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Purification, crystallization and preliminary X-ray diffraction study of a recombinant cytokinin oxidase from *Zea mays*

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Cytokinins are hormones that are involved in plant growth and development. They are irreversibly degraded by cytokinin oxidases/dehydrogenases, flavoenzymes which contain a covalently bound flavine adenine dinucleotide (FAD) cofactor. Cytokinin oxidase from *Zea mays* (*Zm*CKO1) was overexpressed in the yeast *Yarrowia lipolytica*, purified (molecular weight 69 kDa) and crystallized using the hanging-drop method. Crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 250.6$, $b = 50.6$, $c = 51.5$ Å, $\beta = 94.1^\circ$. A complete data set has been collected at 100 K to 1.95 Å resolution on an X-ray synchrotron source.

1. Introduction

Cytokinins are N^6-substituted purine derivatives that regulate cell division as well as a variety of plant developmental processes, such as shoot and root branching, leaf expansion, chloroplast ripening and others. Cytokinins are irreversibly degraded by cytokinin oxidases, which cleave the N^6-unsaturated side chain from the adenine/adenosine moiety, releasing the corresponding aldehyde. The reaction proceeds through the formation of an unstable imine intermediate (Laloue & Fox, 1985), either in the presence of oxygen or other electron acceptors, *e.g.* quinones (Frébortová *et al.*, 2004). The cloning of a maize cytokinin oxidase/dehydrogenase gene (*CKO1*/*CKX1*) was achieved by Houba-Hérin *et al.* (1999) and Morris *et al.* (1999). The deduced amino-acid sequence contained a characteristic flavoprotein domain, a signal peptide and eight possible glycosylation sites. The recombinant enzyme was shown to be secreted by moss and yeast cells. Bilyeu *et al.* (2001) confirmed that a FAD cofactor was covalently linked to the recombinant enzyme produced in *Pichia pastoris*. So far, there is no known three-dimensional structure of a cytokinin oxidase. To gain insight into the substrate binding and conversion, the crystal structure would be highly desirable. It may also reveal the existence of separate or combined binding sites for oxygen and other electron acceptors. Here, we describe the purification, crystallization and preliminary X-ray diffraction study of the recombinant cytokinin oxidase CKO1 from *Zea mays* (516 amino-acid residues, molecular weight 69 kDa).

2. Enzyme expression and purification

The putative 18-amino-acid signal peptide of CKO1 was replaced with the prepropeptide of *Yarrowia lipolytica* alkaline extracellular protease according to Massonneau *et al.* (2004). The *CKO1* DNA fragment was PCR-amplified from an *Escherichia coli* plasmid construct (Houba-Hérin *et al.*, 1999) and cloned into the *Y. lipolytica* expression vector pINA1267. The transformed yeasts were grown in 720 ml minimal PPB medium (Madzak *et al.*, 2000) in 50 m*M* phosphate buffer pH 6.8 at 301 K for 5 d. The recombinant enzyme was recovered from the culture medium. All purification steps were performed at 277 K.

The cultures were centrifuged at 10 000*g* for 2 h. Trypsin inhibitor (400 mg l^{-1}) from soybean (Sigma) was added to the supernatant as a carrier for subsequent precipitation with $(NH_4)_2SO_4$ at 70% saturation. The precipitate was removed by centrifugation (10 000*g* for 2 h). The pellet was then resuspended in T buffer (20 m*M* Tris–HCl pH 8.0) and the solution was centrifuged again at 30 000*g* for 1 h. The supernatant was loaded onto a Sephacryl S-200 column (Pharmacia) equilibrated with T buffer. The collected fractions were assayed for activity using the 2,6-dichlorophenolindophenol test according to Bilyeu *et al.* (2001). Fractions with CKO activity were pooled and loaded onto an FPLC anion-exchange column (ResourceQ, Pharmacia). Elution was performed with a 0–1 *M* NaCl gradient in T buffer. The purity was verified by gel-permeation chromatography on a TSK-G3000SW column (TosoHaas) and further by SDS–PAGE analysis (not shown).

