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Purification, crystallization and preliminary X-ray diffraction analysis of pathogen-inducible oxygenase (PIOX) from *Oryza sativa*

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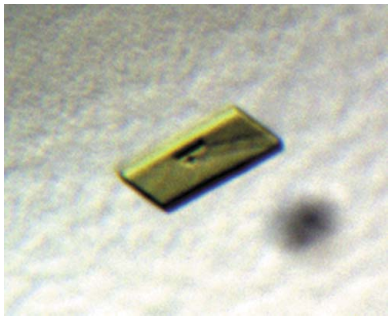

Pathogen-inducible oxygenase (PIOX) is a heme-containing membrane-associated protein found in monocotyledon and dicotyledon plants that utilizes molecular oxygen to convert polyunsaturated fatty acids into their corresponding 2*R*-hydroperoxides. PIOX is a member of a larger family of fatty-acid α-dioxygenases that includes the mammalian cyclooxygenase enzymes cyclooxygenase 1 and 2 (COX-1 and COX-2). Single crystals of PIOX from rice (*Oryza sativa*) have been grown from MPD using recombinant protein expressed in *Escherichia coli* and subsequently extracted utilizing decyl maltoside as the solubilizing detergent. Crystals diffract to 3.0 Å resolution using a rotating-anode generator and R-AXIS IV detector, and belong to space group *P*1. Based on the Matthews coefficient and self-rotation function analyses, there are presumed to be four molecules in the asymmetric unit related by noncrystallographic 222 symmetry.

1. Introduction

Several enzyme systems are activated as a result of pathogen attack on plants, including phospholipase, lipoxygenase, allene oxide synthase and divinyl ether synthase. These enzymes produce oxylipins, bioactive lipid mediators from 18–22 carbon polyunsaturated fatty-acid (PUFA) substrates that play key roles in a plant's defense reaction against pathogens (Hammond-Kosack & Jones, 1996; Creelman & Mullet, 1997). In 1998, Sanz and colleagues identified a 75 kDa oxygenase, termed PIOX (pathogen-induced oxygenase), that was induced upon the infection of tobacco leaves with the bacterium *Pseudomonas syringae* (Sanz *et al.*, 1998). Subsequent analysis of the biological function revealed that PIOX catalyzed a non-lipoxygenase type of PUFA oxidation. Further database searches have identified PIOX in other plants, including other tobacco species (*Nicotiana attenuata*; Hermsmeier *et al.*, 2001), mustard seed (*Arabidopsis thaliana*; Sanz *et al.*, 1998; Liu *et al.*, 2004), rice (*Oryza sativa*; Koeduka *et al.*, 2002), pepper (*Capsicum annuum*; Kim *et al.*, 2002) and tomato (*Lycopersicon esculentum*; Tirajoh *et al.*, 2005). PIOX belongs to a larger family of heme-containing fatty-acid α-dioxygenases that includes the mammalian cyclooxygenases (COX-1 and COX-2; Hamberg *et al.*, 2002), linoleate diol synthase (LDS) from the fungus *Gaeumannomyces graminis* (Brodowsky *et al.*, 1992; Su & Oliw, 1996) and a protein of unknown function encoded by the open reading frame OrfX from *P. alcaligenes* (Gerritse *et al.*, 1998). While it was previously thought that plants do not possess a homolog equivalent to the COX enzymes found in mammals, analysis of the sequence of PIOX and other members of the fatty-acid α-dioxygenase family indicate potential structural and catalytic similarities (Hamberg *et al.*, 2002).

