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Crystallization and preliminary X-ray analysis of recombinant *Glomerella cingulata* cutinase

Mun Peak Nyon,[a,b] David W. Rice,[b]* John M. Berrisford,[b] Huazhang Huang,[c] Arthur J. G. Moir,[b] C. Jeremy Craven,[b] Sheila Nathan,[a,d] Nor Muhammad Mahadi[a,d] and Farah Diba Abu Bakar[a]*

[a]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia, [b]Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, England, [c]Department of Entomology and Cancer Research Center, University of California at Davis, One Shields Avenue, Davis, California 95616, USA, and [d]Malaysia Genome Institute, Heliks Emas Block, UKM–MTDC Smart Technology Centre, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

Correspondence e-mail: d.rice@sheffield.ac.uk, fabyff@pkrisc.cc.ukm.my

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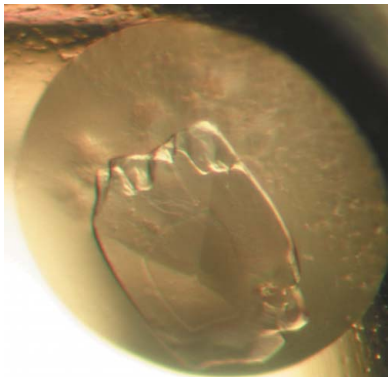

Cutinase catalyzes the hydrolysis of water-soluble esters and long-chain triglycerides and belongs to the family of serine hydrolases. The enzyme is thought to represent an evolutionary link between the esterase and lipase families and has potential applications in a wide range of industrial hydrolytic processes, for which an understanding of the molecular basis of its substrate specificity is critical. *Glomerella cingulata* cutinase has been cloned and the protein has been overexpressed in *Escherichia coli*, purified and subsequently crystallized in a wide range of different crystal forms in the presence and absence of inhibitors. The best crystals are those of the apo cutinase, which diffract to beyond 1.6 Å resolution and belong to space group $P4_12_12$ or $P4_32_12$. Crystals of cutinase with the inhibitors PETFP or E600 belong to space groups $P2_12_12_1$ and $P2_1$, respectively, and diffract to approximately 2.5 Å resolution. All of the crystals are suitable for structural studies, which are currently ongoing.

1. Introduction

Cutinase (EC 3.1.1.74) is an extracellular enzyme secreted by phytopathogenic fungi that catalyzes the hydrolysis of cutin, a waxy polyester that acts as the structural component of the cuticle of higher plants (Purdy & Kolattukudy, 1975), and is capable of hydrolyzing both water-soluble esters and triglycerides (Lauwereys *et al.*, 1991). The cutinase from *Fusarium solani* was the first enzyme of this type to be purified and characterized and is the only one to date for which a three-dimensional structure is available (Longhi *et al.*, 1996; Longhi, Czczek *et al.*, 1997; Longhi, Mannesse *et al.*, 1997*b*; Martinez *et al.*, 1992, 1994; Nicolas *et al.*, 1996). Structural studies of *F. solani* cutinase have confirmed that it contains the α/β-hydrolase fold (Martinez *et al.*, 1992), which is one of the most frequent and widespread protein folds known and is found in proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases (Heikinheimo *et al.*, 1999; Nardini & Dijkstra, 1999). Currently, the structure of cutinase represents the smallest α/β-hydrolase-fold enzyme to be found (Heikinheimo *et al.*, 1999; Egmond & Vlieg, 2000).

The crystal structures of *F. solani* apo cutinase and of a range of inhibitor complexes showed that its active site contains the expected catalytic triad (Ser120, His188, Asp175) and an oxyanion hole that is formed by the main-chain N atoms of Ser42 and Gln121 and the side-chain O atom of Ser42 (Martinez *et al.*, 1994; Nicolas *et al.*, 1996; Longhi *et al.*, 1996). Whilst the activity of cutinase is closely related to that of lipase, they differ in that lipase shows a rate enhancement in the presence of a lipid–water interface (Verger, 1980; Brzozowski *et al.*, 1991) that is not observed for cutinase (Nicolas *et al.*, 1996). The molecular basis of this difference is thought to arise from the fact that cutinase has an accessible active site with an intact preformed oxyanion hole and therefore no significant conformational changes are necessary to bind the substrate (Martinez *et al.*, 1994; Nicolas *et al.*, 1996; Longhi *et al.*, 1996). This is unlike most lipases, which require a

significant conformational change involving movement of a lid over the active site; this exposes a hydrophobic surface, allowing the substrate to bind (Verger, 1980; Brzozowski *et al.*, 1991).

