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Characterization and application of a glycolate dehydrogenase from *Trichoderma harzianum* AIU 353

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ABSTRACT

A glycolate dehydrogenase (GADH), which catalyzes oxidation of glycolic acid to glyoxylic acid, was found from a newly isolated fungus, *Trichoderma harzianum* AIU 353. The dehydrogenase reaction required nitro blue tetrazolium or 2,6-dichlorophenolindophenol, but not NAD^+ and $NADP^+$ as an electron carrier. Methylglycolate, glycolaldehyde, L-lactic acid, glyceraldehyde and dihydroxyacetone were also oxidized, but primary alcohols, dihydric alcohols and aliphatic aldehydes were not. The K_m values for glycolic acid, L-lactic acid and dihydroxyacetone were estimated to be 0.94 mM, 3.6 mM and 49.5 mM, respectively. The GADH activity was optimum at pH 7.0 and 30 °C. The molecular mass of this enzyme was 280 kDa consisting of four subunits with molecular mass of 107, 81, 53 and 38 kDa. These characteristics of the fungal GADH are remarkably differentiated from those of other GADHs. The enzyme and cells were applicable for the production of glyoxylic acid from glycolic acid.

1. Introduction

The enzymes catalyzing oxidation of glycolic acid to glyoxylic acid were studied in photosynthetic organisms for a long time, and presence of two different enzymes, glycolate oxidase (GAOD) (EC 1.1.3.1, now referred to as EC 1.1.3.15, (S)-2-hydroxy-acid oxidase) and glycolate dehydrogenase (GADH) (EC 1.1.99.14), were well established in higher plants and unicellular algae [1–7]. The GAODs, which use inorganic oxygen as an electron acceptor and formed H_2O_2, were demonstrated in higher plants and some algae [1–4]. The properties and structure of GAODs were also elucidated [8–14]. In contrast, GADHs, which exhibit the enzyme activity by incubation with an exogenous electron carrier, were observed in green alga, cryptomonads, and diatoms [4–7], and the GADH genes were isolated from green alga [15–17]. The presence of GADHs was also reported in a glycolate-oxidizing bacterium [18], a glyoxylate-fermenting bacterium [19] and *Escherichia coli* [20,21], but those GADHs have not been purified yet.

During studies of enzymes for the conversion of ethylene glycol into glyoxylic acid, we isolated new microorganisms, which exhibit GADH activity. These isolated strains did not require the light environment for the production of GADH. This cultivation characteristic gets an advantage over photosynthetic organisms to develop a new biochemical method for the production of glyoxylic acid, which is used as a key intermediate in the pharmaceutical or agrochemical industries. We therefore selected one fungal strain, which exhibited high dehydrogenase activity for glycolic acid but not for glyoxylic acid, from our isolated strains, and purified the dehydrogenase. The present paper describes identification of the new isolated strain, optimum conditions for GADH production, and certain properties of the dehydrogenase.

2. Materials and methods

2.1. Chemicals

Glycolic acid sodium salt, glycolaldehyde, glyoxylic acid sodium salt, DL-glyceric acid, DL-glyceraldehyde, propylene glycol, nitro blue tetrazolium (NBT), 2,6-dichlorophenolindophenol (DCPIP), phenazine methosulphate (PMS) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). NAD^+ and $NADP^+$ were from Oriental Yeast Co. Ltd. (Tokyo, Japan). All other chemicals used were of analytical grade and commercially available.

2.2. Screening of microorganism

Microorganisms were first isolated by enrichment culture using propylene glycol as a sole carbon source. The isolates were then incubated in a test tube containing 20 ml of a propylene glycol medium consisting of 3% propylene glycol, 0.1% NaH_2PO_4, 0.1% K_2HPO_4, 0.2% NH_4NO_3, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01%$CaCl_2 \cdot 2H_2O$ and 0.05% yeast extract, pH 7.0, at 30 °C for 3 days, after which a

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cell-free extract was prepared by disrupting the cells at below 5 °C for 8 min by a multi-beads shocker (Yasui Kikai, Osaka, Japan). Then, the dehydrogenase activity for glycolic acid and glyoxylic acid was assayed using the cell-free extract, and GADH-producers were selected. Substrate specificity of the enzyme produced by the selected strains was further confirmed using eluates from a DEAE-Toyopearl column chromatography of each crude enzyme solution, which was prepared from cells from 300 ml of the propylene glycol medium. The strain exhibited high activity for glycolic acid but not for glyoxylic acid was finally selected, and used in this study.

2.3. Taxonomic studies of isolated strain

Identification of a newly isolated strain was performed at TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan) as follows. The isolated strain was incubated on a potato–dextrose agar plate (Nihon Seiyaku, Tokyo, Japan) and an oatmeal agar plate (Becton Dickinson, MD, USA) at 25 °C in the dark, and the morphological characteristics were observed with both a compound microscope and a stereomicroscope. The sequences of 28S rDNA-D1/D2 and ITS-5.8S rDNA were analyzed using a PrimeSTAR HS DNA polymerase (TakaraBio, Otsu, Japan), an ABI BigDye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA, USA), and a ABI PRISM 3130x1 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). The sequence alignment and calculation of the homology levels were carried out using the database of GenBank, DDBJ and EMBL.

