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Purification, characterization, and stabilization of alcohol oxidase from *Ogataea thermomethanolica*

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ABSTRACT

Alcohol oxidase (AOX) functions in oxidation of primary alcohols into the corresponding aldehydes with potential on catalyzing synthesis reactions in chemical industry. In this study, AOX from a thermotolerant methylotrophic yeast, *Ogataea thermomethanolica* (OthAOX) was purified to high homogeneity using a single step chromatographic separation on a DEAE-Sepharose column. The purified OthAOX had a specific activity of 15.34 U/mg with 77.5% recovery yield. The enzyme worked optimally at 50 °C in an alkaline range (pH 9.0). According to kinetic analysis, OthAOX showed a higher affinity toward short-chain aliphatic primary alcohol with the V_{max}, K_m, and k_{cat} of 0.24 nmol/min, 0.27 mM, and 3628.8 min^{-1}, respectively against methanol. Addition of alginic acid (0.35%) showed a protective effect on enhancing thermal stability of the enzyme, resulting in 72% increase in its half-life at 40 °C under the operational conditions. This enzyme represents a promising candidate for conversion of bioethanol to acetaldehyde as secondary chemical in biorefinery.

1. Introduction

Alcohol oxidase (Alcohol: O_2 Oxidoreductase; EC 1.1.3.13, AOX) is a homo-octameric peroxisomal matrix protein consisted of eight identical subunits, each containing a strongly bound flavin adenine dinucleotide (FAD) as the prosthetic group [1,2]. The enzymes have been found in several methanol-utilizing yeasts belonging to genera *Pichia*, *Candida*, *Ogataea*, and *Komagataella* [3,4]. In methylotrophic yeasts, this enzyme is responsible in catalyzing methanol taken as the carbon source to formaldehyde, which is then further metabolized in the yeast's catabolic pathway [5]. The catalytic reaction of AOX is as described by the following scheme:

$$R\text{-}CH_2OH + O_2 \rightarrow R\text{-}CH{=}O + H_2O_2.$$

Various AOXs, particularly from methylotrophic yeasts, which predominantly utilize short chain alcohols as the sole carbon and energy sources, have been characterized and studied for their biochemical characteristics [6–9]. The main biotechnological application of AOXs is in their use as alcohol sensing molecules in alcohol biosensors for measurement of lower primary alcohols and formaldehyde which relies on the quantitation of H_2O_2 released as a co-product [10,11]. Formaldehyde assay in food products have been developed using enzymatic and biosensor approaches based on the use of AOXs [12]. One of the most relevant applications of AOXs is the monitoring of ethanol in beverages, foods, pharmaceuticals and cosmetics to control fermentation process and product quality with high sensitivity and selectivity [13–15]. Other applications of AOXs also include the use of enzymes for diagnostic purposes e.g. quantification of ethanol in biological fluids such as in human breath, saliva, urine, and plasma in clinical and forensic laboratories where accurate and rapid measurement of ethanol are required [16].

Presently, AOX has gained an increasing interest on biochemical synthesis of industrially useful carbonyl compounds in biorefinery industry [17]. Conversion of ethanol to secondary chemicals such as acetaldehyde using AOXs has been reported using either purified AOXs or whole-cells from methylotrophic yeasts [18–21]. Acetaldehyde and other short-chain aldehydes are important intermediates in synthesis of ethyl acetate, isobutyl acetate, pyridine, peracetic acid, butyraldehyde, acetic ether, pentaerythrito, and butadiene [22,23]. This opens up a

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promising way on value-added conversion of ethanol to commodity chemicals replacing those conventionally produced from petro-industry and helps increasing the profitability of the ethanol industry. In this study, an AOX from *O. thermomethanolica* (OthAOX), a thermotolerant methylotrophic yeast, recently reported as an alternative host for heterologous protein production [24,25] has been purified and characterized for basic biochemical and kinetic properties and compared with the enzymes obtained from other sources. This work shows potential of OthAOX as a promising biocatalyst candidate for aldehyde synthesis in chemical industry.

2. Materials and methods

2.1. Materials

2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid (ABTS), and horseradish peroxidase from horseradish roots (*Amoracia rusticana*) were purchased from Sigma-Aldrich. Hiprep DEAE FF 16/10 column was obtained from GE Healthcare Life Sciences (Uppsala, Sweden). *O. thermomethanolica* BCC16875 was obtained from the BIOTEC Culture Collection (www.biotec.or.th/bcc). Organic buffer 3-(*N*-morpholino) propanesulfonate (MOPS) and sodium alginate were purchased from Sigma-Aldrich. PEG 4000 and 8000 and ammonium sulfate were obtained from Fluka (Buchs, Switzerland). All reagents were analytical-grade and purchased from major chemical suppliers.

2.2. Cultivation and preparation of cell-free extracts

O. thermomethanolica was grown in YEPD medium (2% peptone, 2% glucose, and 1% yeast extract) with shaking at 200 rpm at 30 °C for 16–24 h and used as an inoculum. The cells were inoculated at 1% v/v to YP-methanol medium (2% peptone, 1% methanol, 1% yeast extract, and 0.67% yeast nitrogen base without amino acids). The production of alcohol oxidase was induced by addition of 1% v/v methanol as the carbon source. The initial pH of the media was adjusted to 6.0. The culture was grown at 30 °C for 18 h. The supernatant was collected after centrifugation at 10,000 × g at 4 °C for 10 min and used as the crude enzyme preparation.

