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Characterization of the major dehydrogenase related to D-lactic acid synthesis in *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293

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ABSTRACT

Leuconostoc mesenteroides subsp. *mesenteroides* ATCC 8293 is a lactic acid bacterium that converts pyruvate mainly to D-(−)-lactic acid by using D-(−)-lactate dehydrogenase (ldhD). The aim of this study was to identify the gene responsible for D-lactic acid formation in this organism and to characterize the enzyme to facilitate the production of optically pure D-lactic acid. A genomic analysis of *L. mesenteroides* ATCC 8293 revealed that 7 genes encode lactate-related dehydrogenase. According to transcriptomic, proteomic, and phylogenetic analyses, LEUM_1756 was the major gene responsible for the production of D-lactic acid. The LEUM_1756 gene, of 996 bp and encoding 332 amino acids (36.5 kDa), was cloned and overexpressed in *Escherichia coli* BL21(DE3) Star from an inducible pET-21a(+) vector. The enzyme was purified by Ni-NTA column chromatography and showed a specific activity of 4450 U/mg, significantly higher than those of other previously reported ldhDs. The gel permeation chromatography analysis showed that the purified enzyme exists as tetramers in solution and this was the first report among lactic acid bacteria. The pH and temperature optima were pH 8.0 and 30 °C, respectively, for the pyruvate reduction reaction, and pH 11.0 and 20 °C, respectively, for the lactate oxidation reaction. The K_m kinetic parameters for pyruvate and lactate were 0.58 mM and 260 mM, respectively. In addition, the k_{cat} values for pyruvate and lactate were 2900 s^{-1} and 2280 s^{-1}, respectively. The enzyme was not inhibited by Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, or urea, but was inhibited by 1 mM Zn^{2+} and 1 mM SDS.

1. Introduction

As our understanding of global warming is increasing, the need to develop new materials to replace petroleum-based plastics of which the production releases large amounts of CO_2 becomes increasingly important. Polylactic acid (PLA) is a bio-plastic synthesized by direct condensation and polymerization of the lactic acid monomer. In recent years, interest in the use of lactic acid to produce PLA product has rapidly increased [1]. Lactic acid has two optical isomers: D-lactic acid and L-lactic acid [2]. Optically pure L-(+)- or D-(−)-lactic acid, rather than racemic DL-lactic acid, can be polymerized to produce highly crystalline PLA suitable for commercial use [3,4]. Although racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources, optically pure L-(+)- or D-(−)-lactic acid can be obtained by microbial fermentation of renewable resources using appropriate microorganisms [5–7].

D- and L-lactic acids are also produced in lactic acid-fermented foods. For example, D/L proportion in sauerkraut and kimchi are 1.13 and 0.60, respectively, and in yogurt the proportion of D-lactic acid to L-lactic acid is low (0.1–0.81) [8]. *Leuconostoc* spp., used as a starter culture in the manufacture of various fermented foods such as sauerkraut and kimchi, is the main lactic acid bacterium (LAB) producing lactic acid, acetic acid, alcohol, and CO_2. *Leuconostoc* spp. mainly synthesize D-lactic acid through the action of D-lactate dehydrogenase (ldhD) during pyruvate metabolism [8,9]. Recently, strains used in the commercial production of lactic acid have become almost proprietary, and it is believed that most LABs used commercially belong to the *Lactobacillus* and *Streptococcus* genera [5,10]. In previous studies, *ldhD* genes were mainly cloned from *Lactobacillus* species, such as *Lb. rhamnosus*, *Lb. delbrueckii*, *Lb. plantarum*, and *Lb. casei* [11–13]. Until now, although the metabolic process for producing lactic acid from pyruvate in *Leuconostoc* strains has been investigated, no research on the potential use of *Leuconostoc* spp. for biotechnological production of lactic acid has been done, and among *Leuconostoc* species only the *Leuconostoc cremoris ldhD* gene has been studied [14].

The aim of this study was to characterize the gene and encoded enzyme responsible for D-lactic acid formation in *Leuconostoc mesenteroides* ATCC 8293, whose full genome sequence was completed recently. For this, we identified and cloned the

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major gene involved in D-lactic acid synthesis among different dehydrogenase genes present in *L. mesenteroides* ATCC 8293 by transcriptomic, proteomic, and phylogenetic analyses, and expressed and purified the enzyme. We then characterized the purified enzyme by analyzing enzyme activity, substrate specificity, and kinetic parameters, and by performing inhibitor screens.

