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Crystallization and preliminary X-ray diffraction analysis of chloroplastic ascorbate peroxidase of tobacco plants

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The stromal ascorbate peroxidase of tobacco plants was crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 4000 as a precipitant. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 37.2, b = 76.8, c = 98.8 Å. The calculated V_M value based on a monomer in the asymmetric unit is 2.2 Å^3 Da^{-1}. A data set was successfully collected to 1.6 Å resolution from a frozen crystal using synchrotron radiation of wavelength 1.0 Å at KEK-PF, Japan.

1. Introduction

Ascorbate peroxidase (APX; EC 1.11.1.11) is a haem-containing enzyme that plays a central role in scavenging H_2O_2 to protect photosynthetic organisms from oxidative stress (Asada, 1992; Shigeoka *et al.*, 2002). APX isoenzymes are distributed in at least four distinct cellular compartments: the stroma (sAPX) and thylakoid membrane (tAPX) in chloroplasts (Miyake *et al.*, 1993; Asada, 1997), the microbody membrane (mAPX; Yamaguchi *et al.*, 1995; Bunkelmann & Trelease, 1996; Ishikawa *et al.*, 1998) and the cytosol (cAPX; Mittler & Zilinskas, 1991; Ishikawa *et al.*, 1996). Recently, a novel APX isoenzyme was found in mitochondria from potato tuber (Leonardis *et al.*, 2000). Interestingly, two chloroplastic APX isoenzymes, sAPX and tAPX, are encoded by only one gene in various plant species such as spinach (Ishikawa *et al.*, 1997; Yoshimura *et al.*, 1999), pumpkin (Mano *et al.*, 1997), *Mesembryanthemum crystallinum* (accession Nos. AF069315, tAPX; AF069316, sAPX) and tobacco (AB022273, tAPX; AB022274, sAPX). Since the mRNAs corresponding to these chloroplastic APX isoenzymes are generated by the alternative splicing of the 3′-terminal introns, the chloroplast transit sequence and the catalytic active domain of sAPX are identical to those of tAPX except for the C-terminal transmembrane domain. Therefore, chloroplastic APX isoenzymes have the same enzymological properties (Yoshimura *et al.*, 1998).

One of the characteristics of chloroplastic APX isoenzymes is their rapid inactivation under conditions where ascorbate (AsA) is absent (Asada, 1992; Shigeoka *et al.*, 2002). These enzymes lose their activity within several minutes when the concentration of AsA is kept below 20 μ*M* (Miyake & Asada, 1996). In contrast, mAPX and cAPX remain active for at least several hours at these concentrations of AsA (Chen & Asada, 1989; Yoshimura *et al.*, 1998). Inactivation of APX isoenzymes is believed to result from decomposition of compound I, which is a high oxidation state intermediate comprised of an oxyferryl haem and a free radical on a porphyrin molecule, [Fe^{IV}═O(porphyrin$^{\cdot}$)].

The three-dimensional structure of the recombinant cAPX of pea has been determined by X-ray crystallography (Patterson & Poulos, 1995). However, the structure of a chloroplastic APX isoenzyme has yet to be reported. Since chloroplastic APX isoenzymes have the same sequence except for part of the C-terminus, it is likely that the three-dimensional structure of sAPX reflects that of tAPX. Here, we report the crystallization and preliminary X-ray crystallographic analysis of sAPX from tobacco plants. X-ray analysis of the enzyme will provide insight into the cause of the rapid inactivation in the absence of AsA and a basis for further studies of the structure–function relationship of APX isoenzymes.

2. Materials and methods

2.1. Overexpression and purification of the recombinant sAPX

Recombinant sAPX of tobacco plants was overexpressed in *Escherichia coli*. The DNA fragments encoding the mature form were amplified by PCR using primers with overhanging restriction sites. The gene encodes a 295-residue haem enzyme with a molecular mass of 33 kDa. The PCR product was cloned downstream of the T7 promoter in the expression vector pET-3a (Novagen). PCR, restriction digestion, ligation reactions and bacterial transformations were performed according to standard protocols. Competent *E. coli* BL21(DE3)pLysS cells carrying

