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High-level expression and characterization of *Zea mays* cytokinin oxidase/dehydrogenase in *Yarrowia lipolytica*

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Abstract

Cytokinin oxidase/dehydrogenase (CKO/CKX) is a flavoenzyme, which irreversibly inactivates cytokinins by severing the isoprenoid side chain from the adenine/adenosine moiety. There are several genes coding for the enzyme in maize (*Zea mays*). A *Z. mays CKO1* cDNA was cloned in the yeast *Yarrowia lipolytica* to achieve heterologous protein expression. The recombinant ZmCKO1 was recovered from cultures of transformed yeasts and purified using several chromatographic steps. The enzyme was obtained as a homogeneous protein in a remarkably high-yield and its molecular and kinetic properties were characterized. The enzyme showed a molecular mass of 69 kDa, p*I* was 6.3. Neutral sugar content of the molecule was 22%. Absorption and fluorescence spectra were in accordance with the presence of FAD as a cofactor. Peptide mass fingerprinting using MALDI-MS correctly assigned the enzyme in MSDB protein database. The enzyme showed a relatively high degree of thermostability (T_{50} = 55 °C for 30 min incubation). The following pH optimum and K_m values were determined for natural substrates (measured in the oxidase mode): pH 8.0 for isopentenyl adenine (K_m = 0.5 μM), pH 7.6 for isopentenyl adenosine (K_m = 1.9 μM), pH 7.9 for zeatin (K_m = 1.5 μM) and pH 7.3 for zeatin riboside (K_m = 2.0 μM). ZmCKO1, functioning in the oxidase mode, catalyzes the production of one molecule of H_2O_2 per one molecule of cytokinin substrate. This finding represents clear evidence for the existence of dual enzyme functionality (oxygen serves as a cosubstrate in the absence of better electron acceptors).

Keywords: Cytokinin oxidase/dehydrogenase; Hydrogen peroxide; Maize; Protein expression and purification; *Yarrowia lipolytica*

1. Introduction

While cytokinin oxidase/dehydrogenase (CKO/CKX), which irreversibly degrades cytokinins, was partially purified from maize kernels as early as 1974 [1], the first gene in maize (*ZmCKX1*, EMBL/GenBank accession code AF044603) and its mRNA (*ZmCKO1*, EMBL/GenBank accession code Y18377) were isolated only in 1999 [2,3]. The deduced protein sequence comprises 534 amino acids, including eight possible N-glycosylation sites and a putative signal peptide of 18 amino acids. The *ZmCKX1/ZmCKO1*-encoded protein has enzymatic activity, and is secreted and glycosylated [2,3]. Another CKO from *Zea mays* (ZmCKO3) was also shown to be secreted, when expressed in its native form (with its native signal peptide) in the yeast *Yarrowia lipolytica* [4].

ZmCKO1 contains FAD as a cofactor covalently bound to the conserved His105 residue of a GHS motif [5]. Recently Malito et al. [6] published the crystal structure of ZmCKO1 expressed in the yeast *Pichia pastoris* showing that FAD is linked through the 8-methyl group of the flavin ring. The ZmCKO1 molecule exhibits a topology defined by cofac-

Abbreviations: DCPIP, 2,6-dichlorophenol indophenol; GOD, glucose oxidase; HRP, horseradish peroxidase; HVA, homovanillic acid; iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenosine; Z, N^6-(*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenine = zeatin; ZmCKO/ZmCKX, cytokinin oxidase/dehydrogenase from maize (*Zea mays*); ZR, N^6-(*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenosine = zeatin riboside.

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tor and substrate domains. Two active-site residues, Asp169 and Glu288, seem to be crucial for both cytokinin binding and enzyme action.

The enzyme, which oxidatively degrades cytokinins in plants, has been named cytokinin oxidase since the dawn of its study [7]. It was suggested and later demonstrated that the reaction could not proceed without oxygen [1,8]. In 1999, two independent groups reported that CKO is able to reduce 2,6-dichlorophenol indophenol (DCPIP) as an electron acceptor [2,9]. Later, Galuszka et al. [10] reported that oxygen is not required and hydrogen peroxide is not produced during the catalytic reaction of purified CKOs from wheat and barley grains. They also proposed to reclassify the enzyme to cytokinin dehydrogenase and this has been approved (EC 1.5.99.12). Moreover, H_2O_2 could not be detected in activity assays performed with a recombinant maize enzyme [11].

When isopentenyl adenine (iP) undergoes oxidation by CKO, adenine and 3-methyl-2-butenal are formed as reaction products [12]. Substrate degradation proceeds via an unstable imine intermediate [13]. During the first half reaction, the oxidized FAD cofactor (having absorption maxima at around 360 and 450 nm) is reduced to $FADH_2$ via two-electron transfer that leads to a temporary bleaching and the subsequent appearance of a new maximum at 315 nm. Reoxidation of the reduced FAD is achieved in two ways. In the oxidase mode, electrons are transferred to oxygen and hydrogen peroxide is expected to be released. Alternatively, in the dehydrogenase mode, electrons are transferred to other electron acceptors, e.g. quinones [14]. The reductive half reaction is fast ($k = 950\ s^{-1}$ for iP), therefore, the reoxidation of FAD seems to be the rate limiting step in the catalytic cycle. In the presence of different artificial electron acceptors, iP shows the best substrate properties (k_{cat}/K_m) [5,14]. Some diphenylureas, which are known as potent cytokinin agonists, inhibit CKO activity [8,13] in a competitive manner towards substrates [5].