However, subsequent crystallographic studies of the structure of *F. solani* apo cutinase did reveal small conformational changes involving two loops (residues 80–88 and 180–188) delimiting the active-site crevice, suggesting that they might form a mini-lid (Longhi, Mannesse *et al.*, 1997). This suggestion is consistent with nuclear magnetic resonance studies of cutinase, which indicated that these two loops are involved in conformational rearrangements on a timescale in the millisecond range and which may represent the interconversion between open and more closed conformations (Prompers, Groenewegen *et al.*, 1999). Further NMR studies indicated that the enzyme adopts a more ordered conformation upon inhibitor binding (Prompers, Noorloos *et al.*, 1999).

To contribute to a deeper understanding of the nature of the substrate-induced conformational changes in cutinase, we have initiated a structural investigation of cutinase from the fungal phytopathogen *Glomerella cingulata*. This enzyme is composed of a single polypeptide chain and is thought to be processed by removal of an N-terminal signal peptide to give a protein of approximate molecular weight 21 kDa composed of 194 residues with 50% identity to *F. solani* cutinase. In this paper, we describe the crystallization and preliminary X-ray analysis of recombinant *G. cingulata* cutinase and its complexes with the inhibitors 3-phenethylthio-1,1,1-trifluoropropan-2-one (PETFP) and diethyl *p*-nitrophenyl phosphate (E600).

2. Purification of recombinant *G. cingulata* cutinase

A construct for recombinant *G. cingulata* cutinase (GenBank accession No. AF444194), corresponding to residues Glu31–Gly224 and equivalent to the ordered segment of the *F. solani* enzyme observed in the crystal structure, was overexpressed in an *Escherichia coli* strain containing a plasmid with the cloned gene of *G. cingulata* cutinase fused to an N-terminal Trx-His-S tag. *E. coli* cell paste was grown and disrupted to release the soluble protein by breaking cells using the BugBuster Protein Extraction kit (Novagen, Germany). The cell paste was suspended in BugBuster reagent and soluble protein was separated from cell debris by centrifugation at 20 000*g* for 20 min. Subsequently, the protein was loaded onto a nickel-based resin (Ni Sepharose High Performance, GE Healthcare Biosciences, Sweden), washed with 0.5 *M* NaCl, 20 m*M* K_2HPO_4 pH 7.4 and eluted with the same buffer containing 200 m*M* imidazole. The

