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Oxidation of ethylene glycol to glycolaldehyde using a highly selective alcohol dehydrogenase from *Gluconobacter oxydans*

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ABSTRACT

A cytosolic medium-chain alcohol dehydrogenase Gox0313 from *Gluconobacter oxydans* DSM2003 was heterologously expressed in *Escherichia coli* BL21 (DE3) and the resulting proteins were purified and characterized. The recombinant enzyme was confirmed to have a good ability to selectively oxidize the terminal hydroxyl group of varied aliphatic and aromatic diols to the corresponding hydroxyl aldehydes, with no oxidative activities toward hydroxyl aldehydes in the presence of NAD^+. This enzyme could not oxidize *sec*-alcohols. Among the primary diols, ethylene glycol was efficiently converted to glycolaldehyde by Gox0313 coupled with NADH oxidase-2 (NOX-2) from *Lactobacillus brevis* ATCC367 for NAD^+ regeneration. Under the optimal conditions using this enzyme-coupled system (1 U/mL Gox0313 and 4 U/mL NOX-2), 42.32 mM glycolaldehyde was produced from 50 mM ethylene glycol in 8 h with a yield of 83.2%. When the enzyme concentration of Gox0313 was increased to 120 U/mL in the reaction, 500 mM of ethylene glycol was converted to 484.2 mM of glycolaldehyde in 14 h, resulting in a yield of 96.8%. With respect to the superior catalytic activity to primary diols, Gox0313 might be a potentially promising biocatalyst for bioproduction of glycolaldehyde and other hydroxyl aldehydes.

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1. Introduction

Aliphatic hydroxyl aldehydes that contain both an aldehyde group and a hydroxyl group are widely used as precursors and intermediates in organic synthesis. Among them, glycolaldehyde is the smallest possible molecule and useful as an intermediate to synthesize D,L-serine [1], vitamin B_6 [2] and lactic acid [3], as well as used as reducing agent [4] or probe molecule [5] in some special synthesis. Thus, preparation of such compound has been developed through various synthetic methods, including chemocatalysis and biocatalysis.

Chemical method of glycolaldehyde production has been reported using hydroformylation of formaldehyde [6] or an optical–thermal coupling method from glycerin with TiO_2 as a catalyst [7]. Glycolaldehyde production from ethylene glycol have been demonstrated by the dehydrogenation of ethylene glycol using a combination of metals such as copper, zinc, silver, gold rhodium or cobalt as catalyst [8–11]. However, these methods have some drawbacks such as low yield of glycolaldehyde, formation of large amounts of by-products, heavy metal catalysts usage, and high reaction temperature requirement. Therefore, bioproduction of hydroxyl aldehydes including glycolaldehyde becomes more and more attractive.

Alcohol oxidases from methanol yeasts such as *Pichia pastoris* [12] or *Candida* sp. [13] and glycerol oxidase from *Aspergillus japonicas* [14] have been applied to oxidize ethylene glycol to glycolaldehyde. However, low selectivity of these enzymes made easily oxidation of glycolaldehyde to glyoxal; by optimizing the reaction conditions, intermediate glycolaldehyde might be remarkably accumulated [15].

The acetic acid bacterium *Gluconobacter oxydans* is well known for its ability to rapidly incompletely oxidize of numerous carbohydrates and alcohols [16]. The genome of *G. oxydans* DSM2003 and function analysis revealed the presence of oxidoreductases capable of oxidation of alcohols and polyols. A PQQ-dependent alcohol dehydrogenase has been demonstrated its function in the oxidation of primary diols to corresponding hydroxyl aldehydes [17]. However, this enzyme is membrane-bound and difficult to be prepared in large scale; and whole-cell catalysis involved in the further oxidation of glycolaldehyde to glycolic acid due to the presence of aldehyde dehydrogenases in *G. oxydans*.

Herein, we report the cloning, heterologous expression and biochemical characterization of a zinc-dependent medium-chain

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alcohol dehydrogenase (Gox0313) from *G. oxydans*. Gox0313 was heterologously expressed and identified with activity and selectivity for primary diols to produce hydroxyl aldehydes. Furthermore, an alternative route for enzymatic method of glycolaldehyde production from ethylene glycol was developed. During the glycolaldehyde producing process by Gox0313, a stoichiometric amount of NAD^+ would be reduced into NADH. Due to the high price of NAD^+, an efficient cofactor regeneration system is prerequisite for the practical utilization of the alcohol dehydrogenase [18–20]. As a consequence, NADH oxidase-2 (NOX-2) from *Lactobacillus brevis* (*L. brevis*) that converts O_2 to H_2O was used for regeneration of NAD^+ [21–24]. Various reaction conditions of this enzyme-coupled system were systematically investigated for glycolaldehyde production.

2. Materials and methods

2.1. Chemicals and organisms

Diols and glycolaldehyde dimer were purchased from Sigma–Aldrich (America). NAD^+ and NADH were obtained from Roche (Germany). Isopropyl-β-D-1-thiogalactopyranoside (IPTG), ampicillin and kanamycin were provided by Merck (Germany). Methyl alcohol (HPLC grad) was obtained from Tedia (America). PCR primers were synthesized by Generay (Shanghai, China). PCR premix, T_4 DNA ligase and restriction enzymes were obtained from Fermentas (Canada). All other chemicals were commercially available reagents of analytical grade.

Escherichia coli DH5α was used for general cloning, *E. coli* BL21 (DE3) was used for protein expression. Luri–Bertani (LB) medium was used for *E. coli* cultivations. The wild type *G. oxydans* DSM2003 was cultivated in a medium containing (per liter) 80 g sorbitol, 20 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$ and 0.1 g glutamine. DeMan–Rogosa Sharpe (MRS) medium was used for *L. brevis* ATCC367 culture.

