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Purification, crystallization and preliminary X-ray analysis of recombinant betaine aldehyde dehydrogenase 2 (OsBADH2), a protein involved in jasmine aroma, from Thai fragrant rice (*Oryza sativa* L.)

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Fragrant rice (*Oryza sativa* L.) betaine aldehyde dehydrogenase 2 (OsBADH2) is a key enzyme in the synthesis of fragrance aroma compounds. The extremely low activity of OsBADH2 in catalyzing the oxidation of acetaldehyde is believed to be crucial for the accumulation of the volatile compound 2-acetyl-1-pyrroline (2AP) in many scented plants, including fragrant rice. Recombinant fragrant rice OsBADH2 was expressed in *Escherichia coli* as an N-terminal hexahistidine fusion protein, purified using Ni Sepharose affinity chromatography and crystallized using the microbatch method. Initial crystals were obtained within 24 h using 0.1 *M* Tris pH 8.5 with 30%(*w*/*v*) PEG 4000 and 0.2 *M* magnesium chloride as the precipitating agent at 291 K. Crystal quality was improved when the enzyme was cocrystallized with NAD^+. Improved crystals were grown in 0.1 *M* HEPES pH 7.4, 24%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium chloride and diffracted to beyond 2.95 Å resolution after being cooled in a stream of N_2 immediately prior to X-ray diffraction experiments. The crystals belonged to space group $C222_1$, with unit-cell parameters a = 66.03, b = 183.94, c = 172.28 Å. An initial molecular-replacement solution has been obtained and refinement is in progress.

1. Introduction

Betaine aldehyde dehydrogenases [betaine aldehyde:$NAD(P)^+$ oxidoreductases; EC 1.2.1.8; BADHs] belong to the nonphosphorylating aldehyde dehydrogenase (ALDH) superfamily (Muñoz-Clares *et al.*, 2010). BADHs have the ability to catalyze the irreversible $NAD(P)^+$-dependent oxidation of betaine aldehyde to glycine betaine (Muñoz-Clares *et al.*, 2010). In addition, BADHs are capable of oxidizing a variety of aromatic and aliphatic aldehydes (Boyd *et al.*, 1991). Fragrant rice has two betaine aldehyde dehydrogenase gene homologues encoding OsBADH1 and OsBADH2 on chromosomes four and eight, respectively (Bradbury *et al.*, 2008). OsBADH1, which responds to salt tolerance in rice, catalyzes the efficient oxidation of acetaldehyde as well as the oxidation of betaine aldehyde to glycine betaine (Mitsuya *et al.*, 2009). In contrast, OsBADH2 converts γ-4-aminobutyraldehyde (GAB-ald) to γ-aminobutyric acid (GABA), which gives fragrant rice its aromatic properties (Sakthivel *et al.*, 2009).

Fragrant rice has a pleasant aroma owing to accumulation of the volatile compound 2-acetyl-1-pyrroline (2AP) as a principal aroma compound (Sakthivel *et al.*, 2009). With the availability of molecular maps and genome sequences, a major gene for fragrance (*badh2*) has been identified on chromosome 8 (Bradbury *et al.*, 2005). An 8 bp deletion in exon 7 of *badh2* leads to the generation of a premature stop codon and results in the truncation of OsBADH2. Loss of OsBADH2 function accounts for loss of the catalytic conversion of GAB-ald to GABA (Juwattanasomran *et al.*, 2011). GAB-ald is instead processed to 2AP *via* a polyamine pathway, leading to the accumulation of this major aroma compound (Sakthivel *et al.*, 2009).

Although fragrant rice OsBADH2 and OsBADH1 proteins share 75% identity at the amino-acid level and 92% similarity in the catalytic domain (Fitzgerald *et al.*, 2010), their activity is different. In the present study, protein expression, purification and crystallization

of OsBADH2 were carried out. Moreover, a preliminary crystallographic study of OsBADH2 is reported.