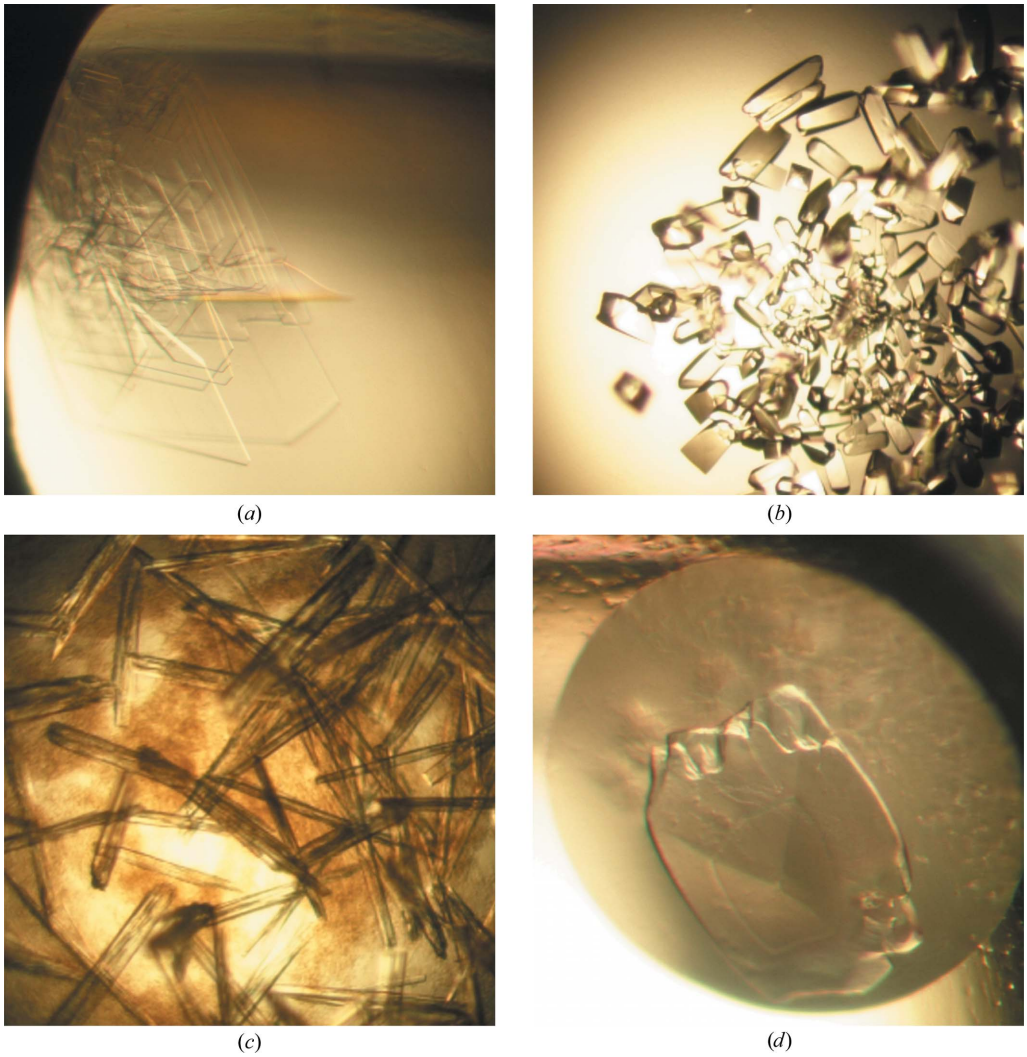

Figure 1
(*a*) Form *A* crystals of apo cutinase (0.15 × 0.10 × 0.05 mm). (*b*) Form *B* crystals of apo cutinase (0.15 × 0.15 × 0.10 mm). (*c*) Crystals of the E600–cutinase complex (0.20 × 0.10 × 0.10 mm). (*d*) A crystal of the PETFP–cutinase complex (0.20 × 0.15 × 0.15 mm).

fractions containing cutinase were desalted against 20 m*M* Tris–HCl pH 7.5 buffer using a HiTrap desalting column (GE Healthcare Biosciences, Sweden) and subsequently cleaved with recombinant enterokinase (Novagen, Germany) according to the manufacturer's specifications, leaving an additional linker sequence of seven residues (AMAISDP) at the N-terminus. The recombinant cutinase was further purified using an anion-exchange resin (Q Sepharose High Performance, GE Healthcare Biosciences, Sweden) and the protein was eluted with a linear gradient of 0–1.0 *M* NaCl in 20 m*M* Tris–HCl pH 8.0. The peak fractions containing recombinant cutinase were pooled and concentrated to 25 mg ml^{-1} (estimated by the method of Bradford, 1976) using a Vivaspin concentrator with a molecular-weight cutoff of 10 kDa (Vivascience, Germany) and buffer-exchanged to 20 m*M* Tris–HCl pH 8.0 prior to crystallization. Full details of the construction of the plasmid and the biochemical characterization of the enzyme will be described elsewhere (Nyon *et al.*, 2008).