pET-3a/sAPX were cultured in 50 ml Luria–Bertani (LB) medium supplemented with ampicillin and chloramphenicol at 310 K. After overnight culture, the cells were inoculated into fresh medium (2 l). Expression was induced by the addition of 0.5 m*M* isopropyl β-D-thiogalactopyranoside when the absorbance at 600 nm reached 0.6; cultivation was continued for a further 8 h at 310 K. The cells were harvested, resuspended in 30 ml 50 m*M* potassium phosphate pH 7.0 containing 1 m*M* AsA and 1 m*M* EDTA (buffer *A*) and sonicated. The cellular debris was removed by centrifugation and the supernatant was loaded onto a HiLoad 16/10 DEAE Sepharose ion-exchange column (Amersham-Pharmacia) equilibrated with buffer *A* and eluted with a linear gradient of NaCl (0–0.3 *M*). The active fractions were subjected to ammonium sulfate precipitation at 30% saturation. The precipitates were removed by centrifugation and the supernatant was loaded onto a HiLoad 16/10 phenyl Sepharose column (Amersham-Pharmacia) equilibrated with buffer *A* containing ammonium sulfate (30% saturation) and eluted with a descending linear gradient (30–0% saturation) of ammonium sulfate. After the ammonium sulfate in the active fractions was removed by excess dialysis against buffer *A*, the resulting enzyme solution was applied to an UNO-Q HR 5/5 column (Bio-Rad) equilibrated with buffer *A* and eluted with a linear gradient of NaCl (0–0.3 *M*). The active fractions were collected and the purity was confirmed by SDS–PAGE.

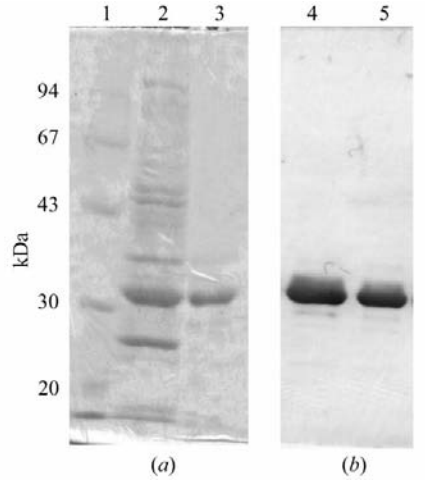

Figure 1
Expression and purification of sAPX of tobacco plants were analyzed by SDS–PAGE and immunoblots. (*a*) Coomassie brilliant blue staining. (*b*) Immunoblots using monoclonal antibody against chloroplastic APX isoenzymes. Lane 1, molecular mass standards (Pharmacia); lanes 2 and 4, crude enzyme; lanes 3 and 5, purified enzyme.

2.2. Crystallization

For crystallization, the purified protein was dialyzed against 50 m*M* HEPES buffer pH 7.0 containing 1 m*M* AsA. All crystallization trials were carried out using the hanging-drop vapour-diffusion method at 277 K. Crystallization conditions were screened using the sparse-matrix screening kits Crystal Screens I and II from Hampton Research (Jancarik & Kim, 1991). Each hanging drop was prepared by mixing 1 µl of 8 mg ml^{-1} sAPX solution with 1 µl of various reservoir solutions and equilibrated against 0.5 ml of the respective reservoir solution. The initial conditions found to be the best were optimized by varying the pH and the concentrations of protein and precipitant. We also optimized the protein: reservoir ratio.

2.3. Data collection and analysis

Preliminary X-ray diffraction studies were carried out using an R-AXIS IIc image-plate area detector mounted on a Rigaku RU-300 rotating-anode source operating at 40 kV, 100 mA with Cu $K\alpha$ radiation. A complete data set was collected at 100 K on an ADSC CCD detector using synchrotron radiation of wavelength 1.0 Å at the BL18B station of KEK-PF, Japan. The crystal-to-detector distance was 80 mm and 180 images were recorded at 1° intervals with an exposure time of 180 s per image. The intensity data were processed with the program *MOSFLM* (Steller *et al.*, 1997) and scaled using the program *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The purification of the enzyme yielded approximately 2 mg of protein from a 2 l culture and the purity was evaluated to be >95% on Coomassie Brilliant-blue stained gels (Fig. 1*a*). The purified enzyme gave a red colour and its absorption spectrum was close to that of the native enzyme. Thus, we considered that the purified enzyme contains a haem as does the native enzyme. The enzyme was identified by immunoblot analysis (Fig. 1*b*) using a monoclonal antibody chl-mAb6 that specifically recognizes the chloroplastic APX isoenzymes (Yoshimura *et al.*, 2001). In the initial crystallization trials, small crystals were obtained from