2. Materials and methods

2.1. Bacterial strains and plasmids

L. mesenteroides subsp. *mesenteroides* ATCC 8293 was grown in MRS medium (Difco, Detroit, MI, USA) at 30 °C without shaking. *Escherichia coli* MC 1061 and *E. coli* BL21 (DE3) Star strains, used for cloning and protein expression, were grown aerobically at 37 °C in Luria–Bertani (LB) medium. *E. coli* transformants were grown in LB medium containing ampicillin (100 μg/mL final concentration) at 37 °C. The commercial pGEM-T vector (Promega, Madison, WI, USA) was used for direct cloning of PCR products. The pET-21a(+) *E. coli* plasmid (Novagen, Darmstadt, Germany) was used for DNA manipulations.

2.2. Transcriptomic, proteomic, and phylogenetic analyses of L. mesenteroides ATCC 8293 ldhD

L. mesenteroides ATCC 8293 was cultured in MRS medium, and cells were harvested from the exponential growth phase occurring at $OD_{600\,nm}$ of 0.7–1.0. For transcriptome analysis, sample preparation and microarray hybridization was performed according to Seo [15]. Hybridized microarrays were scanned using a GenePix 4000B microarray scanner (Axon Instruments, Union City, Alameda, USA) at PMT 500–700, pixel size 5, and focus position 130. Data were analyzed according to Nittler et al. [16].

For proteome analysis, *L. mesenteroides* cells were lysed and soluble proteins were precipitated for pretreatment. Whole lysate proteins were analyzed using an Eksigent Nano LC 2D system coupled to an LTQ ion-trap mass spectrometer (Thermo-Fisher) through a Picoview nano-spray source according to Kim et al. [17]. All MS/MS samples were analyzed using X! Tandem (http://www.thegpm.org; version 2006.04.01.2) and Scaffold. Data were analyzed according to Paoletti et al. [18].

For phylogenetic tree construction, the amino acid sequence of *L. mesenteroides* ATCC 8293 lactate-related dehydrogenase was compared with similar ldhs from published LABs, and ldh enzymes were aligned using the CLUSTAL W program in the DNA Data Bank of Japan (DDBJ).

2.3. Construction of recombinant plasmid

The *ldhD* gene was PCR amplified from *L. mesenteroides* ATCC 8293 genomic DNA using primers designed by sequence information in the genome database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov): *ldhD*-N (5′-TTCATATGAAGATTTTTGCTTAC-3′) and *ldhD*-C (5′-TTCTCGAGATATTCAACAGCAATAGC-3′). The purified PCR product was ligated to the pGEM-T vector to construct the pGEM-T-*ldhD* plasmid (4002 bp). The *ldhD* open reading frame (996 bp) was then excised from pGEM-T-*ldhD* by digestion with *Nde*I and *Xho*I and ligated into a pET-21a(+) backbone to construct the pET*ldhD* (6363 bp) recombinant plasmid. pET*ldhD* was transformed into *E. coli* MC1061 and transformants were selected on LB agar containing 100 μg/mL ampicillin.

2.4. ldhD protein expression

For ldhD expression, *E. coli* BL21 (DE3) Star cells transformed with pET*ldhD* were cultured at 37 °C in LB medium containing 100 μg/mL ampicillin until $OD_{600\,nm}$ of 0.5 was reached. Protein expression was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and cells were grown for an additional 6 h at 30 °C with shaking at 250 rpm. Cells were harvested by centrifugation at 10,786 × *g* for 5 min at 4 °C, resuspended in lysis buffer (10 mM imidazole, 0.5 M sodium chloride, 20 mM sodium phosphate; pH 7.4), and disrupted by sonication. Soluble enzyme was purified and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Purification of recombinant enzyme by Ni-NTA chromatography

Recombinant enzyme containing a C-terminal 6-histidine tag was purified by Ni-NTA chromatography using a HisTrap-FF column (GE Healthcare, Uppsala, Sweden) and eluted with elution buffer (0.5 M imidazole, 0.5 M sodium chloride, 20 mM sodium phosphate; pH 7.4).

Gel permeation chromatography (GPC) analysis was performed to investigate the oligomeric state of recombinant ldhD. Ni-NTA column fractions containing ldhD protein were concentrated by ultrafiltration (Amicon Co., USA), dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and a protein sample (100 μL) was applied to a Superdex 200 column (GE Healthcare, Uppsala, Sweden) on an AKTA FPLC system (Fast Protein Liquid Chromatography; GE Healthcare) and eluted with the same buffer at a flow rate of 0.5 mL/min. Protein molecular weight markers (Sigma–Aldrich Co., St. Louis, MO, USA) were used to estimate the apparent molecular weight of the enzyme.

