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Biochemical characterization of unusual *meso*-2,3-butanediol dehydrogenase from a strain of *Bacillus subtilis*

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ABSTRACT

meso-2,3-Butanediol dehydrogenase (BDH) catalyzes the redox reaction between (*R*)-acetoin and *meso*-2,3-butanediol (*meso*-BD). The BDHs isolated from bacteria are generally characterized as homo-tetramers. In the present work, an isolated and identified high acetoin (AC)-yielding (41.63 g/L) bacteria strain belonging to *Bacillus subtilis* but without 2,3-butanediol (BD) byproduct during its fermentation was selected. To understand the characteristics of BDH activity from this high AC-yielding strain, we cloned, purified and compared the BDH from *Enterobacter aerogenes* (CICC10293) (*E. a*-BDH) with the BDH from this high AC-yielding strain *B. subtilis*. Sequence alignments indicate a non-conservative amino acid substitution from Asp to Gly at site 194 on the *B. s*-BDH compared to that of *E. a*-BDH. Enzymatic analysis of *E. a*-BDH and D194G *B. s*-BDH shows D194G *B. s*-BDH has almost lost its entire enzymatic activity. Moreover, Isothermal titration calorimetry (ITC) measurements detected the substrate binding for the D194G *B. s*-BDH but no reaction was detected. Circular dicroism (CD) spectroscopy characterization revealed an identical secondary structure of *E. a*-BDH and D194G *B. s*-BDH. Remarkably, D194G *B. s*-BDH is highly susceptible to protease digestion, suggesting that the aspartic acid to glycine substitution might cause the proteolytic susceptibility of D194G *B. s*-BDH. Furthermore, by homology modeling with *meso*-2,3-butanediol dehydrogenase from *Klebsiella pneumoniae* (*K. p*-BDH) as a template, Gly194 seems to lose the hydrogen bond interactions with the surrounding residues (Gly206, Gly207 and Thr209), resulting in a putative conformational changes of D194G *B. s*-BDH which might be responsible for the loss of activity.

1. Introduction

Acetoin (AC) is a flavoring compound that is naturally found in fresh apples, milk, wines, etc. It has been widely used in food production as a chemical raw material. In addition, it is the precursor for the synthesis of liquid fuels by microorganisms [1]. So far, AC has been found to be produced by many microorganism fermentation or catalysis or chemical synthesis [2]. Compared to enzymatic conversion or chemical method, microbial fermentation is a cost-effective approach with many advantages including inexpensive raw materials, less environmental pollution and mild reaction conditions.

A high-yielding strain is pivotal for the commercial fermentation of AC. Many microorganisms could produce AC, including *Saccharomyces carlsbergensis* [3], *Saccharomyces cerevisiae* [4], *Hanseniaspora guilliermondii* [5], *Lactococcus lactis* [6], *Leuconostoc citrovorum* [7], and *Zygosaccharomyces bailii* [8], etc. In particular, *Serratia marcescens* H13 (75.2 g/L) [9], *Bacillus licheniformis* MEL 09 (41.2 g/L) [10], and *Bacillus pumilus* DSM 16187 (63.0 g/L, EP 2005081882) exhibited high AC-producing ability from glucose.

AC is synthesized in the bacteria cytoplasm with the catalysis of pyruvate (catabolic pathway) by two enzymes: α-acetolactate synthase, that catalyzes the condensation of two pyruvate molecules to form (*S*)-α-acetolactate and α-acetolactate decarboxylase,

Abbreviations: DTT, dithiothreitol [(2*S*,3*S*)-1,4-bissulfanylbutane-2,3-diol]; PMSF, phenylmethylsulfonyl fluoride; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; IPTG, isopropyl τ-D-1-thiogalactopyranoside; EDTA, 2-[2-[bis(carboxymethyl)-amino] ethyl-(carboxymethyl)amino] acetic acid; NADH, nicotinamide adenine dinucleotide (reduced form); NAD^+, nicotinamide adenine dinucleotide (oxidized form); BD, 2,3-butanediol; AC, acetoin; DA, diacetyl; BDH, 2,3-butanediol dehydrogenase; *budC*, gene encoding BDH; ε_{340}, molar extinction coefficient at 340 nm; ITC, isothermal titration calorimetry; CD, circular dichroism; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time of Flight.