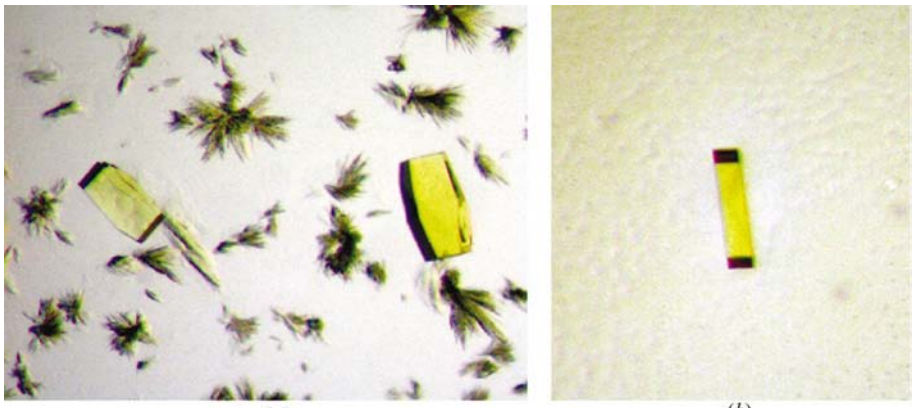

Figure 1
Crystals of a recombinant maize cytokinin oxidase (*Zm*CKO1). (*a*) Typical crystals. (*b*) The crystal (dimensions 0.13 × 0.03 ×0.03 mm) used for X-ray diffraction data collection.

The total yield of purified *Zm*CKO1 was about 12 mg per litre of culture medium as estimated by spectrophotometry at 280 nm. The specific activity was 510 nmol s^{-1} mg^{-1} (further details of the purification procedure will be published elsewhere).

A unique band of ∼69 kDa was detected, in contrast to 63 kDa for the native maize enzyme. After deglycosylation with endo-glycosidase H (Sigma), the 69 kDa band shifted to about 55 kDa (not shown), which corresponds to the size predicted by the amino-acid sequence. Such a high level of glycosylation is frequently observed for proteins expressed in *Y. lipolytica*. Edman degradation yielded an N-terminal sequence of LAAGTPALGDD, as expected after correct processing of the *Y. lipolytica* secretion signal.

3. Crystallization

Prior to crystallization, the protein was desalted and concentrated to 20 mg ml^{-1} using ultrafiltration through a Centricon 10 kDa cutoff device (Amicon). Preliminary crystallization conditions were found using a screening kit (Crystal Screen, Hampton Research). After optimization, the best crystals were grown at 293 K using the hanging-drop vapour-diffusion method. 2 µl of *Zm*CKO1 [20 mg ml^{-1} with 0.5%(*w*/*v*) *n*-octyl-β-D-glucopyranoside] were mixed with 2 µl reservoir solution containing 30%(*w*/*v*) polyethylene glycol 1500 in 0.1 *M* Tris–HCl buffer pH 7.0. After one week, several crystals appeared. They were yellow owing to the presence of FAD cofactor bound to the protein. Most of them were clustered, but a few single crystals also appeared (Fig. 1).

4. Data collection and processing

After soaking in a cryoprotectant solution [30%(*w*/*v*) PEG 1500 and 20%(*v*/*v*) glycerol in 0.1 *M* Tris–HCl buffer pH 7.0] for 5 min, crystals were flash-cooled in cryogenic storage vials containing liquid ethane (185 K). The vials were then stocked in liquid nitrogen. X-ray diffraction data were collected at 100 K on the BM30A-FIP beamline at the European Synchrotron Radiation Facility (Grenoble, France) equipped with a CCD detector and tuned to 0.98 Å wavelength. Data were collected in frames of 1° over 150°, with 20 s exposure per frame. A complete data set could be collected at 1.95 Å from the recombinant glycosylated enzyme. The crystal belongs to the monoclinic space group *C*2, with unit-cell parameters $a = 250.6$, $b = 50.6$, $c = 51.5$ Å, $\beta = 94.1°$. Assuming that the unit cell contains one molecule per asymmetric unit, the calculated Matthews coefficient (V_M) is 2.3 $Å^3$ Da^{-1} (Matthews, 1968) and the corresponding solvent content is 46.2%. The raw data were indexed, processed and scaled using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinoski & Minor, 1997). Data-collection and processing statistics are given in Table 1.

A search for a sequence-related protein of known crystal structure using the *3D-PSSM* program (Kelley *et al.*, 2000) indicated a few proteins with secondary structure similar to that predicted for *Zm*CKO1 (most were enzymes that included an FAD cofactor). However, their overall sequence identity with *Zm*CKO1 was less than 20%, which hindered molecular replacement. As the *Zm*CKO1 sequence contains cysteine residues, we are currently trying to infiltrate crystals of the recombinant enzyme with mercury derivatives in order to solve the structure by SIRAS (single isomorphous replacement with anomalous scattering) method.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.02–1.95 Å).

Space group	*C*2
Unit-cell parameters (Å, °)	$a = 250.6$, $b = 50.6$, $c = 51.5$, $\beta = 94.1$
Resolution (Å)	30.0–1.95
No. observed reflections	286333
No. unique reflections	44513
Completeness (%)	95.0 (93.8)
R_{sym}† (%)	9.4 (14.5)
$I/\sigma(I)$	9.1 (6.6)

† $R_{sym} = \sum_{hkl}[(\sum_i |I_i - \langle I \rangle|)/\sum_i I_i]$ for equivalent observations.

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