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Crystallization and preliminary X-ray diffraction study of lactonohydrolase from *Fusarium oxysporum*

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Abstract

The lactonohydrolase from the fungus *Fusarium oxysporum* AKU 3702 was crystallized by the vapour-diffusion procedure in the presence of polyethylene glycol 4000 as a precipitant. The crystals belong to the monoclinic space group $P2_1$ with unit-cell parameters a = 156, b = 100, c = 94.1 Å, β = 91.7°. Assuming that there are two lactonohydrolase-dimer molecules in the asymmetric unit, the crystal volume per unit molecular mass, V_m, is calculated to be 2.94 Å^3 Da^{-1}.

1. Introduction

Enzymes catalyzing the reversible or irreversible hydrolysis of carboxylic esters to the respective carboxylic acids and alcohols (E.C. 3.1.1) play an important role in the biosynthesis and biodegradation of various compounds. In particular, lipase (E.C. 3.1.1.3) and esterase (E.C. 3.1.1.1) have been well investigated: the genes encoding these enzymes from various organisms have been cloned, their primary amino-acid sequences have been determined and crystallization and X-ray diffraction studies have been performed in order to elucidate their reaction mechanisms from their three-dimensional structures (Beer *et al.*, 1996; Cygler *et al.*, 1993; Schrag *et al.*, 1991; Uppenberg *et al.*, 1995; Winkler *et al.*, 1990). Lactonohydrolases also catalyze the hydrolysis of carboxylic esters, but act on the intramolecular ester bonds of lactone compounds. They are involved in the synthesis and degradation of various lactone compounds *in vivo*. Gluconolactonase (aldonolactonase; E.C. 3.1.1.17) has been suggested as participating in the formation of L-gulono-γ-lactone from L-gulonate in L-ascorbate biosynthesis (Bublitz & Lehninger, 1961). Lactonohydrolases acting on aldonate lactones, such as L-arabinonolactonase (E.C. 3.1.1.15), D-arabinonolactonase (E.C. 3.1.1.30), L-rhamnonolactonase (E.C. 3.1.1.65) and D-xylonolactonase (E.C. 3.1.1.68), are involved in the oxidative degradation of aldoses through the ring-opening of aldonate lactones, which are formed by enzymatic oxidation of aldoses (Buchert & Viikari, 1988; Dilworth *et al.*, 1986; Novick & Tyler, 1982; Rigo *et al.*, 1985). Pyridoxolactonase (E.C. 3.1.1.27), dihydrocoumarin hydrolase (E.C. 3.1.1.35), limonin-D-ring lactonase (E.C. 3.1.1.36) and deoxylimonate-A-ring lactonase (E.C. 3.1.1.46), and actinomycin lactonase (E.C. 3.1.1.39) are involved in the degradation pathways for vitamin B_6 (Jong & Snell, 1986), aromatic compounds (Kosuge & Conn, 1962; Casellas *et al.*, 1997), limonoids (Hasegawa *et al.*, 1980; Merino *et al.*, 1996) and actinomycin (Hou & Perlman, 1970), respectively. As regards molecular biochemical studies, only the gene encoding gluconolactonase from *Zymomonas mobilis* has been cloned and sequenced (Kanagasundaram & Scopes, 1992), and there have been no reports of the three-dimensional structure of any lactonohydrolase.

Recently, we found that *Fusarium oxysporum* produced a novel lactonohydrolase catalyzing the hydrolysis of aldonate lactones and aromatic lactones (Shimizu *et al.*, 1992). The relative molecular mass of the native enzyme is 125000, and the subunit molecular mass is 60000. The enzyme contains 15.4%(*w*/*w*) glucose equivalent of carbohydrate and about one mole of calcium per mole of subunit, and stereospecifically hydrolyzes aldonate lactones which carry 'downward' hydroxy groups at their 2-position C atoms (according to Haworth's system), such as D-galactono-γ-lactone, L-mannono-γ-lactone *etc*. The enzyme was also found to catalyze the enantioselective hydrolysis of D-pantolactone, which resembles aldonate lactones in chemical structure and also possesses a 'downward' hydroxy group at the 2-position C atom, and can be applied to the practical optical resolution of racemic pantolactone (Kataoka *et al.*, 1995*a*,*b*, 1996). All linear esters tested were not hydrolyzed by the enzyme. This selectivity, *i.e.* the recognition of the configuration of hydroxy groups at the 2-position and of the lactone structure of substrates, might depend on the three-dimensional structure of the enzyme. Therefore, elucidation of the three-dimensional structure of the lactonohydrolase from *F. oxysporum* is important for the elucidation of the mechanisms underlying the stereoselectivity and substrate specificity of the enzyme. The lactonohydrolase was easily crystallized by desalting (Shimizu *et al.*, 1992), but the crystals obtained showed poor stability with respect to X-ray irradiation. In this study, we report the crystallization of the lactonohydrolase of *F. oxysporum* to give crystals suitable for atomic resolution X-ray structure analysis, and the preliminary crystallographic characterization of the lactonohydrolase.