2.6. Assay of enzyme activity and the reaction product

The ldhD activity was measured in two reactions: (1) pyruvate reduction and (2) lactic acid oxidation. For pyruvate reduction, 0.01 mL enzyme was incubated in 0.3 mL substrate solution (containing 0.5 mM NADH and 10 mM pyruvate in 0.3 mL 0.1 M Tris–HCl buffer, pH 8.0). For lactic acid oxidation, 0.01 mL enzyme was incubated in 0.3 mL substrate solution (containing 5 mM NAD^+ and 500 mM D-lactic acid in 0.3 mL 0.1 M glycine–sodium hydroxide, pH 11.0). Both types of reaction mixtures were incubated at the temperatures and for the times indicated and absorbance at 340 nm was used to monitor product synthesis. One unit of activity was defined as the oxidation of 1 μmol NADH per min or the reduction of 1 μmol NAD^+ per min. Protein concentration was measured using a Thermo BCA™ protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA).

Product quantitation was performed using a high-performance liquid chromatography (HPLC) system, consisting of a YOUNG-LIN M930 solvent delivery pump (Younglin, Seoul, Korea), a YOUNG-LIN M720 absorbance detector, and an 8.0 mm × 50 mm Shodex ORpac CRX853 chiral column with a CRX-G column (Showa Denco, Tokyo, Japan). The flow rate of the mobile phase (1.00 mM $CuSO_4$ in H_2O) was 1 mL/min and analytes were monitored at 250 nm absorbance using a UV spectrophotometric detector.

2.7. Effects of pH and temperature on ldhD enzyme activity

The effect of pH on ldhD activity was measured using 100 mM phosphate buffer (pH 6.0–7.0), 100 mM Tris–HCl buffer (pH 8.0–10.0), and 100 mM glycine–sodium hydroxide (pH 11.0–13.0) at room temperature. To test the effect of temperature on ldhD activity, reactions were performed at a range of temperatures (10–50 °C).

2.8. Substrate specificity and enzyme inhibition

To investigate substrate specificity, levulinic acid, sodium phenylpyruvate, α-ketobutyric acid, oxaloacetate, 2-oxoglutarate, and malic acid were tested as substrates for the purified enzyme. Enzyme activity for these substrates was analyzed using reaction conditions described above. The activities towards pyruvate and lactate substrates were defined as 100%.

To determine the effects of inhibitors on ldhD activity, purified enzyme was incubated in 0.1 mM or 1 mM concentrations of potential inhibitors (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Na^+, SDS, and urea) in Tris–HCl buffer (pH 8.0) at room temperature for 30 min. Pyruvate reducing activity of enzyme in a control reaction without inhibitor was defined as 100%.

2.9. Kinetic parameters

For the kinetic analysis of ldhD pyruvate reduction activity, various pyruvate concentrations (0.1, 0.2, 0.5, 1, and 5 mM) were tested at 0.5 mM NADH; in addition, various NADH concentrations (0.05, 0.1, 0.2, 0.4, and 0.5 mM) were tested at 10 mM pyruvate. For the kinetic analysis of ldhD lactic acid oxidation activity, various lactate concentrations (25, 50, 100, 250, and 500 mM) were tested at 5 mM NAD^+; in addition, various NAD^+ concentrations (0.25, 0.5, 1, 2, and 4 mM) were tested at 500 mM lactate. Results were analyzed using double reciprocal Lineweaver–Burk plots.

3. Results and discussion

3.1. Transcriptomic, proteomic, and phylogenetic analyses of ldh genes

A genomic analysis of *L. mesenteroides* ATCC 8293 revealed that 7 genes encode lactate dehydrogenases or related enzymes: LEUM_0373, LEUM_1837, LEUM_0445, LEUM_1233, LEUM_0503, LEUM_1756, and LEUM_2043. When transcriptional levels of those genes were measured by microarray analysis, mRNA of 7 genes were detected and among them LEUM_0445 and LEUM_1756 were highly transcribed (Table 1). Additionally, when translational levels of 7 genes were analyzed using LC–MS/MS, 3 proteins were detected and among them the protein corresponding to LEUM_1756 gene product was detected at the highest levels (Table 1). The amino acid sequences encoded by the 7 *L. mesenteroides* ATCC 8293 *ldh* genes were compared with those of other *ldhD* genes previously reported (Table 2). The amino acid sequence of LEUM_1756 has