3. Crystallization and preliminary X-ray analysis of apo cutinase

Initial crystallization trials were carried out on a Matrix Hydra II (Thermo Fisher Scientific, USA) with crystallization kits from NeXtal (Qiagen, Germany) using the standard sitting-drop vapour-diffusion technique by adding 0.2 µl concentrated apo cutinase (in 20 m*M* Tris–HCl pH 8.0) to an equal volume of precipitant and then equilibrating against the same precipitant (100 µl) at 290 K. Two potential crys-

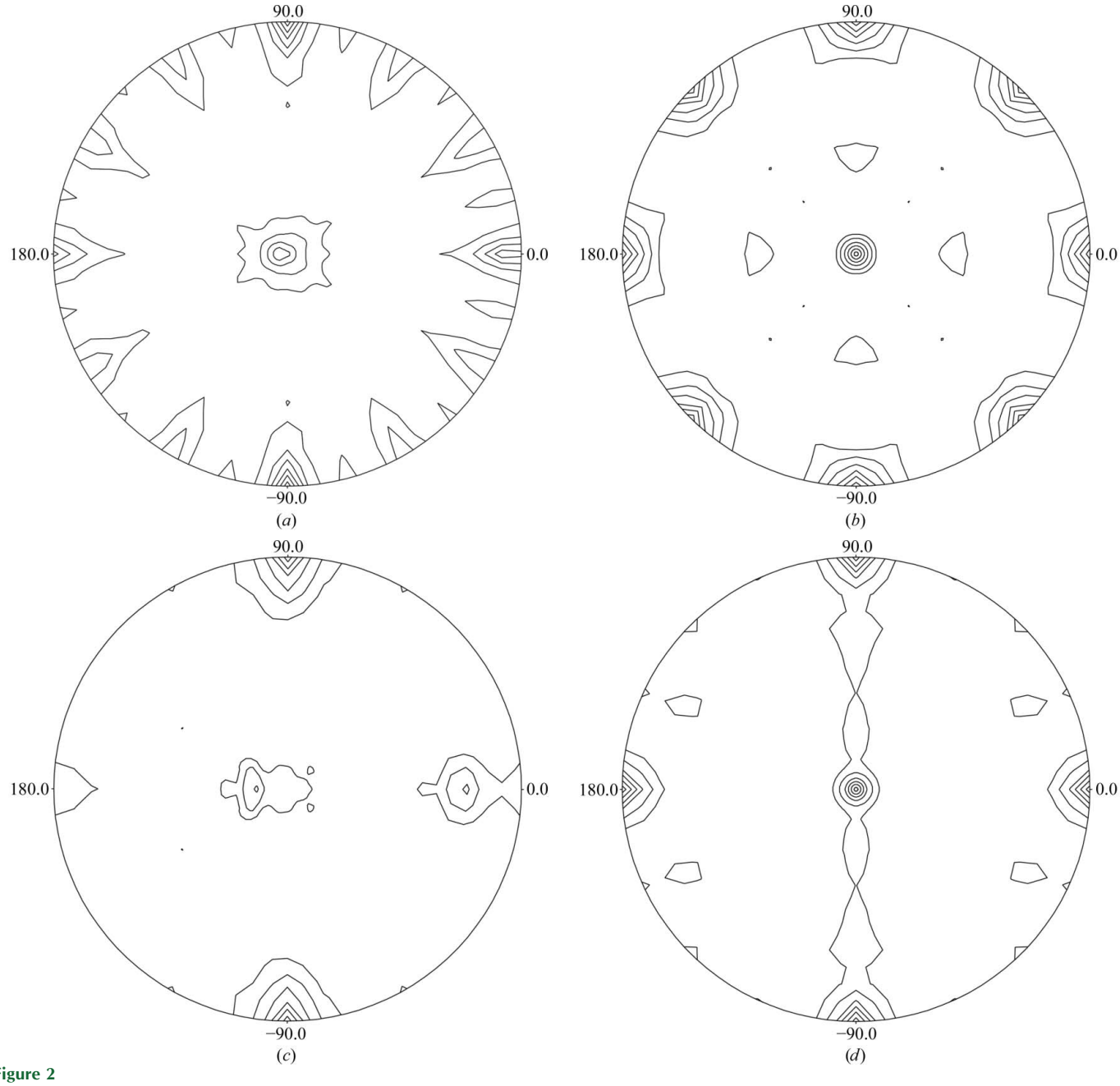

Figure 2
Stereographic projection of the $\kappa = 180^\circ$ section of the self-rotation functions calculated using *POLARRFN* for (*a*) form *A* apo cutinase, (*b*) form *B* apo cutinase, (*c*) the E600–cutinase complex and (*d*) the PETFP–cutinase complex. All the maps are scaled to an origin of 100 and contoured from 15 in intervals of 15.