2. Materials and methods

2.1. *Crystallization*

The enzyme was purified as described by Shimizu *et al.* (1992). The crystallization conditions were preliminarily screened with a Crystal Screen reagent kit (Hampton Research, USA). 2 µl of a protein solution (10 or 20 mg ml^{-1}) in 20 m*M* Tris–HCl buffer (pH 7.4) containing 100 m*M* KCl were mixed with an equal volume of each Crystal Screen reagent on a glass slide for hanging-drop vapour diffusion and then equilibrated against 500 µl of the same reagent. Preliminary screening of the crystallization conditions using the Crystal Screen reagent kit suggested that polyethylene glycol (PEG) 4000 was an effective precipitant for lactonohydrolase. Subsequently, protein solutions of concentration 7 mg ml^{-1} were prepared in the presence of 3–25%(*w*/*v*) PEG 4000 as a precipitant in 100 m*M* buffer solutions in the pH range 5.0–8.9

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containing 1–100 m*M* KCl. Droplets (4 µl) of the protein solutions were pipetted onto glass slides and then equilibrated against 200 µl of a reservoir solution in which the concentration of the precipitant was higher than that of the protein solution. Crystallization was carried out at 277 or 293 K.

2.2. *Data collection*

Crystals were mounted in glass capillaries with a trace amount of mother liquor. For crystallographic characterization, precession and oscillation photographs were taken at room temperature with a Huber precession–rotation camera and a DIP100s imaging-plate camera system (Mac Science Co. Ltd, Tokyo), using graphite-monochromated Cu $K\alpha$ radiation generated by an M18X rotating-anode X-ray generator (MAC Science Co. Ltd) operated at 50 kV and 90 mA, with a fine-focus filament. The crystal-to-film distance was set at 150 mm. The X-ray diffraction patterns of the precession photographs were each recorded on a 125 × 125 mm imaging plate (Fuji Photo Film Co. Ltd, Tokyo), which was digitized at 50 µm intervals with an R-AXIS-DS read-out system (Rigaku Co. Ltd, Tokyo). The unit-cell dimensions were refined on oscillation photographs with the program *DENZO* (Otwinowski, 1986). A preliminary diffraction study was also performed at the Photon Factory at the National Laboratory for High Energy Physics, Tsukuba, using the X-ray beam [1.0 Å with an Si(111) monochromator system] from a 2.5 GeV synchrotron radiation source with a Weissenberg camera for protein crystallography (Sakabe, 1991). The imaging plates were digitized at 100 µm intervals with a Fujix BAS2000 read-out system (Fuji Photo Film Co. Ltd).

3. Results and discussion

Crystals of lactonohydrolase from *Fusarium oxysporum* suitable for an X-ray diffraction study were obtained using the following protocol. A 4 µl protein droplet, with a protein concentration of 7 mg ml^{-1} in 100 m*M* sodium citrate buffer (pH 6.2) containing 3.5 m*M* KCl and 11.0%(*w*/*v*) PEG 4000, was equilibrated against 0.2 ml of the same buffer solution containing 7 m*M* KCl and 21.5%(*w*/*v*) PEG 4000 at 277 K. The plate-like crystals grew to a maximum size of 0.7 × 0.3 × 0.1 mm in three weeks (Fig. 1).

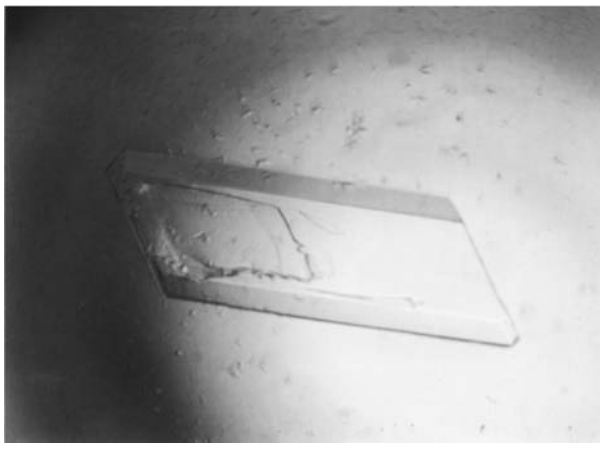

Fig. 1. A single crystal of lactonohydrolase from *Fusarium oxysporum*.

The crystals, which were sufficiently stable in the X-ray beam, diffracted laboratory-source X-rays and synchrotron radiation to about 3.5 Å and beyond 3.0 Å, respectively. The Laue symmetry and systematic absences of reflections on the precession photographs indicated that the crystals belong to the monoclinic space group $P2_1$. The unit-cell parameters were determined to be a = 156, b = 100, c = 94.1 Å, β = 91.7° by refinement of the reflections recorded on the oscillation photographs. Assuming that there are two lactonohydrolase dimer molecules in the asymmetric unit, the crystal volume per unit molecular mass, V_m, was calculated to be 2.94 Å^3 Da^{-1} (Matthews, 1968). A preliminary full set of native intensity data was collected with a DIP100 system using one crystal. The crystal was mounted in a capillary so that it could be rotated about the (010) axis. The total oscillation range of 180° was covered by oscillation photographs with a range of 4.5°. After averaging of symmetry-equivalent reflections, a total of 34535 unique reflections was obtained, which corresponds to 87% of the possible reflections of a crystal in the resolution range 50–3.5 Å. The merging R factor, defined as $R_{\rm merge} = \sum_h \sum_j |I_{hj} - \langle I\rangle_h| / \sum_h \sum_{hj} I_{hj}$, was 0.076 for 181754 measurements, where $\langle I\rangle_h$ is the mean intensity of reflection h and I_{hj} is the jth measurement of reflection h. A search for heavy-atom derivatives is in progress using the crystals obtained in order to establish the three-dimensional structure.

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