2. Materials and methods

2.1. Expression and purification of recombinant OsBADH2

The gene encoding OsBADH2 was amplified from plasmid pUC18-Os2AP (a gift from Associated Professor Apichart Vanavichit, Kasetsart University) by polymerase chain reaction using the synthetic primers OsBADH2F, 5′-GGAATTCCATATGGCCACG-GCGATCCC-3′, and OsBADH2R, 5′-CCGCTCGAGTTACAGCT-TGGAAGGGGATT-3′. The resulting *Nde*I/*Xho*I fragment was subcloned into the *Nde*I/*Xho*I sites of the pET28b expression vector (Novagen) to produce pET28b-OsBADH2 vector, which encodes a fusion protein with an N-terminal His tag followed by a thrombin cleavage site and OsBADH2, resulting in the addition of 20 amino-acid residues (MGSSHHHHHHSSGLVPRGSH) prior to OsBADH2. *Escherichia coli* strain BL21 (DE3) harbouring pET28b-OsBADH2 was cultured in Luria–Bertani (LB) medium at 310 K until the OD_{600} reached 0.6. The cell culture was then placed on ice for 15 min prior to induction with 0.5 m*M* IPTG (final concentration). Induction continued at 293 K for 16 h. Following this, cells were harvested by centrifugation and lysed by sonication in lysis buffer (50 m*M* Tris–HCl pH 8.0, 0.5 m*M* NaCl, 30 m*M* imidazole and 0.5% Triton X-100). Chromatographic separation was performed at room temperature using a pre-packed Ni Sepharose column connected to an ÄKTA FPLC system (Amersham Pharmacia Biotech, Stockholm, Sweden). The column was equilibrated with ten column volumes (CV) of binding buffer (50 m*M* Tris–HCl pH 8.0, 0.5 m*M* NaCl and 30 m*M* imidazole). The supernatant was injected onto the column and washed with 10 CV binding buffer. Bound proteins were then eluted by the application of a linear gradient of elution buffer (50 m*M* Tris–HCl pH 8.0, 0.5 m*M* NaCl and 500 m*M* imidazole). Protein fractions (approximately 1 ml) were analyzed by 12% SDS–PAGE and the fractions containing purified materials of interest were pooled. Protein concentration was determined using an extinction coefficient for OsBADH2 of 88 765 M^{-1} cm^{-1}, which was calculated by deducing the amino-acid sequence using the program *BioEdit* (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The purified protein was concentrated using a 30 kDa cutoff concentrator. The protein was dialyzed against and kept in 50 m*M* monosodium phosphate buffer pH 8.0 containing 0.2 *M* EDTA and 20 m*M* β-mercaptoethanol for protein stability. No attempt was made to remove the His tag. Removal of precipitated protein by centrifugation at 12 000*g* was carried out prior to the protein crystallization process.

2.2. Crystallization

Preliminary crystallization screening for OsBADH2 was performed using Hampton Research Crystal Screen HT by the microbatch method in 60-well plates (Nunc) at 289 K. Crystallization wells contained equal volumes (2 µl) of individual solutions from the kit and protein solution consisting of 20 mg l^{-1} OsBADH2, 50 m*M* monosodium phosphate buffer pH 8.0, 0.2 *M* EDTA and 20 m*M* β-mercaptoethanol and were covered by 10 µl of oil (commercial Babimild baby oil). Initial crystals of OsBADH2 with poor X-ray diffraction quality were grown using 0.2 *M* magnesium chloride hexahydrate, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) polyethylene glycol 4000 as the precipitating agent. Crystallization optimization around this condition yielded single crystals with unchanged X-ray diffraction quality. The precipitant concentration was varied and the salt types and buffer systems were changed for further crystallization optimization. Cocrystallization with NAD^+ (2:1 molar ratio of ligand: protein) was also used to improve crystal quality. The best crystal for X-ray diffraction of OsBADH2 grown in the presence of NAD^+ was obtained in 0.1 *M* HEPES pH 7.4, 24%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium chloride.

Table 1
X-ray data diffraction details and statistics for the OsBADH–NAD^+ crystal.

Values in parentheses are for the highest resolution shell.

X-ray source	Cu $K\alpha$ rotating anode
Wavelength (Å)	1.542
Resolution (Å)	39.84–2.95 (3.11–2.95)
Space group	$C222_1$
Unit-cell parameters (Å)	a = 66.03, b = 183.94, c = 172.28
Observed reflections	86496 (10825)
Unique reflections	22150 (2889)
Multiplicity	3.9 (3.7)
Mean $I/\sigma(I)$	4.6 (2.2)
Completeness (%)	98.1 (89.6)
R_{merge}† (%)	18.8 (48.9)
Matthews coefficient V_M (Å^3 Da^{-1})	2.39
Solvent content (%)	48.6

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value of its unique reflection.

2.3. Data collection and processing

X-ray diffraction data were collected at 100 K in the nitrogen flow from a 700 series Cryostream Cooler (Oxford Cryosystems, Oxford, England) using a Cu $K\alpha$ rotating-anode source mounted on a Microstar generator operating at 45 kV and 60 mA connected to a Rayonix SX-165 CCD detector at the Synchrotron Light Research Institute (SLRI; Nakhon Ratchasima, Thailand). The crystal was cryoprotected by soaking it in crystallization solution containing 30% glycerol for 15 s and flash-cooled. 0.5° oscillation images were obtained with 90 s exposures. Under these conditions, the best X-ray diffraction from an OsBADH2 crystal was obtained to 2.6 Å resolution. However, full data collection from an OsBADH2 crystal grown in the presence of NAD^+ was successfully carried out at the lower resolution of 2.95 Å. Although analysis of the diffraction data using *iMOSFLM* (Battye *et al.*, 2010) indicated that the complex crystal belonged to the centred orthorhombic space group *C*222, with unit-cell parameters a = 66.03, b = 183.94, c = 172.28 Å, subsequent analysis of asymmetric absences clearly indicated that $C222_1$ was the correct space group. A total of 22 150 unique reflections were processed with 98.1% completeness and the poorly scaled data had a high R_{merge} value of 18.8%. A small subset of randomly selected reflections (5%) of the data set were set aside and labelled in order to calculate R_{free}. The Matthews coefficient was 2.39 Å^3 Da^{-1}, which is consistent with two molecules of protein in the asymmetric unit (Winn *et al.*, 2011; Gruez *et al.*, 2004). Data-collection and processing statistics are shown in Table 1.

