(Fitzgerald, 1988) suite of programs were obtained with both subtilisin BPN′ (Bott *et al.*, 1988) ($R = 0.46$) and proteinase K (Betzel, Pal & Saenger, 1988) ($R = 0.40$) as the search model. In both cases, the complete model for the enzyme was employed. Efforts to refine the structure by both simulated annealing [*X-PLOR* (Brünger, Kuriyan & Karplus, 1987)] and restrained least-squares [*PROFFT* (Finzel, 1987)] methods produced poor-quality electron-density maps that were not suitable to fit to the aqualysin I sequence. Work is currently underway to obtain crystals suitable for higher-resolution X-ray data collection and the determination and refinement of the structure of the enzyme.

Note: The protein sample used for the crystallization experiments was recently analyzed by laser desorption mass spectrometry (LDMS) by T. Keough and M. Lacey (Procter & Gamble Company). The measured M_r of the intact protein is 28 296 Da and is approximately 51 Da less than the M_r calculated from the gene sequence of aqualysin I (Kwon, Terada, Matsuzawa & Ohta, 1988). This discrepancy in M_r suggests that the loss of a glycine residue has occurred. Additional LDMS analyses of a CNBr-treated sample and a carboxypeptidase Y-treated fragment from the CNBr treatment of the enzyme have shown the protein specimen is a mixture of the intact enzyme (~ 20% abundance) and the enzyme with its C-terminal glycine removed (~ 80% abundance). The cause of this loss of the C-terminal residue could not be determined.

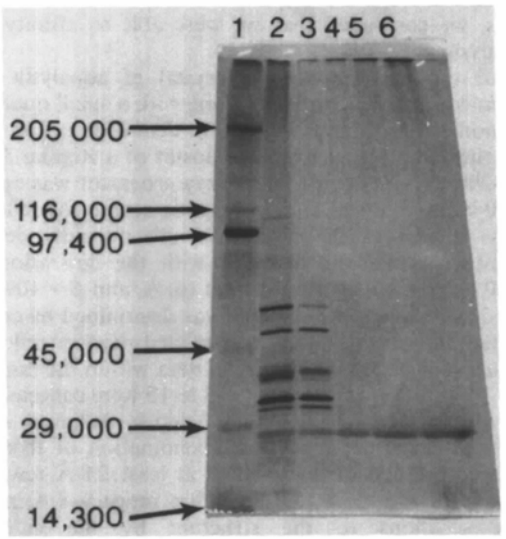

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1, standards: myosin, 205 000; α-galactosidase, 116 000; phosphorylase B, 97 400; ovalbumin, 45 000; carbonic anhydrase, 29 000; lysosyme, 14 300. Lane 2, ammonium sulfate pellet. Lane 3, pooled fractions from the CM-Sepharose column. Lanes 4–6, affinity purified aqualysin I (1, 6 and 12 μg of protein, respectively).

The authors thank Raymond A. Grant of the Procter & Gamble laboratories for performing the sequence analysis experiments.

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Crystallization of a *Humicola lanuginosa* lipase-inhibitor complex with the use of polyethylene glycol monomethyl ether.

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(*Received* 5 *November* 1992; *accepted* 26 *January* 1993)

Abstract

The fungal *Humicola lanuginosa* lipase complexed with the inhibitor *n*-dodecylphosphonate ethyl ester was crystallized in space group $P2_12_12_1$ with pseudotetragonal unit-cell parameters of $a = 131.7\,(2)$, $b = 131.3\,(1)$, $c = 75.4\,(1)$ Å. 92% of X-ray diffraction data to 2.8 Å resolution were collected with a final $R_{merge} = 8.5\%$. The crystals were grown using a new kind of precipitant – polyethylene glycol monomethyl ether (Peg-mme) of molecular weight 5000.

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0907-4449/93/030352-03$06.00

Introduction

Lipases are hydrolytic enzymes, belonging to the group of esterases, which break down triglycerides to di- and monoglycerides, glycerol and free fatty acids. However, they are distinct from esterases in that they generally exhibit a phenomenon described as interfacial activation whereby they are very poorly active against lipids which are in a monodispersed form, but show a significant increase in hydrolytic activity when the lipid concentration exceeds the critical micelle concentration.

