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Purification and characterization of membrane-bound L-sorbose dehydrogenase from *Gluconobacter oxydans* GO112

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Abstract

The bacterial strain *Gluconobacter oxydans* GO112 exhibiting enhanced production of 2-keto-L-gulonic acid (2-KLG) from L-sorbose was bred by this laboratory. L-Sorbose dehydrogenase is one of the key enzymes responsible for the production of 2-KLG. In this study, we purified L-sorbose dehydrogenase from GO112 to homogeneity through sequential chromatographical steps: DEAE-Sepharose Fast Flow, Mono Q anion exchange and Superose 12 gel filtration. The purified L-sorbose dehydrogenase was single subunit protein with apparent molecular weight of about 60 kDa by SDS-PAGE and about 116 kDa by gel filtration chromatography, indicating the L-sorbose dehydrogenase may exist as dimeric molecules under physiological conditions. The optimum pH and temperature for the enzyme were pH 6.86 and 40 °C. It showed good stability at pH 6.2 and temperatures below 30 °C. At 40 °C, the L-sorbose dehydrogenase lost its activity by 37% in the first 1.5 min and then inactivated slowly. The K_m value for L-sorbose was 36 mM. The activity of the L-sorbose dehydrogenase was greatly stimulated by Ca^{2+}, while Mn^{2+}, Fe^{2+}, Cu^{2+}, EDTA and citric acid inhibited the activity of the L-sorbose dehydrogenase to different degrees.

Keywords: 2-Keto-L-gulonic acid; Enzyme characterization; *Gluconobacter oxydans*; L-Sorbose dehydrogenase

1. Introduction

2-Keto-L-gulonic acid (2-KLG) is a key intermediate in commercial production of L-ascorbic acid (L-AA, Vitamin C). The most popular process for synthesizing 2-KLG is the 70-year-old Reichstein's method, in which glucose is transformed to 2-KLG in five chemical steps [1]. Since Gray' description of microbial methods for the conversion of L-sorbose to 2KLG [2], the use of microbial processes to produce 2-KLG has become attractive [3–7] and gradually replaced the chemical method for lower industrial cost and less ecological problems. There are two most commercially promising methods for microbial production of 2-KLG. One is called "2,5-DKG pathway" [8] and the other is called "L-sorbose pathway" [6]. At present, the yield of 2-KLG through the "L-sorbose pathway" is much higher than that through the "2,5-DKG pathway" [9–11]. Generally, the mixture of *Gluconobacter oxydans* and a *Bacillus* genus strain is used in the "L-sorbose pathway" [9,12,13]. The L-sorbose dehydrogenase and L-sorbosone dehydrogenase (SNDH) expressed by *G. oxydans* are responsible for transforming L-sorbose to 2-KLG, while the *Bacillus* genus strain functions as the companion to accelerate the growth of *G. oxydans* [10,16].

The "L-sorbose pathway" was firstly elucidated by Hoshino et al. in 1990 in *Gluconobacter melanogenus* IFO 3293. The membrane-bound L-sorbose dehydrogenase and the cytoplasmic SNDH catalyzed a series of reactions that transformed L-sorbose to 2-KLG [15,16]. Sugisawa et al. further purified and characterized the L-sorbose dehydrogenase from *G. melanogenus* UV10—a mutant of *G. melanogenus* IFO 3293 and indicated that L-sorbose dehydrogenase was the rate-limiting enzyme in the transformation process [17,18]. However, the L-sorbose dehydrogenase in *G. oxydans*, the most commonly used microorganism in the biological production of 2-KLG, is still poorly studied. Only Saito had reported the purification and molecular cloning of the L-sorbose dehydrogenase from *G. oxydans* T-100 [22].

Recently, our laboratory isolated a *G. oxydans* strain GO112 [14]. The mixture of GO112 and *B. megaterium*

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BM302 transformed L-sorbose to 2-KLG at a level of 94.5% in molar transformation rate, the highest that have been reported. Since L-sorbose dehydrogenase is one of the key factors that determine the transformation rate, we focused on purification and characterization of the L-sorbose dehydrogenase expressed by *G. oxydans* GO112 in the present study. The results were presented in this article.

2. Materials and methods

2.1. Microorganisms and chemicals

Gluconobacter oxydans GO112 and *Bacillus megaterium* BM302 were preserved in the Key Laboratory of Ion Beam Bioengineering of Chinese Academy of Sciences. 2,6-Dichlorophenolindopenol (DCIP) was purchased from Sigma–Aldrich. L-Sorbose used for analysis was purchased from Fluka. All other chemicals used in this study were reagent grade.