PIOX converts linoleic acid, α-linolenic acid, arachidonic acid and other PUFAs to their corresponding 2*R*-hydroperoxides *via* stereoselective dioxygenation (Sanz *et al.*, 1998; Hamberg *et al.*, 1999; Koeduka *et al.*, 2002). The resulting 2*R*-hydroperoxides undergo spontaneous decarboxylation to shorter aldehydes and fatty acids (Hamberg *et al.*, 1999; Koeduka *et al.*, 2002). The stereoselective dioxygenation catalyzed by PIOX differs from that observed for COX and other plant lipoxygenases in that an unsaturated region of the PUFA substrate is not targeted for oxygen addition (Hamberg *et al.*, 1999; Liu *et al.*, 2004). Many residues crucial for activity in

Table 1
Data-collection statistics from *SCALA*.

Values in parentheses are for the last shell of data.

Wavelength (Å)	1.54
Resolution (Å)	3.00 (3.10–3.00)
Measured reflections	216724
Unique reflections	54569
Multiplicity	3.97 (3.98)
Average $I/\sigma(I)$	7.79 (3.50)
$R_{\rm merge}$ (%)	9.00 (19.00)
Completeness (%)	97.00 (96.00)

mammalian COX are conserved in PIOX. These residues include the distal and proximal histidine residues involved in heme binding and a catalytic tyrosine which is responsible for the initiation of catalysis (Sanz *et al.*, 1998; Koeduka *et al.*, 2002; Garavito *et al.*, 2002; Liu *et al.*, 2004). The complete conservation of these residues within the fatty-acid α-dioxygenase family suggests that the dioxygenations carried out by PIOX are initiated by the conserved tyrosine and that all of the members bind a heme group (Hamberg *et al.*, 2002).

While there are global differences between PIOX and COX enzymes with respect to their potential overall structural architecture, substrate specificities and signaling pathways, the mechanisms by which these enzymes stereoselectively oxygenate PUFAs are strikingly similar. Therefore, elucidation of the structure of PIOX and the subsequent mechanism controlling catalysis will enhance the existing knowledge of how PUFAs are stereoselectively oxygenated and how this oxygenation is controlled. This information will also provide useful insights into other systems besides COX enzymes which discriminate between the saturation and chain length of a PUFA substrate, such as lipoxygenases (Brash, 1999; Kuhn, 2000; Schneider & Brash, 2002; Coffa & Brash, 2004), cytosolic phospholipase A_2 (Clark *et al.*, 1995) and lysophospholipid acyl transferases (Reitz *et al.*, 1969). We report here the purification, solubilization, crystallization and data collection of rice PIOX.

2. Methods

2.1. Protein purification

The expression vector containing the cDNA encoding for PIOX from rice (*O. sativa*) was obtained from Dr Kenji Matsui of the Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan. PIOX was expressed and purified according to the procedure reported previously (Koeduka *et al.*, 2002) with significant modifications to the purification protocol which allowed the isolation of larger quantities of homogeneous protein required for subsequent crystallization trials. Briefly, decyl maltoside (C_{10}M) was added to the cell lysate from a 6 l growth to a final concentration of 0.7%(*w*/*v*) and stirred for 60 min at 277 K to solubilize the protein. The detergent-solubilized mixture was then ultracentrifuged for 45 min at 277 K, followed by passing the clarified supernatant through a 0.22 µm filter. The filtered supernatant was applied onto a Talon immobilized metal-affinity column (10 ml bed volume) equilibrated with buffer *A* [50 m*M* sodium phosphate pH 7.0, 300 m*M* NaCl, 0.7%(*w*/*v*) C_{10}M]. PIOX was eluted from the column in buffer *A* containing 100 m*M* EDTA, which strips the metal (and subsequent bound protein) from the column support matrix. Fractions containing O_2-uptake activity (measured as described in Koeduka *et al.*, 2002) were pooled and dialyzed overnight at 277 K against 50 m*M* Bis-Tris pH 7.0, 300 m*M* NaCl and 0.7%(*w*/*v*) C_{10}M to remove cobalt and EDTA and to initiate detergent exchange. At this point, either 0.1%(*w*/*v*) C_{10}M or 0.5%(*w*/*v*) β-octylglucoside (β-OG) can be used as the crystallization detergent. Both have been used successfully to generate diffraction-quality crystals. The dialyzed protein was then concentrated to ∼2 ml using a Millipore Ultrafree-15 spin concentrator with a 50 kDa nominal molecular-weight cutoff and loaded onto a HiPrep Sephacryl S-300 HR 16/60 gel-filtration column (16 mm diameter and 120 ml bed volume) equilibrated with 50 m*M* Bis-Tris pH 7.0, 150 m*M* NaCl and either 0.1%(*w*/*v*) C_{10}M or 0.5%(*w*/*v*) β-OG to finalize detergent exchange and remove aggregates.

