PURIFICATION AND CHARACTERIZATION OF MEMBRANE-BOUND GLUCOSE DEHYDROGENASE FROM MUTANT *Gluconobacter frateurii* THD32N

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Abstract

Acetic acid bacteria are aerobic Gram-negative rod which able to oxidize various substrates such as sugar and sugar alcohol to corresponding oxidative products. These oxidation reactions are catalyzed by the membrane-bound dehydrogenases which located on the outer surface of the cytoplasmic membrane. Glucose dehydrogenase has been widely used for enzymatic determination of glucose in food industry as well as in medicinal field. Glucose dehydrogenase purified from cytosolic fraction, so called cytosolic glucose dehydrogenase is dependent on nicotinamide adenine dinucleotide as the coenzyme. While, membrane-bound glucose dehydrogenase has PQQ as the prosthetic group and it is independent from nicotinamide adenine dinucleotide. Membrane-bound glucose dehydrogenase is more useful for glucose determination due to its stability. This work, membrane-bound glucose dehydrogenase from the thermotolerant mutant *Gluconobacter frateurii* THD32N was purified and characterized. Membrane fraction was solubilized with 1% n-octyl β-glucoside in the presence of KCl for 1 h. Solubilized fraction was obtained by centrifugation at 40,000 rpm for 1 h. After dialysis, the enzyme solution was applied onto DEAE-Toyopearl column chromatography and subsequence onto ceramic hydroxyapatite column chromatography. The purified enzyme had molecular mass of 90 kDa and had the optimum pH at 5.0. The purified enzyme did not inhibited by EDTA at the concentration up to 350 mM. The purified enzyme had narrow substrate specificity toward glucose and xylose. The residual activity of purified enzyme when incubating at 37°C remained 80% to that of the standard assay at 30°C. Michealis constant and maximum velocity of the purified enzyme was measured to be 2.03 mM and 21.27 U/ml, respectively.

Keywords: purification, membrane-bound glucose dehydrogenase, acetic acid bacteria, *Gluconobacter frateurii*

Introduction

Acetic acid bacteria are obligate aerobes and well known as oxidative bacteria (Matsushita *et al*., 1994). There are several genera of biotechnologically significant acetic acid bacteria that currently known to produce oxidative fermentation products with quantitative yields after incomplete oxidation of substrate and accumulate the corresponding oxidative products in the culture medium. Oxidative fermentation in acetic acid bacteria is carried out by membrane-bound dehydrogenase located in the outer surface of cytoplasmic membrane or periplasmic space of the organisms. Membrane-bound dehydrogenases have either pyrroloquinoline

quinone (PQQ) or flavin adenine dinucleotide (FAD) as the primary coenzyme linked to the respiratory chain and generate bioenergy for growth (Matsushita *et al*., 1994).

There are two types of glucose dehydrogenase harboring PQQ as their prosthetic group. The first type is the membrane-bound glucose dehydrogenase, which has been isolated from *Acinetobacter calcoaceticus*, *Pseudomonas* sp., and *Gluconobacter suboxydans* (Matsushita et al., 1988; Matsushita et al., 1982; Ameyama et al., 1981a, 1981b; Matsushita et al., 1989). The purified enzymes are single peptide of the molecular mass of 87 kDa. Their physiological role in the bacterial cell is the terminal oxidation of glucose coupled with the respiratory chain by ubiquinone (Igarashi et al., 2004). The second type of glucose dehydrogenase containing PQQ as the prothetic group is the water-soluble glucose dehydrogenase isolated from *A. calcoaceticus*. The enzyme does not share the primary structure with the previous membrane-bound glucose dehydrogenase. Water-soluble glucose dehydrogenase from *A. calcoaceticus* is a homodimeric composted of 55 kDa monomer containing one PQQ molecule (Matsushita et al., 1995). Glucose dehydrogenase has the most industrially attractive which is now become the major enzyme used in sensor systems for food process monitoring and the clinical diagnosis of diabetes as well as amperometric DNA sensor which is specifically detect the DNA sequence of the virulence gene from the pathogenic bacterium *Salmonella* (Ikebukuro et al, 2002). However, glucose oxidase has been used wildly in industrially for glucose determination, but the enzyme requires oxygen as the electron acceptor. Membrane-bound glucose dehydrogenase is independent or insensitivity to oxygen as electron acceptor and several glucose sensors employing membrane-bound glucose dehydrogenase have been reported (Laurinavicius et al., 2002; 2004). In this study, purification and characterization of the properties of membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N was performed. The purified enzyme derived in this work will be further used for glucose biosensor preparation.

