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Characterization of a thermostable mannitol dehydrogenase from hyperthermophilic *Thermotoga neapolitana* DSM 4359 with potential application in mannitol production

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ABSTRACT

Mannitol-2-dehydrogenase (MtDH) (E.C. 1.1.1.67) gene was cloned from *Thermotoga neapolitana* DSM 4359 and expressed in *Escherichia coli* BL21. The purified enzyme showed a predicted clear band of 36 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), native molecular mas was 135 kDa. K_m and V_{max} values for reduction of D-fructose to D-mannitol were 20 mM and 200 U mg-1 respectively. k_{cat} for reduction direction was 180 s^{-1} and k_{cat}/K_m were 9 mM^{-1} s^{-1}. The enzyme showed optimal pH at 6.5 and the optimum temperature was 90 °C with 100% relative activity. The purified enzyme was quite stable at 75 °C and had half of initial activity after 1 h of incubation at 90 °C. (TnMtDH) showed no activity with xylitol, inositol, sorbitol, rahmanose, mannose and xylose, and with NADPH and $NADP^+$ as co factors. The presence of some divalent metals in the reaction enhanced the enzyme activity. The enzyme might be utilizing to produce mannitol without other sugar conformation under high temperature.

1. Introduction

Mannitol is a type of sugar alcohol occurring naturally in bacteria, fungi, algae, and higher plants [1]. It has desired low calorie and high relative sweetness with low hygroscopicity and antioxidants properties, therefore it is widely used in food and nutraceuticals manufacturing [2]. Industrially, mannitol is produced by chemical hydrogenation of glucose and fructose mixture with yield of 25% (W/W) mannitol and 75% (W/W) sorbitol mixture [3]. In addition, mannitol can be produced from 50% fructose and mannitol 50% (W/W) for both sugars with sorbitol combination [4].

Recently, enzymatic production of mannitol from fructose attracted much attention because theoretically, the bioconversion ratio of fructose to mannitol reached 100% [1]. The co-enzyme responsible for conversion of fructose to mannitol is NADH-dependent mannitol 2-dehydrogenase (M2DH, EC 1.1.1.67). The MDH family mainly contributes in microbial metabolism of fructose and mannose. Consequently, MDH distributed in a wide species of microorganisms. Microbial production of mannitol and the related biosynthesis pathway have been widely studied, and lactic acid bacteria (LAB) were the most studied microorganisms [1]. MDH has been characterized from different LAB strains, including *L. sanfranciscensis* [5], *L. intermedius* [6], *L. brevis* [7], *L. reuteri* [8], *L. mesenteroides* [9], and *L. pseudomesenteroides* [10]. MDH was used to continuously produce mannitol from fructose with NADH cofactor regeneration through formate dehydrogenase (FDH) co-expression [11,12], moreover, the NADH recycling system together with co-expression of glucose isomerase (GI) was also developed for mannitol production directly from glucose as cheaper substrate [12].

MDH has also been characterized for industrial potential from other mesophilic microorganisms, including *C. magnolia* [13], *P. fluorescens* [14], and *R. sphaeroides* [15]. Generally, numerous groups have reported the purification and characterization of MDH from plants and microbial origins [31–34]. However, for industrial applications, the biocatalysts are preferably to be highly thermostable. Thermophilic or hyperthermophilic microorganisms are generally regarded as good sources for the thermostable enzymes. So far, the highly thermostable MDH only identified from *Thermotoga maritima* as hyperthermophilic strain [16]. In this study, biochemical characterization of a recombinant MDH from the hyperthermophilic *T. neapolitana* was studied, and the enzyme dis-

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played much higher thermostability than that reported form *T. maritime* MDH, showing a good potential for industrial application in mannitol production.

2. Materials and methods

2.1. Chemicals and reagents

Column resin for recombinant TnMtDH purification, the chelating Sepharose Fast Flow was ordered from GE Healthcare (Uppsala, Sweden). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA). Isopropyl- β-D-1-thiogalactopyranoside (IPTG), all chemicals for the assay were obtained from Sigma-Aldrich (St Louis, MO, USA). Sangon Biological Engineering Technology and Services (Shanghai, China) synthesized the reconstructed plasmid. *E. coli* strains used in this study were DH5α (Invitrogen, Carlsbad, CA, USA) for cloning and plasmid propagation.

2.2. Gene cloning and expression

T. neapolitana DSM 4359 whole genome sequence (GenBank accession number: ACM22561.1) revealed a putative MDH-encoding gene (locus_tag: CTN_0385, protein ID: WP_015918880.1). The target gene was commercially synthesized (Generay Biotech Co., Ltd, Shanghai, China) and cloned into the *Nde*I and *Xho*I sites of pET-22b (+) expression vector (Novagen, Darmstadt, Germany) with in-frame fusion 6 × histidine-tag sequence at the C-terminus. The reconstructed plasmid was transformed into *Escherichia coli* BL21 (DE3). The recombinant strain was grown in Luria-Bertani medium supplemented by 100 μg mL^{-1} ampicillin by shaking at 200 rpm and 37 °C for 12 h. After measuring the optical density 0.6 at 600 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and cultures were further grown for overexpression at 30 °C for 6 h.