2.2. Crystallization

Peak fractions were pooled based on O_2-uptake activity and sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then concentrated to 5–10 mg ml^{-1} for crystallization trials. Initial crystallization screening was carried out using the microbatch-under-oil method in conjunction with the High Throughput Crystallization Screening Robot located within the Hauptman–Woodward Institute (Luft *et al.*, 2003; http://www.hwi.buffalo.edu/). To supplement the search for initial crystallization leads, commercial screens from Hampton Research (Crystal Screens 1 and 2, MembFac, PEG/Ion, PEG/LiCl and MPD) were set up using the sitting-drop vapor-diffusion method. For optimization, the sitting-drop vapor-diffusion method was employed with experimental drop volumes of 5 µl and reservoir solution volumes of 1 ml. Drops were prepared with a 1:1(*v*:*v*) ratio of protein:precipitant solution. All crystallizations were carried out and stored at 294 K.

2.3. Data collection and analysis

Diffraction data were collected on a Rigaku R-AXIS IV image-plate detector mounted on an RU-H3RHB 18 kW rotating-anode X-ray generator (Cu target) equipped with Osmic Max-Flux confocal multi-layer mirrors and an X-Stream cryogenic system. Crystals were removed from sitting drops and directly flash-frozen in the nitrogen stream (100 K). Individual 1° oscillation frames were collected over a range of 360°. Data were initially processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and subsequently with *MOSFLM* (Leslie, 1992) combined with *SCALA* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). Self-rotation function and Matthews coefficient calculations were performed using *MOLREP* (Vagin & Teplyakov, 1997) and *Cell Content Analysis* in *CCP*4. Data-collection statistics are given in Table 1.

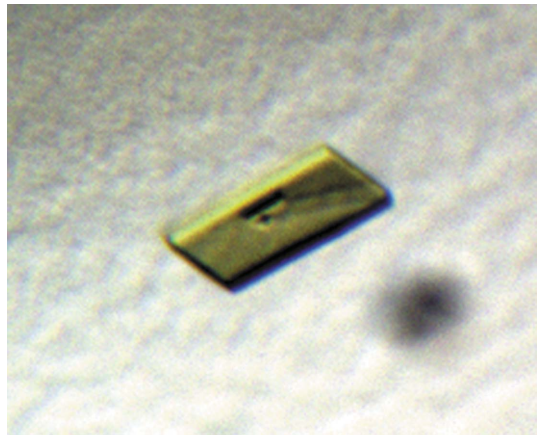

Figure 1
Crystals of rice PIOX grown using C_{10}M as the crystallization detergent.