We recently reported the crystallization of recombinant ZmCKO1 expressed in the yeast *Y. lipolytica* [15]. Since its expression and its purification were reported only briefly, we describe here in detail all steps concerning cloning, high-level expression and purification. Additionally, other enzyme characteristics such as p*I*, thermostability (T_{50}), pH optima and K_m values for natural substrates were determined. The recombinant ZmCKO1 was also digested by trypsin or chymotrypsin and the respective peptide fragments were analyzed by mass spectrometry. For the first time, the reaction stoichiometry of CKO functioning in the oxidase mode (i.e. in the absence of any artificial electron acceptor) was determined. It emerged that the obtained stoichiometric ratio was significant for the confirmation of dual enzyme functionality.

2. Materials and methods

2.1. Chemicals and proteins

Isopentenyl adenine (iP), isopentenyl adenosine (iPR), zeatin riboside (ZR), 4-aminophenol, bicinchoninic acid–protein assay kit, 2,6-dichlorophenol indophenol (DCPIP) and FAD sodium salt were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Zeatin (Z) was from ICN Biochemicals (Cleveland, OH, USA). All other chemicals were of analytical purity grade.

Bovine serum albumin (BSA), catalase from bovine liver, glucose oxidase (GOD) from *Aspergillus niger*, horseradish peroxidase (HRP), soybean trypsin inhibitor and endoglycosidase H (from *Streptomyces griseus*) were from Sigma-Aldrich. Bovine chymotrypsin was from Novo Nordisk (Copenhagen, Denmark), raffinose-modified trypsin was prepared from bovine trypsin (ICN Biomedicals, Aurora, OH, USA) according to a protocol, which will be published elsewhere. Glycoprotein Deglycosylation Kit (Cat. No. 362280) was supplied by Calbiochem (La Jolla, CA, USA).

2.2. Cloning and transformation procedures

The cDNA sequence corresponding to mature ZmCKO1 was inserted into pINA1267 vector for expression/secretion in *Y. lipolytica* [16]. The *ZmCKO1* ORF (in pBluescript KS$^-$ clone 16, [3]) was PCR-amplified to remove its putative signal sequence (18 codons). The CKY02 upstream primer, 5′-ATGCACTA**GGCC**TCTTC**GGCC**GCCAAGCGA*CTAGCGGCAGGCACGCCT* contains a *Sfi*I restriction site compatible with a *Sfi*I site in *XPR2* prepro region of the pINA1267 vector (bold), the codons for a dibasic Lys-Arg cleavage site (underlined) and the N-terminal sequence of mature ZmCKO1 (italics). The CKY01 downstream primer, 5′-CGG**GGTACC**AAA*CTAAAACATGCATGGGCTAT* contains a *Kpn*I restriction site (bold), and the 3′-untranslated Zm*CKO1* cDNA (italics) with the stop codon (underlined). PCR conditions were as previously described [4]. The amplified fragment and the vector were digested with *Sfi*I and *Kpn*I restriction endonucleases and ligated together. The ligation products were used to transform *E. coli* DH10B competent cells by electroporation. One clone (pINA6703), corresponding to the insertion of mature ZmCKO1 sequence into pINA1267, was entirely sequenced over the vector/insert junctions and *ZmCKO1* ORF. It contained two silent mutations compared to *ZmCKO1* sequence (Y18377). The plasmid pINA6703 was linearized using *Spe*I restriction endonuclease to direct its integration at the pBR322 docking platform of the recipient *Y. lipolytica* Po1g strain (Leu$^-$, ΔAEP, ΔAXP, Suc$^+$, pBR322) [16]. Yeasts were transformed by the lithium acetate method and Leu$^+$ transformants were selected on minimal YNB medium [17]. Yeast culture media were as follows. PPB medium: 20 g l^{-1} sucrose, 1.32 g l^{-1} yeast extract, 1.32 g l^{-1} NH_4Cl, 0.32 g l^{-1} KH_2PO_4, 0.13 g l^{-1} $MgSO_4$ and 0.33 mg l^{-1} thiamine in 50 mM phosphate buffer, pH 6.8; YNB medium: 1.7 g l^{-1} yeast nitrogen base (without amino acids and ammonium sulfate), 5 g l^{-1} ammonium sulfate and 10 g l^{-1} glucose; YPD medium: 10 g l^{-1} yeast extract, 10 g l^{-1} glucose and 10 g l^{-1} bacto-peptone.