2.3. Purification of the recombinant TnMtDH

All purification steps were performed at 4 °C. Cells were harvested by centrifugation, re-suspended onto 50 mM phosphate buffer (pH 7.5), and disrupted by sonication using a Vibra-Cell™ 72405 Sonicator. The disrupted cells were centrifuged at 20,000*g* for 30 min and the supernatant was loaded onto a HisTrap HP column (GE Healthcare, Uppsala, Sweden) for rapid affinity purification. The column was washed by using washing buffer (50 mM phosphate buffer, 500 mM NaCl, 50 mM imidazole, pH 7.0), and eluted by elution buffer (50 mM phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.0). Then eluted fraction was dialyzed in 50 mM phosphate buffer (pH 7.0) for overnight.

2.4. Determination of molecular mass

The pureness of TnMtDH was analyzed under denaturing conditions using SDS-PAGE, 5% stacking gel and 12% separating. Gels were staining by immersing in (Coomassie Brilliant Blue 250) and destained with aqueous mixture of acetic acid and methanol 10% (v/v). To determine the molecular mass of the native enzyme, 300 μL of purified enzyme was loaded onto a TSK G3000SW high-performance liquid chromatography (HPLC) column (Tosoh Co., Ltd,Tokyo, Japan) and, using a differential refractometer (Optilab® T-rEX™, CA, USA) with a light-scattering detector (Dawn® Heleos™ II, CA, USA) and UV detector (Waters 2489, USA), eluted with 50 mmol L−1 Tris buffer (pH 7.5) containing 300 mmol L^{-1} NaCl at a flow rate 0.4 mL min^{-1}.

2.5. TnMtDH assay

Enzyme activity was routinely assayed for both reduction and oxidation direction depending on fructose and mannitol as a major substrates, and NADH and NAD^+ as co-factors by measuring the degradation and production of the reactants at 340 nm. UV/visible spectrophotometer (Varian Instruments, Walnut Creek, Calif., USA) was used to measure the absorbance. The reaction mixture (1 mL) consisted of 50 mM phosphate buffer (pH 6.5), 500 mM fructose, 0.25 mM NADH, and 50 μL purified enzyme. The reaction mixture was incubated at 90 °C. The reaction was started by the addition of substrate, and the rate of NADH production was measured. One unit of enzyme activity was defined as the amount of TnMtDH required to convert 1 μmol NADH to NAD^+ min^{-1} at 90 °C.

2.6. Determination of optimal pH and optimal temperature of TnMtDH

Different buffer solutions with different pH values ranged from (5 to 9) were used to detect the optimal media for enzyme activity. The buffers were included sodium citrate buffer (50 mM, pH 4–5.5), sodium phosphate buffer (50 mM, pH 6.0–7.0), and Tris-, HCl buffer (50 mM, pH 7.5–8.5). The optimum temperature for enzyme activity was assaying in range of (50–100 °C) at pH 6.5. The thermal stability of TnMtDH was studied after incubating the enzyme in sodium phosphate buffer 50 mM, pH 6.5 in different temperature degrees 75, 80, 85, and 90 °C within specific interval times.

2.7. Effect of metal ions on recombinant TnMt2DH activity

The enzyme solution was incubated with the divalent metal ions Co^{2+}, Ba^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} at a final concentration of 1 m mol L^{-1}. The measured activities were compared with enzyme activity without metal ion addition (control) under the optimal conditions.

2.8. Substrate specificity of recombinant TnMtDH

To examine the substrate specificity of TnMtDH, enzyme activity was measured in presence of different substrates (xylitol, inositol, sorbitol, rahmanose, mannose and xylose.) at final concentration of 50 mmol L^{-1}. The measured activities were compared with the activity of the enzyme with fructose under the optimal conditions (control value 100%).

2.9. Kinetic analysis

Kinetic parameters of TnMtDH were determined at pH 6.5 and 90 °C using 0.25 to 2.5 mM NADH as cofactor and fructose as substrate from 10 to 500 mM. Kinetic parameters, including Michaelis-Menten constant (K_m) and turnover number (k_{cat}), were calculated based on the Lineweaver-Burk equation.

2.10. Analytical methods

Mannitol production was measured using HPLC system equipped with a Ca^{2+}-carbohydrate column (Waters Sugar-Pak 1, Waters Corp., Milford, MA, USA). The column was eluted by deionized water at 85 °C and 0.4 mL min^{-1} and a refractive index detector was equipped to detect the sugars.

2.11. Sequence similarity

The TnMtDH amino acids sequences released from the gene sequence was compared with similar enzymes from other organisms using the sequence alignment tool and