Table 1
Data-processing statistics for *G. cingulata* cutinase crystals.

Values in parentheses are for the highest resolution shell.

	Apo cutinase		Inhibitor complexes	
	Form *A*	Form *B*	E600	PETFP
Space group	$P2_1$	$P4_12_12$ or $P4_32_12$	$P2_1$	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 60.0, b = 34.7, c = 71.1, β = 92.9	$a = b$ = 60.0, c = 86.4	a = 73.5, b = 117.4, c = 95.2, β = 103.6	a = 43.3, b = 88.3, c = 123.7
Resolution range	20.0–3.1 (3.27–3.1)	28.0–1.8 (1.9–1.8)	20.0–2.6 (2.7–2.6)	37.0–2.3 (2.4–2.3)
Unique reflections	13332 (1841)	15325 (2182)	47279 (6694)	21478 (3049)
Completeness (%)	97.1 (98.8)	99.9 (100.0)	98.0 (95.9)	98.8 (97.7)
Multiplicity	2.5 (2.3)	6.5 (6.4)	2.9 (2.4)	5.4 (5.2)
$\langle I/\sigma(I)\rangle$	8.0 (4.7)	24.4 (3.6)	9.4 (2.3)	16.8 (2.7)
$R_{\rm merge}$† (%)	14.1 (19.8)	6.2 (43.7)	12.2 (41.6)	8.8 (53.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

tallization conditions for apo cutinase were optimized using the hanging-drop vapour-diffusion technique (manually) by mixing 1 µl apo cutinase (25 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0) with an equal volume of precipitant and then equilibrating against the same precipitant (500 µl) at 290 K and led to the production of different crystal forms. Crystals of form *A* have approximate dimensions of 0.15 × 0.1 × 0.05 mm and grew from a solution containing 0.05 *M* trilithium citrate and 20%(*w*/*v*) PEG 3350; those of form *B* have approximate dimensions of 0.15 × 0.15 × 0.1 mm and were grown in 16–30%(*w*/*v*) PEG 4000 in 0.1 *M* sodium acetate pH 4.6 (Fig. 1).

A form *A* crystal of apo cutinase was loop-mounted and placed in a cryosolution consisting of 0.05 *M* trilithium citrate, 30%(*w*/*v*) PEG 3350 for approximately 1 min at room temperature prior to cooling in a nitrogen-gas stream at 100 K (Oxford Cryosystems Cryostream, UK). Diffraction images were collected with 1° rotations using a MAR Research MAR345 image-plate detector mounted on a Rigaku MM007 rotating copper-anode generator. Examination of the diffraction pattern revealed that the crystal diffracted to approximately 3.0 Å resolution. Processing of the X-ray diffraction data using *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1997) from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994) and analysis of the merging statistics indicated that the crystals belonged to a primitive monoclinic system with unit-cell parameters a = 60.0, b = 34.7, c = 71.1 Å, β = 92.9°. The absence of reflections with $k = 2n + 1$ suggested that the crystals belonged to space group $P2_1$. Consideration of the unit-cell volume suggested that the crystal contained one subunit or two subunits in the asymmetric unit, with a corresponding $V_{\rm M}$ of 3.29 or 1.64 Å^3 Da^{-1} and a solvent content of 63% or 25%, respectively (Matthews, 1977). Analysis of a self-rotation function calculated with *POLARRFN* (Collaborative Computational Project, Number 4, 1994) using data in the resolution range 20–3 Å and with a radius of integration of 30 Å revealed strong peaks equivalent to 67% of the origin at $\omega = 87°$ and $\omega = 177°$, both at $\varphi = 0°$ on the $\kappa = 180°$ section (Fig. 2*a*). This indicated the presence of noncrystallographic twofold axes perpendicular to the b axis and suggested that the asymmetric unit contains two molecules related by twofold symmetry. However, gel filtration suggested that *G. cingulata* cutinase was most likely to be monomeric in solution and therefore the two subunits are not thought to represent a biological dimer.