Methodology

Microorganism and culture conditions

Thermotolerant *Gluconobacter frateurii* THD32 was previous isolated by Moonmangmee (Unpublished data) and the strain was mutated with NTG in which assigned as *G. frateurii* THD32N. Culture medium used for enzyme production was composed of 2% sodium gluconate, 0.5% glucose, 0.3% yeast extract and 0.3% peptone. The cultivation was done aerobically at 35-37°C in Erlenmeyer flask.

Preparation of membrane and cytosolic fraction

G. frateurii THD32N was used throughout this study. The bacterial cells were harvested with a high speed centrifuge at 10,000 rpm for 10 min and suspended in 10 mM potassium phosphate buffer (pH 6.5). The bacterial cell suspension chilled in ice-cold water was passed twice through a French pressure cell press at 16,000 psi. To remove intact cells and cell debris, the suspension was centrifuged at 9,000 rpm at 4°C for 15 min. Separation of the membrane fraction from the cytosolic fraction was done by ultracentrifugation at 40,000 rpm at 4°C for 60 min.

Assay of enzyme activity

All enzyme assays were done in a final volume of 1.0 ml at 30°C. The assay for measurement of membrane-bound glucose dehydrogenase activity was done using potassium ferricyanide or PMS-DCIP as the artificial electron acceptors. The enzyme assay mixture contained 10 µmol potassium ferricyanide or 0.2 µmol PMS in the presence of 0.11 µmol DCIP, 100 µmol glucose, and enzyme solution in 0.8 ml of McIlvaine buffer (pH 5.0). The reaction was started by the addition of enzyme solution. The resulting Prussian-blue color with potassium ferricyanide was measured colorimetrically at 660 nm and PMS-DCIP assay was traced by measuring the decreasing absorbance at 600 nm, respectively (Moonmangmee et al., 2001). One unit of the enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 µmol of glucose per min under these conditions. The specific activity was defined as units of enzyme activity per mg of protein. Protein content was measured according to a modified of Lowry's method (Dully and Grieve, 1975) using bovine serum albumin as the protein standard.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was done on 12.5% (w/v) slab gel by the methods described by Laemmli (1970). Before application, samples were treated with 6% (w/v) SDS and 0.1% dithiothreitol at 60°C for 30 min. The following calibration proteins with the indicated molecularmass were used as the references: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.1 kDa), and lysozyme (14.4 kDa). Gels were stained with Coomassie brilliant blue (CBB R-250).

Results and discussion

Localization of glucose dehydrogenase

Glucose dehydrogenase activity of thermotolerant mutant *G. frateurii* THD32N was found to be located in the cytoplasmic membrane the same as other typical membrane-bound dehydrogenases in acetic acid bacteria (Matsushita et al., 1994). Glucose dehydrogenase used potassium ferricyanide as well as PMS-DCIP as electron acceptors *in vitro*.