2.2. Cloning, expression and purification of Gox0313 and NOX-2

The genomic DNA of *G. oxydans* DMS2003 was extracted by using TINamp Bacteria DNA Kit (TIANGEN, Shanghai, China). The gox0313 gene was amplified by PCR using forward primer g1: 5′-TTTGGATCCATGGCTGATACAATGCTCGC-3′ with a *Bam*HI restriction site and reverse primer g2: 5′-ATGAAGCTTTCAGGACCGGAAGTCGAGCACT-3′ with a *Hin*dIII restriction site. The amplified gene was inserted into vector pET32a (+) (Novagen, USA) with His-tag at the N-terminal, generating expression plasmid pET32a-gox0313. Using the same process that described above, the *nox*-2 gene fragment was obtained from the genome of *L. brevis* ATCC367 using forward primers n1: 5′-GACGGATCCATGAAAGTCACAGTTGTT-3′ with the *Bam*HI restriction site and reverse primer n2: 5′-GAGGAATTCTTAAGCGTTAACTGATTGGG-3′ with the *Eco*RI restriction site, the resulted fragment was inserted to vector pET28a(+) (Novagen, USA). Then transformed into *E. coli* DH5α (Novagen, USA) and *E. coli* BL21 (DE3) (Novagen, USA) for plasmids cloning and protein expression, respectively.

Recombinant *E. coli* cells were grown at 37 °C on a rotary shaker (200 rpm) in LB medium containing ampicillin (100 μg/mL) for Gox0313 or kanamycin (50 μg/mL) for NOX-2. When OD_{600} reached 0.6 (approximately 2 h), 0.15 mM IPTG was added to induce protein expression. After induced for 10–12 h at 20 °C, the recombinant cells were harvested by centrifugation at 7000 g for 10 min at 4 °C, and washed twice with distilled water. Resuspended cell pellets in phosphate buffer solution (PBS, 20 mM, pH 7.4) were disrupted by the ultrasonic cell disruptor in an ice bath at 300 W for 99 cycles (working 5 s and intervals 5 s as one cycle). Disrupted cells were centrifuged for 25 min at 20,000 *g* at 4 °C, and the supernatant was purified with a pre-pack Ni-NTA column (5 mL, GE Health, America) that was pre-treated with binding buffer (20 mM imidazole, 50 mM PBS, 500 mM NaCl, pH 7.4), and eluted with the washing buffer (50 mM imidazole, 50 mM PBS, 500 mM NaCl, pH 7.4) to remove the untagged proteins, and then the elution buffer (500 mM imidazole, 50 mM PBS, 500 mM NaCl, pH 7.4) to wash the target proteins. The fractions containing Gox0313 or NOX-2 were concentrated by ultrafiltration, and stored in 4 °C for further experiments.

2.3. Protein measurements, gel electrophoresis and apparent molecular mass determination

The protein concentration was measured by Bradford assay [25]. SDS-PAGE was used to verify the expression and purification of Gox0313 and NOX-2 with protein molecular weight marker from TaKaRa (Dalian, China).

The native molecular weight was determined by gel filtration chromatography using TSK-GEL G3000SW (TOSPH, Japan) equilibrated with 100 mM PBS (pH 7.0) buffer containing 100 mM Na_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min. Protein molecular weight standards were horse-spleen apofemtin (443 kDa), sweet potato β-amylase (200 kDa), L-lactic dehydrogenase (140 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa). The native molecular mass of Gox0313 and NOX-2 were calculated based on the standard curve of log MW and retention time.

2.4. Enzyme assay of Gox0313 and NOX-2

Gox0313 and NOX-2 activities were assayed by measuring the change in absorbance at 340 nm (ε = 6.22 $mM^{-1}\,cm^{-1}$) corresponding to the oxidation of NADH or the reduction of NAD^+ using a UV/visible spectrophotometer (SpectraMax 190, Molecular devices, USA).

The reaction mixtures (200 μL) for Gox0313 assay contained 5 mM ethylene glycol, 50 mM buffer (Tris–HCl, pH 8.5), 0.5 mM NAD^+, and Gox0313 in limited amounts. Reaction was started by addition of substrate or enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme activity that consumed 1.0 μmol of NAD^+ per min.

The reaction solution for NOX-2 assay contained 0.1 mM NADH, 0.1 M Tris–HCl buffer (pH 8.5), and enzyme in limited amounts. The assay was carried out at 30 °C. One unit enzyme activity was defined as the amount of enzyme that consumed 1 μmol NADH per min.

2.5. Optimal conditions and kinetics

The optimum pH was determined under various pH values (sodium phosphate buffer: pH 6.5–8.0, Tris–HCl buffer: pH 8.0–9.5). The optimum temperature was determined at different temperatures in the range of 20–80 °C. The residual activity was estimated at 30 °C in 50 mM Tris–HCl buffer, pH 8.5, as described above.

To determine pH stability, the enzyme was pre-incubated in buffers at different pH values (sodium phosphate buffer at pH 6.5–8.0 and Tris–HCl at pH8.0–9.5) over 24 h at 4 °C. Thermo stability was evaluated by incubating the enzyme at 4–80 °C for over 24 h. The residual activity was determined at 30 °C as described above.

Nonlinear regressions of the Michaelis–Menten data were used to calculate kinetic constants at pH 8.5 (50 mM Tris–HCl buffer) and 30 °C. Varied substrate concentrations ranging from 0.5 to 20 mM were applied to investigate K_m toward ethylene glycol or glycolaldehyde with NAD^+ (0.5 mM) or NADH (0.1 mM) as cofactor. K_m toward NAD^+ and NADH were determined under varied cofactor

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