Lipases have recently become the subject of intensive studies because of their industrial and medical significance. The structures of several lipases have been determined by X-ray crystallography (Winkler, D'Arcy & Hunziker, 1990; Brady *et al.*, 1990; Schrag, Li, Wu & Cygler, 1991). The results of these investigations have shown that despite very low sequence homology, there are several features common to all lipases: (*a*) they possess at least one α/β domain containing a central β-sheet, five strands of which form a closely superimposable core in all structures; (*b*) the Ser-His-Asp(Glu) amino acids create a trypsin-like catalytic triad with a nucleophilic active serine; (*c*) most lipases contain a Gly-*X*1-Gly-*X*2-Gly motif around the active serine.

So far only one lipase, from the fungus *Rhizomucor miehei*, has been crystallized in the so-called 'active' form, with the catalytic triad fully exposed and inhibitors covalently bound to the active serine (Brzozowski *et al.*, 1991; Derewenda, Brzozowski, Lawson & Derewenda, 1992). In the 'inactive' form of this lipase the active centre is buried under a short helix, referred to as the lid. In two 'active' structures, one complexed with a substrate analog, *n*-hexylphosphonate ethyl ester (C6), the second with diethyl *p*-nitrophenyl phosphate (E600, a typical serine protease inhibitor), the lid is displaced by 8 Å, thereby exposing the catalytic triad. In the lipase–C6 complex structure, the six C atoms of the inhibitor chain occupy a hydrophobic pocket. However, the likely location of an additional six C atoms, part of a natural 12 C-atom-long substrate, is unknown and difficult to predict. Therefore, in order to further characterize this substrate-binding pocket, crystallization trials were undertaken to obtain crystals of lipase complexed with a more substrate-like fatty-acid moiety, *n*-dodecylphosphonate ethyl ester (C12) lipase inhibitor. This paper describes the crystallization and preliminary X-ray studies performed on a related fungal lipase from *Humicola lanuginosa*.

Materials and methods

Pure recombinant *Humicola lanuginosa* (SP400) lipase (MW = 30 kDa) and C12 inhibitor were supplied by Novo Nordisk A/S (2880 Bagsvaerd, Denmark). To inhibit the enzyme, 12 mg of lipase were dissolved in 1.9 ml 50 m*M* Tris/HCl buffer pH 8.0; 5 µl of C12 inhibitor were dissolved in 95 µl of dimethyl sulfoxide (DMSO) and 100 µl of this solution were added to the enzyme solution. This mixture was incubated for 18 h at 310 K; inhibited protein was washed several times in a Centricon (Amicon) concentrator (10 kDa MW cut off), and finally concentrated in 50 m*M* Tris/HCl pH 8.0 to give a 60 mg ml^{-1} solution. Inhibited enzyme was subsequently crystallized by the vapour diffusion hanging-drop method (Falcon 3047 multiwell plate). 1.5 µl of reservoir solution were mixed with an equal volume of protein solution. All trials were performed at 291 K. The resulting crystals were mounted in capillaries and X-ray data were collected using a Rigaku R-AXIS IIC image-plate detector on a Rigaku rotating anode RU-200 X-ray generator with a Cu target and an Ni filter (operated at 50 kV and 100 mA). The crystal-to-image plate distance was set at 160 mm and 72 × 1.2° oscillation images were recorded (exposure time 45 min), with the crystal rotated about *b**. Subsequently blind-region data were collected. The data were processed by *MOSFLM* (Leslie, Brick & Wonacott, 1986) and *CCP*4 programs (SERC Daresbury Laboratory, 1979) (details of the data processing and structure solution will be described elsewhere).

Results and discussion

Despite the use of practically all conventional precipitants under a wide range of conditions (different pH, buffers, additives, detergents) no crystals were obtained. Consequently, a number of novel precipitants were tried. One of them – polyethylene glycol monomethyl ether (Peg-mme) (Fluka, Aldrich) – gave positive results. Good quality crystals of *Humicola lanuginosa* lipase (SP400) complexed with the 12 C-atom-chain inhibitor (C12) were obtained using a new kind of precipitant – Peg-mme 5000 MW (Fig. 1). The tetragonal and orthorhombic prism-shaped crystals (average dimensions 0.4 × 0.4 × 0.25 mm) grew after 3–4 weeks from 15–17% *w*/*v* Peg-mme solutions in 50 m*M* Tris/HCl pH 8.0 buffer. 2.8 Å resolution X-ray data were collected using these crystals. Preliminary inspection of crystal morphology, and results of initial indexing (based on three still images at 0, 45 and 90°