2.2. Production of L-sorbose dehydrogenase

Seeding medium (%): 2.0 L-sorbose, 0.2 glucose, 0.5 corn steep liquor, 0.1 carbamide, 0.2 $CaCO_3$. Production medium (%): 8.0 L-sorbose, 1.0 cornsteep liquor, 1.2 carbamide, 0.1 KH_2PO_4, 0.01 $MgSO_4{\cdot}7H_2O$, 0.5 $CaCO_3$. Isolation medium (%): 0.5 L-sorbose, 0.3 beef extract, 0.3 yeast extraction, 1 peptone, 0.2 $MgSO_4{\cdot}7H_2O$, 2 agar. These media were adjusted to pH 6.7 before autoclaving. *G. oxydans* GO112 on isolation medium was transferred to agar slant (0.3% yeast extract, 0.3% beef extract, 1% peptone, 0.5% L-sorbose, 0.2% $MgSO_4$ and 2% agar) and mixed with *B. megaterium* BM302 at 29 °C for 24 h. A loopfull of agar slant culture was inoculated into 20 ml of seeding medium, and cultivated at 29 °C on a rotary shaker at 180 rpm for 24 h. Then 9 ml of this culture was inoculated into 200 ml of production medium and cultivated at 33 °C on a rotary shaker at 200 rpm. After 24 h cultivation, the culture was harvested. The biomass of BM302 decreased sharply in 24 h from 9 to 4×10^6 CFU/ml while GO112 increased from 1.2 to 3.9×10^8 CFU/ml. For the collection of *G. oxydans* GO112 cells, the culture was firstly centrifuged at 2000 rpm and 4 °C for 10 min to remove *B. megaterium* BM302 cells. Then the supernatant was centrifuged at 8000 rpm and 4 °C for 20 min to precipitate the GO112 cells. The collected cells were washed twice with PBS and frozen at −20 °C until use.

2.3. Enzyme assay

L-Sorbose dehydrogenase activity was detected at 25 °C by measuring the decrease in the absorbance of DCIP at 600 nm. The basal reaction mixture was prepared just before the assay. It consisted of 100 ml of 0.1 M potassium phosphate buffer (pH 7.0), 15 ml of 2.5 mM DCIP, 15 ml of 0.3% Triton X-100 and 150 ml water. A cuvette with 1 cm light path containing 3.2 ml of basal mixture and 0.8 ml 1 M L-sorbose was incubated at 25 °C for 5 min. Then the reaction was started by adding the enzyme. One unit of L-sorbose dehydrogenase activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of DCIP/min. The extinction coefficient of DCIP and the employed wavelength at pH 5.8, 6.2, 6.4, 6.6, 6.8 was 10.3, 11.7, 12.6, 13.4, 14.2 mM^{-1}, respectively, at pHs 7.0–7.6 was 14.5 mM^{-1}, at pH 8.0 was 15 mM^{-1}, and the employed wavelength is 600 nm.

2.4. Purification of L-sorbose dehydrogenase

The frozen GO112 cells were thawed and resuspended in 10 mM potassium phosphate buffer (pH 7.0, containing 0.3% Triton X-100). This suspension was sonicated on an ice bath and centrifuged at 12,000 rpm and 4 °C for 30 min to remove the cell debris. Then the supernatant that contained L-sorbose dehydrogenase was subjected to following chromatographical purification steps: (1) the crude extract was loaded on a DEAE-Sepharose Fast Flow column which was pre-equilibrated with buffer A (10 mM potassium phosphate buffer, pH 7.0, containing 0.3% Triton X-100). A linear gradient of NaCl from 0 to 0.6 M in buffer A was used to elute L-sorbose dehydrogenase. The fractions showing L-sorbose dehydrogenase activity were pooled, dialyzed against buffer A and concentrated in an Amicon ultrafiltration stirred cell equipped with a 10,000 Da weight cut-off membrane. (2) The concentrated sample was applied to a Mono Q HR 5/5 preloaded column which was also equilibrated with buffer A. Then the same elution condition used in DEAE-Sepharose FF was performed. Fractions containing the L-sorbose dehydrogenase were pooled and concentrated by ultrafiltration. (3) The sample obtained from step 2 was loaded on Superose 12 10/300 GL column equilibrated with buffer B (10 mM potassium phosphate buffer, pH 7.0, containing 0.3% Triton X-100 and 0.2 M NaCl). The column was run with the same buffer. The active fractions were pooled and dialyzed against buffer A. The L-sorbose dehydrogenase purity was assessed by SDS-PAGE.

2.5. Enzyme characterization

The protein concentration was estimated by the method of Lowry et al. [19]. SDS-PAGE was performed on 12% polyacrylamide gels according to standard protocol [20] and stained with Coomassie Brilliant Blue R-250. The molecular weight of the denatured L-sorbose dehydrogenase was determined by SDS-PAGE. The apparent molecular weight of native L-sorbose dehydrogenase was estimated on a preloaded Superose 12 10/300 GL column. The standard proteins kit used to calibrate the column was from Sigma. The optimal pH and pH stability were tested in potassium phosphate buffer at various pH values. For determining the pH stabilities, the purified L-sorbose dehydrogenase was added into potassium phosphate buffer of various pH values and kept at 4 °C for 8 days. The optimal temperature for L-sorbose