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Crystallization of a novel esterase which inactivates the macrolide toxin brefeldin A

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Abstract

A novel esterase obtained from *Bacillus subtilis* and capable of hydrolyzing the phytotoxin brefeldin A was crystallized using the hanging-drop technique. The crystals have two forms and both are monoclinic: form I, space group $P2_1$ with $a = 101.7$, $b = 64.1$, $c = 55.4$ Å and $\beta = 102.5$, and form II, space group $C2$ with $a = 140.7$, $b = 82.6$, $c = 81.5$ Å and $\beta = 112.5$. There are two molecules related by a pronounced non-crystallographic dyad per asymmetric unit in both crystal forms. The crystals diffract to 2.3 Å using a rotating-anode X-ray source.

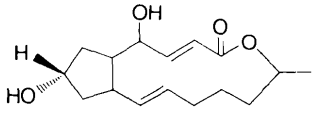
1. Introduction

Brefeldin A (BFA) is a phytotoxin produced by *Alternaria carthami* Chowdhury, the causal agent of a blight disease in safflower (Chowdhury, 1944). Although BFA is a non-host-specific toxin, it has been proposed to be the determining factor for pathogenicity of the fungus (Tietjen, Hammer & Matern, 1985). The mechanism by which this toxin acts in safflower is not known (Kneusel, Matern, Wray & Kloppel, 1990), but the results from other plant cells point to the structural and functional disruption of the Golgi complex (Driouich, Zhang & Staehelin, 1992). Since BFA at very low concentrations specifically inhibits the accumulation of phytoalexins in safflower (Tietjen & Matern, 1984), it appears to impair the endoplasmic reticulum–Golgi flux and processing, an effect that has also been observed in cultured mammalian cells (Hurtley, 1992). Traditional breeding of safflower for resistance has been unsuccessful, and adaptation of safflower cells to BFA failed (Matern & Tietjen, 1989). Another approach to introduce blight disease resistance into safflower would be the *in situ* elimination of BFA (Duering, Porsh, Fladung & Loerz, 1993). This requires identification and characterization of possible detoxifying enzymes. A novel esterase from *Bacillus subtilis* (BG3) was found (Kneusel, Schiltz & Matern, 1994) to be capable of hydrolyzing brefeldin A to non-toxic brefeldin A acid (see Fig. 1). The 40 kDa *B. subtilis* esterase does not require a cofactor for its activity and the presence of metal ions (Zn^{2+}, Mg^{2+}, Ca^{2+} or Fe^{2+}) has no effect on the catalytic rate. The estarase exists as a monomer and reaches its maximal catalytic activity, *in vitro*, at 310 K with physiological pH between 7.5 and 8.0 (Kneusel, Schiltz & Matern, 1994). The primary structure of the *B. subtilis* esterase, deduced from the DNA sequence, shows no similarity to any other known protein sequence (Kneusel, Schiltz & Matern, 1994). It is particularly noteworthy that the *B. subtilis* esterase is the first purified hydrolase that exhibits a specificity towards a macrolide (matrocyclic ester) substrate.

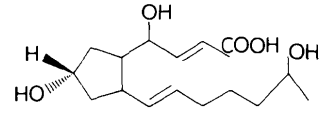
2. Experimental and results

The molecular characterization and cloning of the *B. subtilis* esterase have been reported previously (Kneusel, Schiltz & Matern, 1994). The purified protein was precipitated with 80% saturated ammonium sulfate and dialyzed against water prior to crystallization. The protein concentration of 7.0 mg ml^{-1} was determined using the BioRad protein assay (Bio-Rad kit II). Single crystals were obtained using the hanging-drop technique: 3 µl of the dialyzed protein solution were added to 3 µl of reservoir solution (45% saturated ammonium sulfate, 100.0 m*M* ammonium acetate, pH 5.7) and suspended over 1.0 ml of reservoir solution. The crystals took three weeks to reach 0.4 × 0.05 × 0.05 mm in size. A second crystal form, form II, was obtained under similar experimental conditions. Crystals of form II were grown in hanging drops at room temperature from 3.0 µl protien solution at 1.0 mg ml^{-1} and 3.0 µl of reservoir solution [40% saturated ammonium sulfate, 100.0 m*M* ammonium acetate, pH 6.0, 5.0 m*M* protassium phosphate, 0.5%(*w*/*v*) polyethylene glycol (PEG) 4000], suspended over 1.0 ml of reservoir solution. Crystals grew to a size of 0.5 × 0.4 × 0.3 mm within a few days.