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that decarboxylates (*S*)-α-acetolactate to (*R*)-AC [11]. Besides, α-acetolactate is converted to diacetyl (DA) by a non-enzymatic oxidative decarboxylation. Then, DA is reduced to AC, followed to 2,3-butanediol (BD) by 2,3-butanediol dehydrogenase (BDH, also named diacetyl reductase or acetoin reductase) [12]. Further, different BD isomeric forms (i.e., (2*R*,3*R*)-BD, (2*S*,3*S*)-BD and *meso*-BD) can be produced by various BDHs with different stereospecificity [12].

Noteworthy, fermentation productions of AC by bacteria always accompany a large number of downstream reductive 2,3-butanediol (BD) byproduct [12]. Dai's group isolated and identified a high AC productivity (41.63 g/L) bacteria strain from soil. Intriguingly, this strain does not produce the byproduct BD. According to its 16S rDNA gene sequence, the *gmuG* sequence encoding β-mannanase (EC 3.2.1.78) and the physiological/biochemical experiment results, the strain was characterized as *Bacillus subtilis*, which is a non-pathogenic bacterium used extensively in the large-scale fermentation industry. In fact, a high AC yielding *B. subtilis* mutant (53.9 g/L) blocked in BDH can be obtained by UV irradiation coupled with diethyl sulfate [13]. A nonsense mutation (p.Tyr118X) of the *B. subtilis* mutant was detected prohibiting the synthesis of a full-length functional BDH [13].*meso*-BDH belongs to the short-chain dehydrogenase/reductase (SDR) family judging from its amino acid sequence [14]. A number of crystal structures of SDR enzymes have been reported [15] and all of them have an N-terminal coenzyme-binding motif of GXXXGXG, an active site motif of YXXXK and a core structure similar to the Rossmann fold [16]. In addition, there exists a common catalytic tetrad formed by "Asn-Ser-Tyr-Lys" in SDR family enzymes based on biochemical and crystallographic studies [17]. To date, there have been two X-ray crystal structures of BDHs solved [12,18]. The crystal structure of *K. p*-BDH (*meso*-BDH) was a homo-tetramer with a molecular weight of approximately 100 kDa [19]. The substrates of BDH vary widely in molecular size and properties, including 2,3-pentanedione, pyruvic acid methyl ester and methyl glyoxal [20]. Mutagenesis of active site residues of *meso*-BDH dramatically diminished (the double mutant Q140I/N146F or the triple mutant Q140I/N146F/W190H) its activity toward *meso*-BD but for the triple mutant, it still maintained ~50% and ~67% activity toward (2*S*,3*S*)-BD compared to the wild-type *meso*-BDH and the wild-type (2*S*,3*S*)-BDH, respectively [18].

In our present work, to dissect why the enzymatic activity of D194G *B. s*-BDH was almost abolished, we investigated D194G *B. s*-BDH by ITC and thereby determined its thermodynamic parameters. In addition, a comparative CD spectra and trypsin digestion experiment were performed for *E. a*-BDH and D194G *B. s*-BDH. We show that the D194G *B. s*-BDH almost lost its entire enzymatic activity. Moreover, D194G *B. s*-BDH at native condition was cleaved by trypsin within 1 h. Collectively, we conclude that the conformational change of D194G *B. s*-BDH is the main reason for the loss of enzymatic activities. Namely, this conformational change may restrict the reaction between the protein and its substrate and result in the enzymatic activity losing of D194G *B. s*-BDH.

2. Experimental

2.1. Enzymes and chemicals

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs Inc. (USA). DNA polymerase was purchased from TaKaRa Bio Inc. (China). DNA oligomers were synthesized and purified by TaKaRa Bio Inc. (China). NativeMARK Unstained Protein Standard (Cat.LC0725) was supplied by Life Technologies (USA). Ready Gel® Tris–HCl Gel (Cat. No. 161-1158) by BIO-RAD (USA). DA, AC, (2*R*,3*R*)-BD (98.0%), (2*S*,3*S*)-BD (99.0%), and *meso*-BD (98.0%), NADH/NAD^+ were purchased from Aldrich or Sigma. IPTG, DTT, EDTA and PMSF were obtained from Merck (Germany). All other chemicals were analytical grade and commercially available.