2.4. Cultivation of selected microorganism

The selected strain was incubated in a 500-ml of culture flask containing 150 ml of the propylene glycol medium, at 30 °C for 2 days with shaking (120 strokes/min). The culture (300 ml) was then transferred into a 5-l of culture flask containing 3-l of the propylene glycol medium, and it was incubated at 30 °C for 3 days with shaking.

2.5. Assay of enzyme activity

The GADH activity was assayed using NBT as an exogenous electron carrier as follows. Standard reaction mixture contained 50 μmol glycolic acid sodium salt, 0.12 μmol of NBT, 0.2 mmol of potassium phosphate, pH 7.0, containing 0.05% Triton X-100, and an appropriate amount of enzyme, in a final volume of 1.0 ml. The reaction mixture was incubated at 30 °C, and the rate of formazan dye formation was followed at 550 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of one micromole of the reduced NBT per minute under the above conditions. The molar absorptivity for the dye formed under the above conditions was $13.5 \times 10^3\ M^{-1}\ cm^{-1}$.

2.6. Purification of GADH

All procedures were carried out at 5–10 °C using buffer A composed of 50 mM potassium phosphate buffer, pH 6.0, containing 1 mM 2-mercaptoethanol and 5 mM EDTA, unless otherwise stated.

In the first step, mycelia from 10 l of culture broth (108 g of wet weight) were disrupted with glass beads in buffer A by a multi-beads shocker, and the supernatant solution was collected by centrifugation at 20,000 × *g* for 10 min.

To the supernatant solution (2310 ml), 559 g of solid ammonium sulfate was added to 40% saturation, and resulting precipitate was discarded by centrifugation at 20,000 × *g* for 10 min. Solid ammonium sulfate was further added to the supernatant fraction to reach 65% saturation. The resulting precipitate was collected by centrifugation at 20,000 × *g* for 10 min, and dissolved with buffer A.

The enzyme solution was dialyzed against buffer A, and applied to a DEAE-Toyopearl column (2.6 × 20 cm) equilibrated with same buffer. After the column was washed with 1000 ml of buffer A, the enzyme was eluted by a linear gradient with buffer A and buffer A containing 0.2 M NaCl (500 ml each). The active fractions were combined and deionized to 15 mS/cm by ultrafiltration.

The deionized enzyme solution was then applied to a GigaCap Q-Toyopearl column (1.6 × 20 cm) equilibrated with buffer A containing 0.1 M NaCl. After the column was washed with 400 ml of same buffer, the enzyme was eluted by a linear gradient with buffer A containing 0.1 M NaCl and 0.3 M NaCl (200 ml each). The active fractions were combined and deionized to 15 mS/cm by ultrafiltration.

The deionized enzyme solution was then applied to a hydroxyapatite column (1.1 × 20 cm) equilibrated with 10 mM phosphate buffer A. After the column was washed with 200 ml of 0.2 M phosphate buffer A, the enzyme was eluted by a linear gradient with 0.2 M phosphate buffer A and 0.5 M phosphate buffer A (100 ml each). The active fractions were combined and deionized to 15 mS/cm by ultrafiltration.

The deionized enzyme solution was applied again to a GigaCap Q-Toyopearl column (1.1 × 16 cm) equilibrated with buffer A containing 0.1 M NaCl. After the column was washed with 100 ml of same buffer, the enzyme was eluted by a linear gradient with buffer A containing 0.1 M NaCl and 0.25 M NaCl (75 ml each). The active fractions were combined and concentrated to 0.8 ml by ultrafiltration.

The concentrated enzyme solution was applied to a Toyopearl HW-55 column (1.3 × 53 cm) equilibrated with buffer A.

2.7. Identification of reaction product

Fifty millimolar glycolic acid was incubated with 0.02 units of the purified enzyme and 3 mM NBT at pH 6.0 and 25 °C for 24 h. Then, MBTH derivatives 1 and 2 were prepared using the reaction mixture to identify the aldehyde group of the reaction product according to the method of Isobe and Nishise [22].

The hydrogen peroxide formation was assayed by measuring color development of following reaction mixture at 555 nm. Fifty millimolar glycolic acid was incubated with 0.02 units of the purified enzyme and color development reagent consisting of 0.6 μmol of 4-aminoantipyrine, 1.94 μmol of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt dihydrate and 6.7 units of peroxidase, at pH 6.0 and 25 °C for 24 h.

2.8. Production of glyoxylic acid by resting cell reaction

The cells (180 mg) grown at 30 °C for 3 days in the propylene glycol medium or the glycolic acid medium were incubated with 75 μmol of glycolic acid in a reaction volume of 1.5 ml at pH 6.0 and 20 °C for 6 h. The amount of glyoxylic acid produced by this reaction was determined using the absorbance values at 630 nm of the MBTH derivative 2 of the reaction supernatant.

2.9. Other analytical methods

Protein concentration was spectrophotometrically determined by measuring the absorbance value at 280 nm. The $E^{1\%}_{1\,cm}$ value of 10.0 was used throughout this work.

Native- and SDS-PAGE was performed according to the method of Laemmli [23], and proteins in the gel were stained with Coomassie Brilliant Blue R-250 or silver staining method. Sample for SDS-PAGE analysis was prepared by incubation with 54 mM Tris–HCl, pH 6.8, containing 1.7% SDS, 4.3% 2-mercaptoethanol and 6% glycerol at 100 °C for 3 min. Molecular mass was estimated by gel