For X-ray analysis, a crystal of form *B* was mounted in a sealed glass tube and exposed at room temperature using the same X-ray setup as used for the form *A* crystals. Data were collected using 1° rotations and processed to 1.8 Å resolution (Fig. 3). Analysis of the merging statistics and the pattern of systematic absences indicated that the crystals belonged to a primitive tetragonal system, point group 422; they most probably belong to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b$ = 60.0 Å, c = 86.4 Å. A self-rotation function calculated as above revealed no indication of

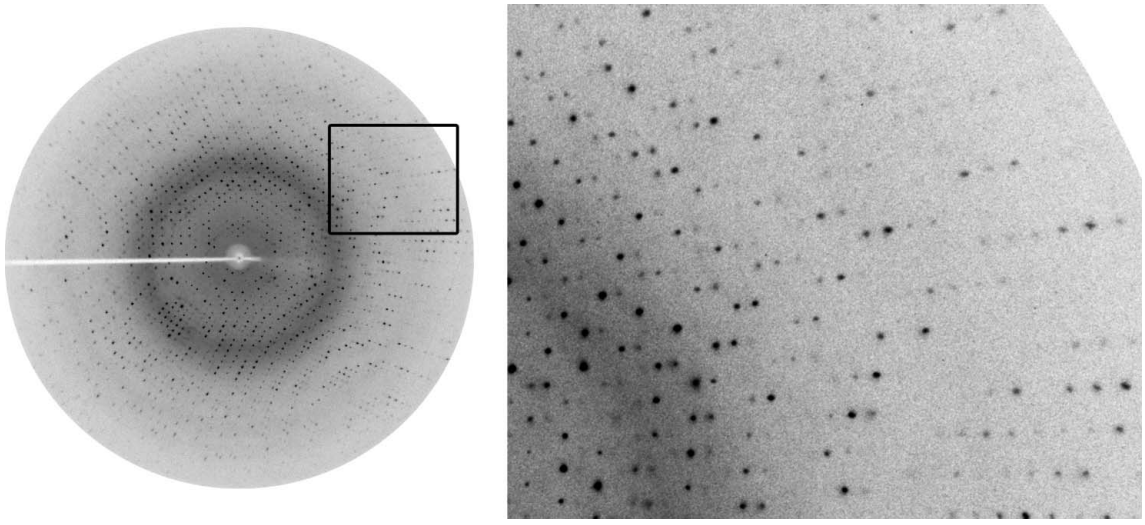

Figure 3
A representative 1° oscillation image of data collected from a form *B* crystal of apo cutinase on a MAR345 detector mounted on a Rigaku MM007 generator. An enlarged view of the region indicated by the square is shown on the right. The edge of the image corresponds to a resolution of 1.6 Å.

noncrystallography symmetry (Fig. 2*b*), suggesting that the asymmetric unit contains a single subunit with a V_M of 1.89 Å^3 Da^{-1}. Data-collection and processing statistics are shown in Table 1.