Solubilization conditions

Since glucose dehydrogenase was located on the cytoplasmic membrane, solubilization of the enzyme was done using 1% various detergents in the presence of 0.1M KCl as the chaotropic agent for 1 h at 4°C. Enzyme solubilization was done with the original native membrane. Among detergents, n-octyl-β-glucoside was selected for the purpose. Like the other membrane-bound glucose dehydrogenase from *A. calcoaceticus* and *Pseudomonas* was solubilized with Triton X-100 (Matsushita et al., 1988; 1982). In this study, glucose dehydrogenase could be solubilized with recovery of 87-90% from the membrane fraction. Enzyme activity assayed with solubilized enzyme using potassium ferricyanide was much lower than that of PMS-DCIP. It was suggested that the detergent used might be discociated heme component in the membrane during enzyme solubilization, since potassium ferricyanide seemed to be less effective as the electron acceptor once the enzyme has been solubilized. Thus, the following steps after enzyme solubilization were required to use only PMS-DCIP for enzyme activity measurement.

Purification of membrane-bound glucose dehydrogenase

Membrane-bound glucose dehydrogenase from thermotolerant mutant *G. frateurii* THD32N was purified to homogeneity as below. All operations were done at 4°C. The membrane fraction of the microorganism was homogenized with 10 mM potassium phosphate buffer (pH 6.5) giving a protein concentration of about 10 mg/ml. Solubilization was done using 1% n-octyl-β-glucoside in the presence of 0.1 M KCl. The membrane suspension was stirred gently for 1 h and centrifuged at 40,000 rpm for 1 h. The solubilized fraction was then dialyzed overnight against 3 mM Tris-HCl pH 7.0 containing 0.3% Mydol 10 for 7 h. Dialyzed enzyme was put only DEAE-Toyopearl column (3.5 x 10 cm) previously equilibrated with the same buffer and the column was washed with the same buffer with the flow rate of 1.5 ml/min. Elution of the enzyme was done by stepwise elution using the same buffer containing 0.05-0.2 M NaCl. The fraction containing enzyme activity was put into the dialysis tube to concentrate with sucrose powder. The enzyme was three-times diluted with 1 mM potassium phosphate buffer pH 7.0 before applied only Ceramic hydroxyapatite column chromatography (1.8 x 5 cm) which had been equilibrated with 1 mM potassium phosphate buffer containing 0.3% Mydol 10. After the column was washed with the same buffer, elution of the enzyme was done with a linear gradient by increasing concentration of potassium phosphate to 300 mM at the flow rate of 0.5 ml/min. Fraction having enzyme activity eluted at about fraction number 140-170 were combined and put into a dialysis tube to concentrate the enzyme by embedding in sucrose powder. A summary of the enzyme purification is shown in Table 1. The purified enzyme had specific activity of 55.25 units/mg with glucose as the substrate. The purified glucose dehydrogenase gave one band on SDS-PAGE and the molecular mass measurement by SDS-PAGE gave a relative molecular mass of 90 kDa (Figure 1)

Table 1 Purification summary of membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Membrane	1379	1470	0.94	100	1
Solubilization	1080	444	2.43	78	2.59
DEAE-Toyopearl	822	213	3.86	60	4.11
Ceramic-Hydroxyapatite	121	2.19	55.25	9	58.78

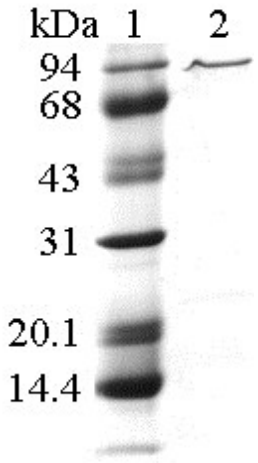

Figure 1 SDS-PAGE of membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N. Lane 1, marker protein; lane 2, purified glucose dehydrogenase from Ceramic-hydroxyapatite. Each lane was loaded by protein 10 µg.