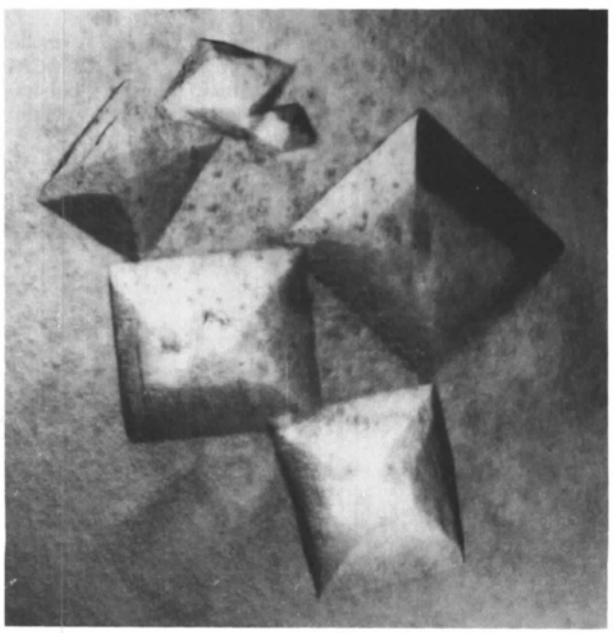

Fig. 1. *Humicola lanuginosa* lipase–C12 inhibitor pseudotetragonal crystals grown from 15% *w*/*v* Peg-mme 5000 MW, 50 m*M* Tris/HCl buffer pH 8.0. The largest crystal has dimensions 0.4 × 0.4 × 0.25 mm.

using Rigaku data-processing software) which gave a = 131.1, b = 131.2, c = 75.0 Å and $\alpha = \beta = \gamma = 90°$, strongly suggested tetragonal symmetry. However, data were initially processed in space group $P1$ (triclinic) and a plot of the $hk0$ reciprocal lattice plane showed only *mm* symmetry (see Fig. 2). Analysis of systematic absences showed the space group to be $P2_12_12_1$. The data were subsequently reprocessed in this space group to give a final R_{merge} = 8.5% [$R_{merge} = 100(\sum|I_i - w\langle I\rangle|/I_i)$], with 92% completeness to 2.8 Å. During processing, all cell parameters were refined independently and their final values were a = 131.7 (2), b = 131.3 (1), c = 75.4 (1) Å, $\alpha = \beta = \gamma = 90°$. Assuming that there are four lipase molecules per asymmetric unit gives V_m = 2.71 Å^3 Da^{-1}, corresponding to a solvent content of 54%, which falls within the range of 1.68–3.53 Å^3 Da^{-1} observed for most protein crystals (Matthews, 1968). The crystal structure analysis is in progress.

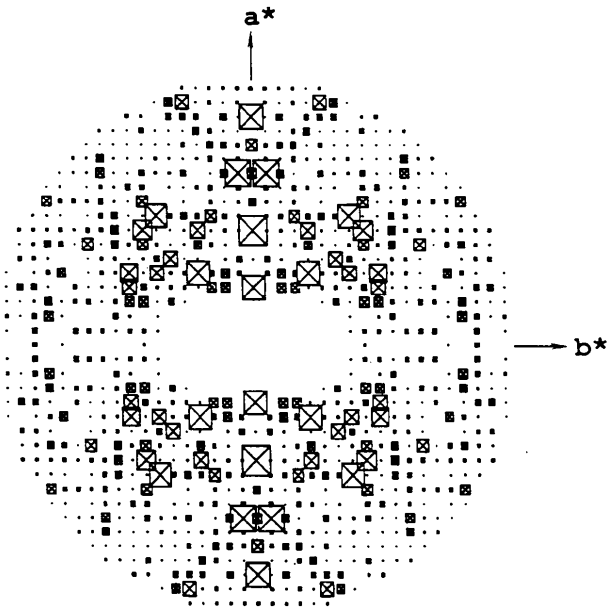

Fig. 2. The pseudoprecession plot of the $hk0$ zone to 7 Å resolution [calculated by the *HKLPLOT* program from the *CCP*4 crystallographic programs package (SERC Daresbury Laboratory, 1979)], showing *mm* symmetry despite a^* and b^* parameters being approximately equal.

During the crystallization trials, Peg-mme compounds of molecular weights 350, 550, 750, 2000 and 5000 were tested. They can be used alone or as an additive with other precipitants, especially because of their chemical similarity to the polyoxyethylene ether-like detergents. Peg-mme compounds represent new useful alternative tools for protein crystallographers.

I am very grateful to B. Huge-Hensen, S. Patkar and F. Bjorkling (Novo Nordisk A/S) for the provision of both proteins and inhibitors and to D. M. Lawson (University of York) for his assistance during data collection and processing.

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