2.4. Molecular replacement and structure refinement

The polyalanine side chain of the OsBADH2 model, generated by *ESyPred3D* (Lambert *et al.*, 2002) using the structure PDB entry 3iwk chain *A* (Tylichová *et al.*, 2010) as a template, was used to calculate an initial phase solution in *MOLREP* (Vagin & Teplyakov, 2010). *REFMAC*5 (Murshudov *et al.*, 2011) and *Coot* (Emsley & Cowtan, 2004) were used for structure refinement, structural building and inspection. After removal of poorly defined loops from the structure, automated side-chain tracing by *ARP*/*wARP* (Perrakis *et al.*, 2001) and refinement were performed, with R and R_{free} factors of 33.4%

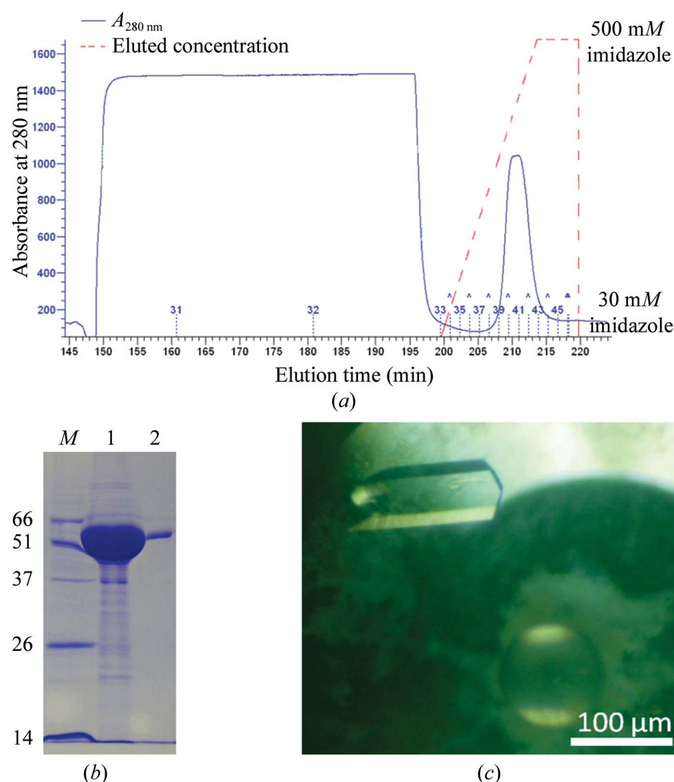

Figure 1
(*a*) Chromatogram of OsBADH2 purification using an Ni-Sepharose affinity column. (*b*) SDS–PAGE of purified recombinant OsBADH2. Lane 1, molecular-weight marker (labelled in kDa); lane 2, 50 µg protein; lane 3, 0.5 µg protein. The molecular weight of the purified protein is 55 kDa. (*c*) A single crystal of OsBADH2 was grown in the presence of NAD^+ with dimensions of 0.2 × 0.05 × 0.05 mm in 0.1 M HEPES pH 7.4, 24% PEG 4000, 0.2 M ammonium chloride.

and 45.7%, respectively. Structure refinement of OsBADH2 is now under way.

3. Results and discussion

Recombinant OsBADH2 was successfully expressed in *E. coli* as an N-terminal hexahistidine fusion protein and was purified using Ni Sepharose affinity chromatography (Fig. 1*a*). The amount of purified protein was 50 mg per litre of cell culture, as shown in Fig. 1(*b*). The protein was subsequently crystallized by the microbatch method (Chayen, 1998) in 60-well plates (Nunc) at 289 K. Initial crystals of OsBADH2 were obtained within 1 d from Hampton Research Crystal Screen HT solution A6 [0.2 M magnesium chloride hexahydrate, 0.1 M Tris–HCl pH 8.5, 30%(*w*/*v*) polyethylene glycol 4000 as precipitating agent]. Optimization around this condition yielded rod-shaped 100 µm single crystals; however, the best diffraction at this stage was to around 6 Å resolution. Crystal quality was further optimized by varying the precipitant concentration and the salt and buffer systems. To improve the crystal quality, addition of NAD^+ to the crystallization droplet was successfully carried out. Orthorhombic crystals of OsBADH2 grown in the presence of NAD^+ were obtained in 0.1 M HEPES pH 7.4, 24%(*w*/*v*) PEG 4000 and 0.2 M ammonium chloride (Fig. 1*c*). The crystal structure of OsBADH2 was solved by molecular replacement. Work is in progress to improve the diffraction quality of the crystals and to produce apo OsBADH2 and OsBADH2–cofactor and OsBADH2–substrate complex crystals in order to understand the apparent dual substrate specificity.

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