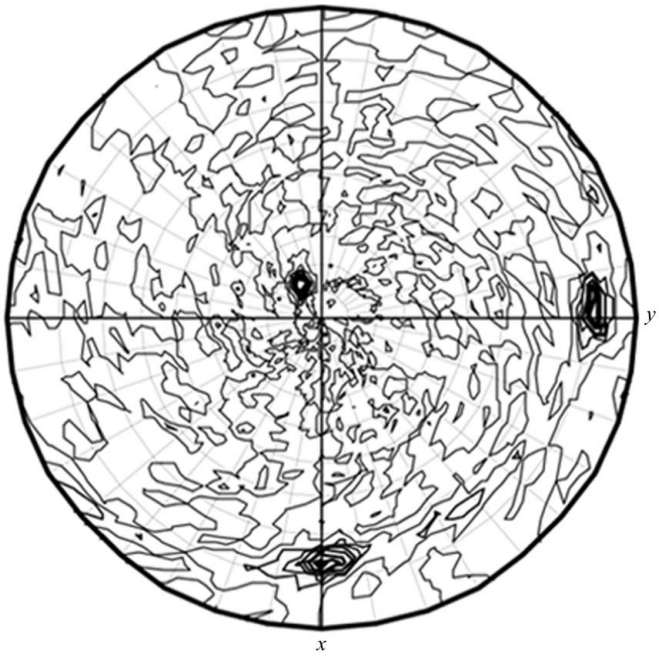

Figure 2
Stereographic projection of the self-rotation function in spherical polar angles plotting θ/φ at $\chi = 180^\circ$. The three orthogonal peaks $\sim 90^\circ$ apart indicate the presence of noncrystallographic 222 symmetry and four PIOX monomers in the asymmetric unit.

3. Results and discussion

Purified PIOX was found to be homogeneous as analyzed by SDS–PAGE and size-exclusion chromatography (data not shown). Over 50 hits were identified in the initial crystallization screening experiments carried out using the HWI crystallization robot. Analysis of the hits revealed common features: (i) low-molecular-weight polyethylene glycol (PEG 400 or PEG 1K) or 2-methyl-4-pentanediol (MPD) as the precipitant, (ii) a pH range of 8.5–10.5 and (iii) 50–150 m*M* of a monovalent salt (NaCl, KCl, LiCl). The conditions were further refined through the construction of standard grid screens using the above identified variables. The largest single crystals could be reproducibly grown in sitting drops by combining 2.5 µl protein solution (5–10 mg ml^{-1}) with 2.5 µl of 45, 50 or 55%(*w*/*v*) MPD supplemented with 100 m*M* Bicine pH 9.0, 100 m*M* KCl and either C_{10}M or β-OG [at final concentrations of 0.05 and 0.33%(*w*/*v*), respectively] as the crystallization detergent. These 5 µl drops were subsequently equilibrated over reservoir solutions consisting of 50–65%(*w*/*v*) MPD, 100 m*M* Bicine pH 9.0 and 50–150 m*M* KCl. PIOX crystals routinely grow to maximum dimensions of 0.2 × 0.2 × 0.05 mm over the course of four weeks (Fig. 1). The optimized crystallization conditions proved ideal for cryopreservation owing to the high concentration of MPD used as the precipitating agent. Hence, crystals could be easily removed from the sitting drops and directly flash-frozen in a stream of cold nitrogen gas.

The cryocooled PIOX crystals diffracted weakly to 3.0 Å, but were sufficiently stable in the X-ray beam to allow a complete data set to be collected. The indexing and scaling routines of both *DENZO*/*SCALEPACK* and *MOSFLM*/*SCALA* identified the space group as *P*1, with unit-cell parameters $a = 74.70$, $b = 90.42$, $c = 119.44$ Å, $\alpha = 69.67$, $\beta = 73.64$, $\gamma = 88.40^\circ$. Unsuccessful attempts at processing the data in other crystal systems and space groups further validated this solution. Self-rotation functions indicate that there are likely to be four PIOX molecules in the asymmetric unit related by 222 noncrystallographic symmetry (Fig. 2), which is in agreement with the calculated values of 2.5 Å^3 Da^{-1} and 51% for the Matthews coefficient and solvent content, respectively. Initial attempts to solve the structure by molecular replacement using a monomer of ovine COX-1 (PDB code 1diy; Malkowski *et al.*, 2000) as the search model proved unsuccessful. Both COX isoforms share ∼12% sequence identity with PIOX and to date are the most homologous search models available. Structure solution utilizing multiple isomorphous replacement and multiwavelength anomalous dispersion methods are currently being pursued.

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