2.3. AOX activity assay (ABTS-POD method)

AOX activity was determined spectrophotometically by determining the increase in absorbance at 405 nm resulting from the oxidation of ABTS™ [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] by a coupled peroxidase-catalyzed reaction (ABTS-POD) according to Tani et al. [26] with some modifications. The stock mixture contained 2.8 mL of 2 mM ABTS solution (in 100 mM potassium phosphate buffer, pH 7.5), 0.01 mL of 250 units/mL peroxidase enzyme solution, and 0.1 mL of 1% (v/v) methanol solution. The assay reaction was started by addition of 10 µL of OthAOX to 190 µL of the stock solution in a 96-well microplate and incubated at 25 °C for 10 min. The reaction was stopped by adding 13.3 µL of 4 N HCl. The color produced was measured spectrophotometically at 405 nm. The reading was corrected for the blank containing no enzyme.

The unit activity of AOX was determined according to the equation below.

$$\text{Units/mL enzyme} = \frac{(Abs\ Rx - Abs\ B) \times 0.2 \times Df}{Rxn\ time \times 36.8 \times 0.01 \times 0{,}6}$$

where

0.2 = Total volume of assay (mL)
Df = Dilution factor
36.8 = Millimolar extinction coefficient of ABTS at 405 nm
0.01 = Volume of enzyme used (mL)
0.6 = Path length (cm)

One unit of AOX was defined as the amount of enzyme that oxidized of 1 µmole of methanol to formaldehyde per min at pH 7.5, 25 °C. Protein concentration was determined according to Bradford's method [27] using Biorad's Reagent with bovine serum albumin (BSA) as a standard.

2.4. Chromatographic purification of OthAOX

OthAOX was isolated from the cell-free extract by a single step purification process on an AKTA Explorer chromatography system (GE Biosciences, Uppsala, Sweden) at 4 °C. The crude extract was applied on a Hiprep DEAE FF 16/10 column containing DEAE-Sepharose (GE Bioscience, Uppsala, Sweden) pre-equilibrated with 0.1 M potassium phosphate buffer, pH 7.5 at the flow rate of 5 mL/min. The column was washed with the same buffer, and the enzyme was eluted with a NaCl gradient of 0.1–0.5 M in the phosphate buffer operated at the same flow rate. Fractions that exhibited the enzyme activity were combined, and the enzyme solution was dialyzed using Amicon Ultra-15 centrifugal filter units MWCO 10 kDa (Merck Millipore, Darmstadt, Germany) then further used as the purified alcohol oxidase.

Native molecular weight of OthAOX was determined by gel filtration using an AKTA pure FPLC system (GE Healthcare). The protein was separated on a HiPrep Sephacryl S-500HR column pre-equilibrated with 50 mM sodium phosphate pH 7.0 supplemented with 0.15 M NaCl. Separation of proteins were performed using the same buffer at the flow rate of 0.5 mL/min. Molecular weight of native OthAOX was estimated based on a standard curve generated using gel filtration high molecular weight calibration kit (GE Healthcare, data file 28-4073-84 AA).

2.5. Enzymatic characterization of purified OthAOX

The temperature profile of OthAOX activity was determined based on formation of formaldehyde at different temperatures. The reactions (1.2 mL) contained 10 mM methanol in 100 mM phosphate buffer, pH 7.5 with an appropriate enzyme dilution and incubated for 10 min at different temperatures (30–70 °C). The reaction was stopped by addition of 0.04 mL of 4 N HCl and the formaldehyde formed was determined by Nash method using freshly prepared reagent (0.02 M acetylacetone, 0.1 M acetic acid and 3.89 M ammonium acetate) with the addition of 0.6 mL [28] and further incubated at 60 °C for 10 min. The color developed was determined spectrophotometically at 412 nm compared to the formaldehyde standard curve.

The effects of pH on OthAOX activity was determined by using 10 mM methanol as a substrate at different pH in a range of 4.0–11.0 under the standard assay condition (50 °C). The buffers used were 100 mM of sodium acetate (pH 4.0 to 6.0), potassium phosphate (pH 6.0 to 8.0), Tris-HCl (8.0–10.0), glycine-base (8.0–11.0). The formaldehyde formed was quantitated using Nash reagent as described above. One unit of AOX was defined as the amount of enzyme forming 1 µmole of formaldehyde per min under the experimental conditions. The relative activity was calculated by comparing the activity for each treatment to that of the maximal activity, which was designated 100%.

2.6. Substrate specificity

The enzyme's substrate specificities were determined using ABTS-POD assay by incubating the purified enzyme with 1% (v/v) of different alcohols: methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-octanol, 2-propanol, *tert*-butanol, 3-pentanol, 3-methyl-1-butanol, and glycerol in phosphate buffer pH 7.5 at 30 °C for 10 min. The relative activity was calculated by comparing the activity for each substrate to that of the maximal activity, which was designated 100%.

2.7. Kinetic study

The kinetic parameters (K_m, V_{max}, and k_{cat}) were determined using