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Crystallization and preliminary X-ray diffraction studies of tulip aryl acylamidase: a key enzyme in plant herbicide detoxification

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Abstract

Crystals of aryl acylamidase (E.C. 3.5.1.13) from tulip bulbs have been obtained by the hanging-drop vapor-diffusion method using polyethylene glycol (PEG) 8000 as a precipitant. The crystals belong to space group $P2_12_12_1$ with unit-cell dimensions $a = 68.7$, $b = 80.1$ and $c = 112.9$ Å. Assuming two molecules of molecular weight of 34 kDa in the asymmetric unit, V_m is 2.28 Å^3 Da^{-1}, indicating a solvent content of approximately 46%. The intensity data have been collected to 2.5 Å resolution with an $R_{\rm merge}$ of 0.067.

1. Introduction

Propanil (3′,4′-dichloropropionanilide) is an amide herbicide which has been utilized in some parts of the world for the control of weeds in paddy fields. The use of propanil in the field selectively kills weeds without damaging rice plants because rice can efficiently degrade the toxin by the action of the enzyme aryl acylamidase (AAA, E.C. 3.5.1.13). AAA catalyzes the hydrolysis of propanil to the non-toxic products 3,4-dichloroaniline (DCA) and propionic acid (see below) (Akatsuka, 1979).

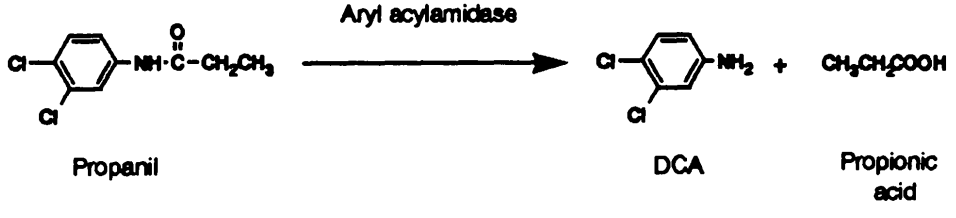
Rice AAA was first identified by McRae *et al.* in 1964 (McRae, Yih, & Wilson, 1964), but has proved to be unsuitable for X-ray crystallographic studies because it is a particle-bound enzyme which aggregates easily even with the use of detergents (Akatsuka, 1979) and because its amino-acid sequence has not been reported yet. Hoagland *et al.* have also reported the existence of an AAA in tulip bulbs (Hoagland & Graf, 1972). Hagiwara recently purified this enzyme, cloned the cDNA, and deduced the amino-acid sequence (Hagiwara, 1996, 1997). Tulip AAA shows no significant sequence homology to any protein sequences available from the Swiss-Prot, PIR, GenPept and PDB databases.

AAA's which utilize amide compounds other than propanil as substrates have been identified in mammalian tissues and in microorganisms. In mammals, AAA's have been partially purified from monkey brain (Oommen & Balasubramanian, 1979) and from human liver (George & Balasubramanian, 1981), whilst in microbes AAA's from *Pseudomonas pickettii* (Hirase & Matsunaka, 1991), *Pseudomonas fluorescens* (Hammond, Price, & Scawen, 1983), and *Nocardia globerula* (Yoshioka, Nagasawa, & Yamada, 1991) have been isolated. However, to date no three-dimensional structures of AAA's have been determined, and little is known about their detailed mode of action, their amino-acid sequences and in particular about the identity of their active-site residues. Hydrolysis of propanil by rice AAA is, however, known to be inhibited by acetylcholinesterase inhibitor insecticides such as parathion and paraoxon (Matsunaka, 1968), thus indicating that there is a serine residue in the active site. In order to facilitate clarification of the catalytic mechanism of AAA's it is first essential to determine the three-dimensional structure of one of the enzymes and to identify its active-site residues. To this end we here report the crystallization of tulip AAA, together with preliminary X-ray crystallography results and intensity data measurements.

2. Methods

2.1. *Protein purification*

Tulip (*Tulipa gesneriana*) bulbs were purchased from a local garden center, and AAA was purified from them using the following procedure. Tulip bulbs (2 kg) were homogenized in 5 l of 30 m*M* sodium phosphate buffer (pH 7) containing 4%(*w*/*v*) polyclar SB100 using an Ultra Turrax blender. The homogenate was filtered through two layers of cheesecloth, then centrifuged at 6 800*g* for 30 min. Solid ammonium sulfate was added to the supernatant to 35% saturation and the resultant precipitate was removed by centrifugation. The supernatant was treated by further addition of ammonium sulfate to 60% saturation and the precipitate was collected by centrifugation and resuspended in the homogenization buffer. This was dialyzed against the buffer before initial purification using a DEAE-Cellulose DE52 column (5 × 24 cm) equilibrated with the same buffer. Elution was performed using a linear gradient of 0–1 *M* NaCl in homogenization buffer, and the active fractions were pooled and treated with solid ammonium sulfate to 35% saturation. This preparation was applied to a Butyl - Toyopearl column (2.5 × 18 cm) equilibrated with homogenization buffer saturated with 35% ammonium sulfate, and the enzyme was eluted with a linear gradient of 35–0% ammonium sulfate in homogenization buffer. Active fractions were pooled to yield pure AAA which was homogeneous as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis



(SDS–PAGE) analysis (Fig. 1). This procedure typically yielded approximately 200 mg of enzyme from 2 kg of tulip bulbs, and details of the protein purification will be described elsewhere (Hagiwara 1996, 1997).