Figure 2
A typical crystal of the sAPX of tobacco plants. Crystal dimensions are approximately 0.3 × 0.1 × 0.05 mm.

drops containing polyethylene glycol (PEG) 4000 as a precipitant. The crystallization conditions finally established were a protein solution of 6 mg ml^{-1} in 50 m*M* HEPES buffer pH 7.0 containing 1 m*M* AsA equilibrated against a reservoir solution consisting of 28%(*w*/*v*) PEG 4000, 0.2 *M* $MgCl_2$ and 0.1 *M* Tris–HCl pH 7.8. A 3 µl drop consisting of 2 µl protein solution and 1 µl reservoir solution was equilibrated against 0.5 ml of the above-mentioned reservoir solution. Crystals were formed within 4 d and grew to maximum dimensions of approximately 0.3 × 0.1 × 0.05 mm in one week (Fig. 2).

Crystals mounted in thin-walled glass capillaries were easily damaged and only diffracted to about 2.4 Å resolution when exposed to Cu $K\alpha$ radiation at room temperature. To avoid this damage, the

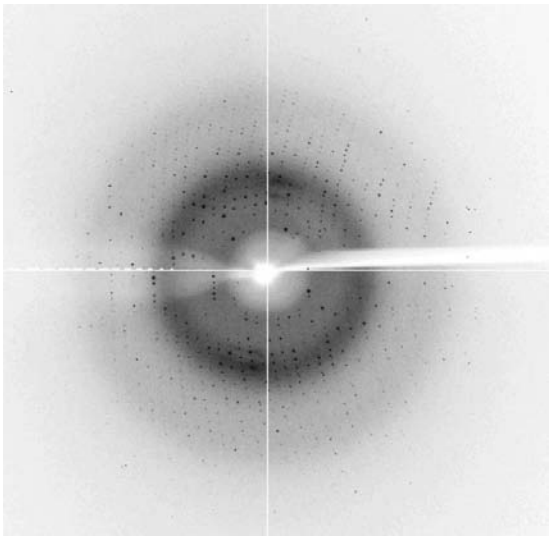

Figure 3
A diffraction image of the sAPX of tobacco plants. Measurements were performed at 100 K with synchrotron radiation at BL18B, KEK-PF, Japan.

Table 1
Summary of crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell (1.69–1.60 Å).

Resolution (Å)	1.6
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 37.2, b = 76.8, c = 98.8
R_{merge} (%)	6.6
$I/\sigma(I)$	8.0 (3.0)
No. of reflections	1018666
Unique reflections	38177
Completeness (%)	99.9 (99.9)
Mosaicity (°)	0.3

crystals were loop-mounted in a cryoprotectant solution containing 20% glycerol as a cryosolvent and frozen in a nitrogen-gas stream at 100 K. As a result, a complete data set was collected to a resolution of 1.6 Å using synchrotron radiation. A typical X-ray diffraction pattern of the sAPX is shown in Fig. 3. The crystals were determined to belong to the orthorhombic space group $P2_12_12_1$ based on the symmetry and the systematic absences of reflections, with unit-cell parameters a = 37.2, b = 76.8, c = 98.8 Å. Detailed crystal parameters and data-collection statistics are shown in Table 1. Assuming a monomer in the asymmetric unit, the V_M value as defined by Matthews (1968) was 2.2 Å^3 Da^{-1}, corresponding to a solvent content of 44%.

Molecular-replacement calculations were carried out using the program *MOLREP* implemented in *CNS* (Brünger *et al.*, 1998), using the complete structure of the cAPX monomer from pea (249 residues; PDB code 1apx) as a search model. The amino-acid sequence of sAPX from tobacco was 44.1% identical to cAPX from pea. A clear peak was found with a correlation coefficient of 36.7 (8–4 Å) after translation-function calculations. Subsequently, rigid-body refinement (8–1.6 Å) resulted in an R factor of 40.2% and an R_{free} of 44.3%, where R_{free} is the R factor based on 10% of the native data withheld from refinement. There were no unfavourable molecular contacts observed in the crystal packing. Model building by manual fitting to the electron-density map using the program *O* (Jones *et al.*, 1991) is now under way.

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