Catalytic properties of purified membrane-bound glucose dehydrogenase

The purified membrane-bound glucose dehydrogenase had the optimum pH at 5.0 for glucose oxidation, the same optimum pH as observed with membrane fraction. PMS and DCIP can be available as electron acceptors for membrane-bound glucose dehydrogenase like other membrane-bound dehydrogenases (Shinagawa et al., 1999; Adachi et al., 2001a, 2001b; Moonmangmee et al., 2001, 2002), while potassium ferricyanide was not as electron acceptor suggesting that the enzyme was heme-free. The apparent Michaelis constant for glucose and the maximum velocity of the enzyme were measured to be 2.03 mM and 21.27 U/ml, respectively. The purified membrane-bound glucose dehydrogenase showed a narrow substrate specificity toward glucose, xylose and trace with fructose (Table 2). The results shown in this work was distinct from membrane-bound glucose dehydrogenase purified from *A. calcoaceticus* in the respect of substrate specificity. Membrane-bound glucose dehydrogenase from *A. calcoaceticus* had substrate specificity toward D-glucose, D-fucose, D-galactose, D-xylose, D-ribose, maltose and lactose (Matsushita et al., 1988).

Table 2 Substrate specificity of membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N

Substrate	Enzyme activity (U/ml)	Relative activity (%)
D-Glucose	5.98	100
D-Gluconate	0	0
D-Fructose	0.27	4.51
D-Xylose	1.09	18.22
L-Arabinose	0	0
L-Sorbose	0	0
D-Mannose	0	0
maltose	0	0

Effects of EDTA treatment on membrane-bound glucose dehydrogenase activity

EDTA (ethylenediamine-N,N,N',N'-tetraacetic acid, disodium salt) is a kind of chelating agent and enzyme inhibitor. In this work, the effect of EDTA on the purified membrane-bound glucose dehydrogenase was performed by incubation the enzyme with various concentration of EDTA for 2 h. After EDTA treatment, the enzyme activity was measured using glucose as the substrate compared to that of purified enzyme without treated with EDTA. As the results, the purified membrane-bound glucose dehydrogenase was not inhibited with EDTA at the concentration from 0-350 mM. However, the enzyme activity was decreased when the concentration of EDTA increased at 450 mM in which the enzyme activity remained 80% of the original activity (Table 3). It was suggested that the purified enzyme had highly tolerance to EDTA.

Thermal stability of the purified membrane-bound glucose dehydrogenase activity

The purified membrane-bound glucose dehydrogenase was put into glass tube and dipped into water bath and incubated for 5 min at temperature between 25-65°C. Then immediately put the glass tube containing the enzyme into ice-cold water. The residual enzyme activity was measured at the standard conditions using PMS-DCIP as electron acceptor. The enzyme activity was gradually decreased when temperature increased (Table 4). At the temperature at 37°C, the enzyme activity remained only 80% to that of the original enzyme activity and the enzyme activity could not be detected when increasing temperature up to 43°C.

Table 3 Effect of EDTA treatment on membrane-bound glucose dehydrogenase activity from thermotolerant mutant *Gluconobacter frateurii* THD32N

Concentration of EDTA (mM)	Enzyme activity (U/ml)
0	2.27
100	2.25
150	2.22
250	2.21
350	2.17
450	1.09

Table 4 Effect of temperature on the activity of purified membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N

Temperature (°C)	Enzyme activity (U/ml)	Relative activity (%)
27	5.57	100
32	5.16	93
37	4.48	80
39	2.79	50
41	0.63	11
43	0	0

Conclusion

Acetic acid bacteria are strict aerobe and well known as oxidative bacteria. The oxidation products derived from oxidation reactions which carried out by dehydrogenase enzyme located in the outer surface of cytoplasmic membrane. This study, membrane-bound glucose dehydrogenase from thermotolerant mutant *Gluconobacter frateurii* THD32N was solubilized and purified to homogeneity. The purified enzyme showed a single band in SDS-PAGE of which the molecular mass corresponded to 90 kDa. The optimum pH of the purified enzyme was found at pH 5.0. The Michaelis constant and the maximum velocity of the enzyme were found to be 2.03 mM and 21.27 U/ml, respectively. The purified enzyme had narrow substrate specificity and was not inhibited by EDTA at the concentration up to 350 mM.

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