2.2. Bacterial strains and plasmids

Eschrichia coli DH5a and BL21 (DE3) were used for general cloning and expression procedures, respectively. The pMD19-T vector (TaKaRa) was used as a vector to subclone the gene, and pET-21b with a T7 promoter was used for protein expression. *Enterobacter aerogenes* was purchased from the China Center of Industrial Culture Collection (CICC10293). D194G *B. s*-BDH-expressing *B. subtilis* was donated by Associate Professor Jian-ying Dai. Luria broth (LB) medium was used for all *E. coli* cultivations. Ampicillin was used at a final concentration of 100 μg/ml.

2.3. Cloning of budC

To obtain the *budC* encoding BDH from *E. aerogenes* and D194G *B. s*-BDH-expressing *B. subtilis*, the primers, P1 and P2, were designed using the genomic sequence of *E. aerogenes* KCTC 2190 as a target. P1 with Nde I restriction site insertion and P2 with Hind III restriction site insertion were as follows: P1, 5′CGCCATATGATGAAAAAAGTCGCACTTGT CACCGG3′ and P2, 5′CCCAAGCTTTTAGTTAAACACCATCCCGCCGTCGA3′. The genes were amplified by polymerase chain reaction (PCR) from the genome of *E. aerogenes* and *B. subtilis* using P1 and P2, and then were ligated to the pMD19-T vector. The constructed vectors were named pMD19-T-*budC-E. a* and pMD19-T-*budC-B. s*, respectively and then sequenced (TaKaRa, Dalian, China).

2.4. Expression, purification and molecular mass of BDH

To construct the *budC* expression vector under the control of promoter T7, the pMD19-T-*budC-E. a* and pMD19-T-*budC-B. s* were digested with Nde I and Hind III, and the gel-purified *budC* fragments were inserted into pET-21b vectors. The resulting plasmids were designated as pET-21b-*budC-E. a* and pET-21b-*budC-B. s*, respectively, and then transformed to *E. coli* BL21 (DE3). For protein production, *E. coli* cells harbored these vectors were grown at 37 °C, 180 rpm in LB medium containing ampicillin (100 μg/ml) to an absorbance at 600 nm of 0.6. Expression of recombinant proteins was induced by the addition of 1 mM IPTG at 16 °C for 16 h. Cells were harvested by centrifugation at 6000 × *g* for 5 min at 4 °C and then washed twice with 0.85% NaCl. The cell pellet was harvested by centrifugation at 10,000 × *g* for 5 min at 4 °C and resuspended with 10 mM Tris–HCl buffer, pH 8.0, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF. Cells were lysed by sonication with an ice bath. The homogenate was centrifuged at 18,000 × *g* for 1 h at 4 °C. Clarified cell lysate was loaded onto a 5 ml HiTrap Q FF anion exchange column (GE Healthcare, USA), equilibrated with 10 mM Tris–HCl buffer, pH 8.0, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF. Active fractions were eluted with 10 mM Tris–HCl buffer, pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF. The active fractions recovered were combined and concentrated by ultrafiltration using Ultracel-30 ultrafiltration membrane centrifuged at 5000 × *g* for 30 min at 4 °C (Millipore, Milford, MA) and subjected to gel filtration on Superdex 200 column (GE Healthcare, USA) in 20 mM potassium phosphate buffer, pH 7.6, for final purification. The column was pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.6 at 25 °C for two-column volume at 1 ml/min. Protein sample loaded volume was 1 ml, and UV detection wavelength was 280 nm.

Protein concentration was determined according to the method of Bradford. Protein purity and identity was assessed by SDS-PAGE, with 200 V applied voltage and visualized by staining with Coomassie Brilliant Blue R-250. The molecular weight of proteins