asymmetric unit.

The model was finally subjected to rigid-body refinement, in the resolution range 10–4 Å, using *AMoRe*. The resulting *R* factor was 51.3% and correlation was 48.4%. Inspection of the crystal packing revealed no bad contacts between neighbouring molecules.

Table 2. *Translation search results*

Peak number	Fractional coordinates *X*	*Y*	*Z*	Peak height
(*a*) Looking for molecule *A*				
1	0.087	0.0	0.328	11.95
2	0.338	0.0	0.079	7.09
3	0.217	0.0	0.448	3.47
4	0.111	0.0	0.218	3.47
5	0.360	0.0	0.470	2.95
(*b*) Looking for molecule *B*				
1	0.303	0.0	0.137	8.88
2	0.050	0.0	0.386	6.64
3	0.324	0.0	0.171	3.33
4	0.323	0.0	0.481	3.33
5	0.459	0.0	0.078	3.21
(*c*) Non-crystallographic search results keeping molecule *A* fixed and searching for molecule *B*				
1	0.801	0.461	0.635	14.14
2	0.300	0.462	0.133	11.80
3	0.267	0.436	0.536	6.09
4	0.987	0.965	0.494	5.82
5	0.252	0.253	0.446	5.92
(*d*) Non-crystallographic search results keeping molecules *A* and *B* fixed and searching for molecule *C*				
1	0.087	0.000	0.328	57.37
2	0.621	0.001	0.812	7.83
3	0.291	0.497	0.285	5.66
4	0.812	0.503	0.914	5.58
5	0.570	0.008	0.483	5.64
(*e*) Non-crystallographic search results keeping molecules *A*, *B* and *C* fixed and searching for molecule *D*				
1	0.800	0.464	0.636	37.68
2	0.293	0.438	0.147	9.76
3	0.311	0.984	0.674	5.96
4	0.629	0.364	0.882	5.36
5	0.342	0.693	0.643	4.57

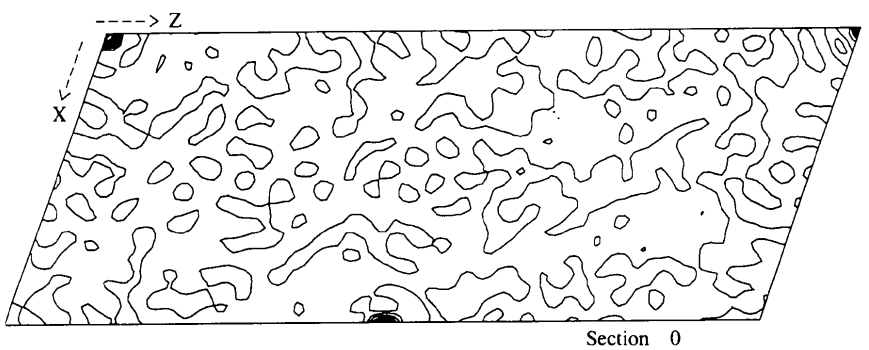

Fig. 2. Self Patterson map, showing a peak at 0.5, 0.5, 67% of origin height.

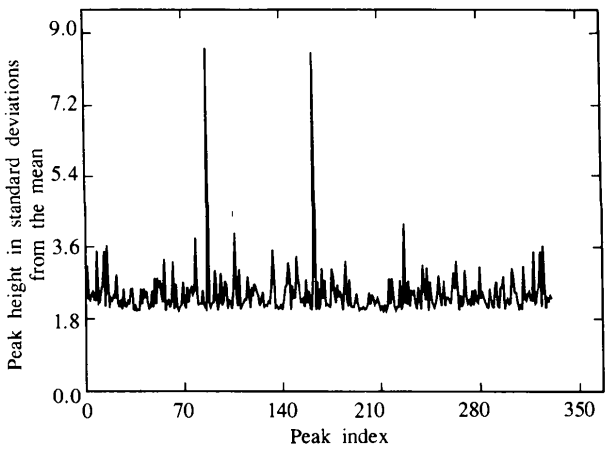

Fig. 3. The results of the real-space cross-rotation search.

Refinement was undertaken using the *X-PLOR* package (Brünger, 1992) using the sequence for triaglycerol lipase from *Geotrichum candidum* with each subunit refined individually. The sequence was then replaced with that from *Candida cylindracea* lipase 1 (Lotti *et al.*, 1993) and further refinement is now under way. The current R factor is 30% ($R_{free} = 39\%$). Higher resolution data is now being collected with and without substrates of special interest such as butryate esters. These will be soaked into the enzyme crystals or co-crystallized to investigate the change in conformation of the enzyme on substrate binding and catalysis. Initial comparisons of this *Candida cylindracea* lipase with the *Candida rugosa* lipase (Grochulski *et al.*, 1994) would suggest that both are in the 'closed-lid' conformation. Further studies are required for detailed comparison of the two structures.

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