The crystals were mounted and sealed in glass capillaries. They were further characterized using a Siemens X-1000 area detector mounted on a Siemens rotating-anode generator with a copper target and a graphite monochromator operating at 45 kV and 90 mA. Crystals of form I and form II diffract to approximately 2.8 and 2.3 Å, respectively. Preliminary X-ray data were collected from both forms of crystals for the purpose of identifying the crystal lattice and determining the unit-cell parameters. Two single crystals from each crystal form were used to collect X-ray experimental data at room temperature. The data were indexed automatically using the



(*a*)



(*b*)

Fig. 1. Schematic diagrams of the structure of (*a*) macrolide toxin brefeldin A (BFA) and (*b*) brefeldin A acid.

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XENGEN package (Howard, Gilliland, Finzel, Poulos, Ohlendorf & Salemme, 1987) and further verified by visually analyzing the reciprocal lattice using the *CCP*4 software *LCFPREC*. Form I crystals are monoclinic, space group $P2_1$, with cell dimensions $a = 101.7$, $b = 64.1$, $c = 55.4$ Å and $\beta = 102.5$. The standard crystallographic merging R factor for the data is 0.11 (on intensities). The unit cell of the form II crystals is monoclinic, space group $C2$, with $a = 140.7$, $b = 82.6$, $c = 81.5$ Å and $\beta = 112.5$. The data of crystal form II were processed using the *XENGEN* package (Howard, Gilliland, Finzel, Poulos, Ohlendorf & Salemme, 1987) giving an R_{merge} factor of 8.9%.

The calculation of the Matthews coefficient (Matthews, 1968) yields values of $V_m = 2.20$ and 2.72 Å^3 Da^{-1}, respectively, suggesting the presence of two molecules per asymmetric unit in each of the two crystal forms. We have analyzed the self-rotation function in search of any non-crystallographic symmetry elements. The calculation was carried out in spherical polar coordinates using the *CCP*4 program *POLARRFN* (Collaborative Computational Project, Number 4, 1994). The results presented in Fig. 2 show that a non-crystallographic twofold symmetry axis can easily be identified for the section of $\kappa = 180$. It is perpendicular to the crystallographic dyad and 40.5 away from the c axis of the form I. A similar non-crystallographic dyad was located 24.8 away from the crystal axis b in form II crystals.

The crystals of form II are more promising because of their reproducibility and better quality of X-ray diffraction. We are pursuing structure determination for the crystal form II by multiple isomorphous replacement method. A search for heavy-atom derivatives so far identified three useful derivatives. The resulting electron-density map is interpretable, and the model building is in progress.

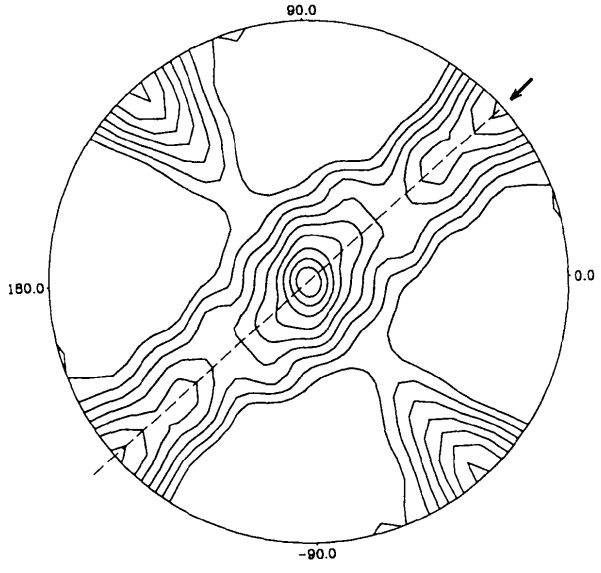

Fig. 2. A stereographic projection of the self-rotation function calculated using the *CCP4* program *POLARRFN*. The peak at the center corresponds to the crystallographic twofold symmetry along the b axis. The peak indicated by an arrow at $\varphi = 40.5$ and $\omega = 90.0$ is the non-crystallographic dyad and has a peak height 74% of the origin peak. The map was calculated using data in the resolution range 12–4.5 Å and a Patterson integration sphere of 4.0–12.0 Å.

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