2.2. *Crystallization methods*

Crystallization trials were performed using the hanging-drop vapor-diffusion method (McPherson, 1982), with a droplet consisting of 2 µl of protein solution and 2 µl of precipitant reservoir solution. Reservoir solutions were initially screened using complete reagent kits (Crystal Screen I and Grid Screen A/S, both from Hampton Research), and a reservoir solution comprising 0.1 *M* sodium cacodylate buffer (pH 7), 0.2 *M* sodium acetate, and 30% PEG 8000 was selected. Protein solution concentration was varied from 13 to 104 mg ml^{-1} using ultrafiltration of samples, assuming an OD_{280} of 1.0 for a 1 mg ml^{-1} solution. In this way a number of screening trials were perfomed.

2.3. *X-ray diffraction methods*

Preliminary X-ray intensity data were collected at beamline 6A2 of the Photon Factory synchrotron facility of the National Laboratory for High Energy Physics, Tsukuba, Japan. The X-ray beam used gave 1.00 Å radiation and the collimator was 0.1 mm. Intensity data were collected on 200 × 400 mm imaging plates (Fuji Film) using a Weissenberg camera for macromolecules (Sakabe, 1991) with radius size of 430 mm. The data were digitized using a Fuji BA100 image analyzer, and the digital data were processed by *DENZO* (Otwinowski, 1993), and scaled with *SCALEPACK* (Otwinowski, 1993).

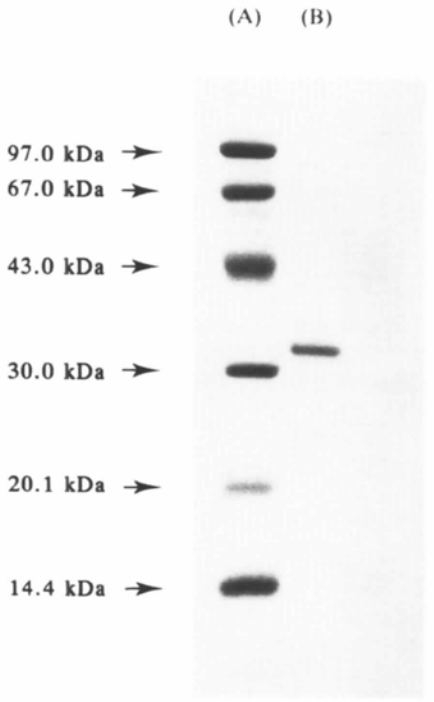


Fig. 1. SDS–PAGE of purified tulip AAA. Electrophoresis of standard proteins (lane *A*) and tulip AAA (lane *B*) was performed using 12.5% polyacrylamide gels.

3. Results and discussion

Initial trials for the crystallization of tulip AAA using ammonium sulfate as the precipitating reagent were unsuccessful. The results of screening experiments with PEG 8000 as the precipitant led, for the first time, to the growth of thin crystals after three weeks at 292 K. Further screening resulted in the production of thicker crystals up to 0.06 × 0.08 × 0.28 mm in size (Fig. 2). Crystals grew in approximately three weeks at 292 K, and the optimal protein concentration was found to be approximately 65 mg ml^{-1}.

X-ray intensity data for the crystals were measured through 14 oscillation photographs covering a total of 98.5° rotation. The crystals diffracted efficiently, considering their small size, to 2.5 Å resolution. The autoindexing routine of *DENZO* (Otwinowski, 1993) indicated that the crystals belong to the orthorhombic crystal system with unit-cell dimensions of $a = 68.7$, $b = 80.1$ and $c = 112.9$ Å. Based on systematic absences observed for the reflections $h00$, $h2n$, $0k0$, $k2n$ and $00l$, $l2n$, the space group was determined to be $P2_12_12_1$. The 46 430 intensities were merged into a set of 16 588 unique reflections with an R_{merge} of 0.067 [$R_{merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $\langle I(h)\rangle$ is the average intensity of the i observations of reflection h]. The native data with $I > \sigma(I)$ were 74.6% complete in the resolution range 20–2.5 Å. Heavy-atom screenings to obtain useful heavy-atom derivatives are now in progress.

Tulip AAA is an enzyme with a molecular weight of 34 kDa consisting of 349 amino-acid residues. Assuming two molecules in the asymmetric unit, the crystal volume per molecular weight (V_m) was calculated as 2.28 Å^{3}Da^{-1} indicating a solvent content of approximately 46% in the unit cell (Matthews, 1968).

Few crystallographic studies of herbicide detoxifying enzymes have been reported. Reinemer *et al.* recently determined the three-dimensional structure of glutathione S-transferase from the plant *Arabidopsis thaliana*, and provided a structural characterization of this herbicide-conjugating enzyme (Reinemer *et al.*, 1996). The present crystallographic study forms the basis for the elucidation of the mechanism of another herbicide detoxification system in plants, and should thus provide a useful insight into the structure-based design of ecologically tolerated herbicides.

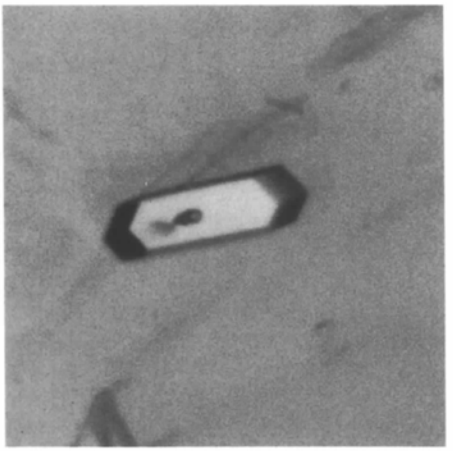


Fig. 2. Typical crystal of tulip AAA.

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