4. Crystallization and preliminary X-ray analysis of cutinase–inhibitor complexes

Like other esterases, cutinase is known to be inhibited by a range of active-site-directed inhibitors that covalently modify the catalytic serine (Martinez *et al.*, 1994; Wheelock *et al.*, 2001). Covalent modification of cutinase by E600 (Sigma, USA; added from a stock solution of 50 m*M* E600 in 20 m*M* Tris–HCl pH 8.0) was confirmed by mass spectrometry using a Voyager-DE STR MALDI mass spectrometer (Applied Biosystems, USA; Fig. 4). Following complete modification of the enzyme by incubation with a fourfold excess of E600, the resulting complex was crystallized as previously described. Plate-like crystals of cutinase–E600 complex with approximate dimensions of 0.2 × 0.10 × 0.1 mm grew from solutions containing 0.1 *M* magnesium acetate and 18%(*w*/*v*) PEG 3350 as precipitant (Fig. 1). Crystallization of the PETFP complex (PETFP dissolved in 20 m*M* Tris–HCl pH 8.0) followed a similar procedure, yielding plate-like crystals with approximate dimensions of 0.2 × 0.15 × 0.15 mm within 2 d in a drop containing 0.1 *M* HEPES pH 7.5 and 70%(*v*/*v*) MPD (Fig. 1).

For data collection, a cutinase–E600 complex crystal was soaked in a cryosolution containing 0.1 *M* magnesium acetate and 30%(*w*/*v*) PEG 3350 for approximately 1 min before cooling. Data were collected to 2.6 Å resolution using 1° rotations and analysis of the merging statistics suggested that the crystals belonged to space group $P2_1$, with approximate unit-cell parameters a = 73.5, b = 117.4, c = 95.2 Å, β = 103.6°. Consideration of the unit-cell volume suggested that the crystal contained between five and ten subunits, corresponding to V_M values between 3.76 and 1.87 Å^3 Da^{-1}. A self-rotation function calculated as above revealed the presence of strong peaks equivalent to 48% of the origin at $\omega = 73°$ and $\omega = 163°$, both at $\varphi = 0°$ on the $\kappa = 180°$ section, indicative of the presence of additional noncrystallographic twofold symmetry in the crystal and suggesting that the asymmetric unit contains an even number of subunits, possibly eight (Fig. 2*c*).

A crystal of the cutinase–PETFP complex was loop-mounted without further cryoprotection and data were collected to 2.3 Å resolution using 0.5° rotations. Analysis of the merging statistics indicated that the crystals belonged to the primitive orthorhombic space group $P2_12_12_1$, with approximate unit-cell parameters a = 43.3, b = 88.3, c = 123.7 Å. Considerations of possible values of V_M suggested that the asymmetric unit contained two or three subunits, with a V_M of 2.82 or 1.86 Å^3 Da^{-1}, respectively. Analysis of a self-rotation function calculated as above gave no indication of additional noncrystallographic symmetry and therefore the ambiguity concerning the number of molecules in the asymmetric unit cannot be resolved (Fig. 2*d*). A summary of the X-ray data-processing statistics for the complexes of cutinase with E600 and PETFP is given in Table 1.

The structure determination of *G. cingulata* apo cutinase and its inhibitor complexes is currently under way using molecular replacement. Once completed, it is hoped that the structure will contribute to a better understanding of catalysis by this class of enzyme, allowing their relationship to lipases to be defined.

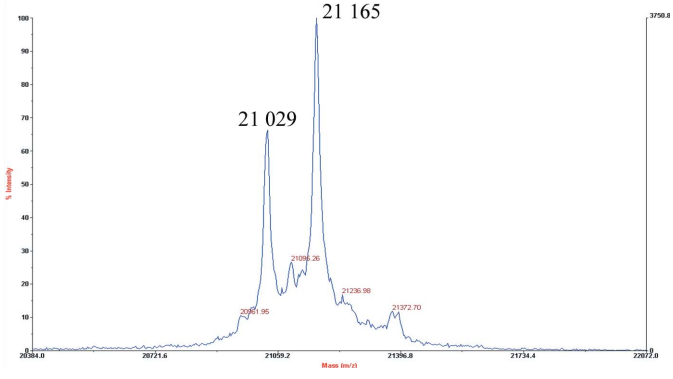

Figure 4
A mass spectrum showing the covalent modification of apo cutinase by E600. The modification was carried out at pH 8.0 in Tris–HCl buffer; cutinase was pre-incubated with a twofold excess of E600 at room temperature for 60 min. Two peaks separated by 136 Da can be seen, which represent the nonmodified and modified enzyme, respectively. Incubation with a fourfold excess ensured complete modification (data not shown).

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