Purification and Characterization of D-Sorbitol Dehydrogenase from Membrane of *Gluconobacter suboxydans* var. *α*

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D-Sorbitol dehydrogenase was solubilized from the membrane fraction of *Gluconobacter suboxydans* var. *α* IFO 3254 by a procedure involving solubilization of the enzyme with Triton X-100 in the presence of KCl and d-sorbitol. Purification of the enzyme was performed by fractionation with polyethylene glycol 6000, and column chromatographies on DEAE- and CM-cellulose in the presence of Triton X-100. The enzyme was purified about 240-fold from the membrane fraction of the organism. The purified enzyme was tightly bound to a c-type cytochrome existing as a dehydrogenase-cytochrome complex. The dehydrogenase was found to be a flavoprotein, and its flavin moiety was covalently bound to the dehydrogenase protein. The optimum pH of the enzyme activity was 4.5, and the optimum temperature was 25°C. D-Sorbitol was specifically oxidized by the purified enzyme, and D-mannitol was also oxidized to 5% of the reaction rate with D-sorbitol.

Today, vitamin C is produced industrially by the method of Reichstein and this manufacturing process involves one fermentation step, oxidation of D-sorbitol to L-sorbose, which is called "sorbose fermentation." The chemical oxidation of D-sorbitol results in racemic modification of sorbose and the yield of L-sorbose is reduced by half. Sorbose fermentation was discovered first in the so-called ketogenic fermentation by oxidative bacteria such as *Acetobacter*, *Gluconobacter* and *Pseudomonas* sp. The first report of sorbose fermentation was made by Bertrand, and *Acetobacter xylinum* was found to be a useful bacterium. Since then the sorbose fermentation has been studied by many workers and many strains of acetic acid bacteria having a strong oxidative activity for d-sorbitol have been found. Nevertheless, studies on the enzyme participating in the sorbose fermentation have been scarce. According to Cummins et al. the cell free extract of *A. suboxydans* (syn. *G. suboxydans*) contains the enzyme participating in three pathways of d-sorbitol oxidation. In addition to a particulate dehydrogenase, there are two enzymes, NAD- and NADP-dependent dehydrogenases, in the soluble fraction. One in the soluble fraction forms fructose in the presence of NAD and the other sorbose in the presence of NADP. The NAD-dependent D-sorbitol dehydrogenase has been partially purified and its characteristics studied, and the NADP-dependent dehydrogenase has been reported to be very fragile. However, concerning the particulate dehydrogenase, it was reported only that D-sorbitol was oxidized by the particulate fraction. The D-sorbitol oxidizing system in the particulate fraction seems to actually participate in sorbose fermentation because L-sorbose is accumulated in the medium as a fermentation product in common with other oxidation products produced by membrane-bound dehydrogenases.

In this paper we describe the solubilization and purification of D-sorbitol dehydrogenase from the membrane fraction of *G. suboxydans* var. *α* which has a high enzyme activity. Characteristics of the enzyme are also described.
MATERIALS AND METHODS

Chemicals. All chemicals used in this work commercial products. D-Sorbitol for the culture medium was a gift from Takeda Pharmaceutical Co. Yeast extract was supplied by the Oriental Yeast Co.

Microorganism and cultivation. G. suboxydans var. z IFO 3254 was supplied by the Institute for Fermentation, Osaka (IFO), and used throughout this work. The medium consisted of 10 g of D-sorbitol, 10 g of sodium D-gluconate, 3 g of yeast extract, and 3 g of Polypepton in 1 liter of tap water. Cultivation was carried out aerobically with rotary shaking or in a 50-liter jar fermentor at 30°C.

Preparation of membrane fraction. Cells were harvested with a Sharples centrifuge and washed twice with water. The cell paste was suspended in 0.01 m sodium acetate buffer (pH 5.0) and passed through a French pressure cell press at 20,000 psi. After centrifugation to remove intact cells, the supernatant was centrifuged at 80,000 × g for 90 min. The resultant precipitate was designated as the membrane fraction.

Enzyme assay. Enzyme activity was measured by a routine assay according to the method employed by Wood et al.16) using ferricyanide as an electron acceptor. The reaction mixture contained 10 μmol of potassium ferricyanide, 0.5 ml of McIlvaine buffer (pH 4.5), 0.05% Triton X-100, 100 μmol of D-sorbitol and enzyme solution in a total volume of 1.0 ml. The reaction was carried out at 25°C with the addition of enzyme, and stopped by adding 0.5 ml of ferric-Dupanol reagent. Then, 3.5 ml of water was further added to the reaction mixture which was left standing for 10 min. After standing, the resulting stabilized Prussian blue color was measured spectrophotometrically at 660 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1 μmol of D-sorbitol per min under these assay conditions, and 4.0 absorbance units equaled 1 μmol of D-sorbitol oxidized.

Determination of protein. Protein concentration was determined according to the method of Lowry et al.17) with bovine serum albumin as the standard. A modified method described by Dulley and Grieve18) was employed for samples which contained Triton X-100.

Electrophoresis. Polyacrylamide gel electrophoresis was performed under essentially the same conditions as described by Davis19) with 7.5% polyacrylamide gel and Tris-glycine buffer (pH 8.3). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Weber and Osborn.20) Protein was stained with Coomassie brilliant blue.

Determination of covalently bound flavin. The assay of bound flavin in the purified enzyme was performed by essentially the same method as that of Wilson and King.21) The enzyme solution (about 0.2 mg protein) was incubated with 1 mg of pronase and 1 mg trypsin at 37°C for 4 hr. After adding trichloroacetic acid to 10%, the suspension was incubated again at 37°C overnight. The fluorescence of the hydrolysate was measured with an AMINCO-BOWMAN spectrofluorometer JA-8962 with excitation at 465 nm after adjusting the pH.

RESULTS

Solubilization

Since D-sorbitol dehydrogenase is a membrane-bound protein, purification was initiated by attempts to solubilize the enzyme with a wide variety of detergents. Membrane fraction suspended in 0.01 m sodium acetate buffer (pH 5.0) to give about 10 mg/ml of protein was treated with 1% of various detergents for 2 hr at 5°C, and after centrifugation at 80,000 × g for 90 min enzyme activity of the supernatant was assayed. Triton X-100 solubilized D-sorbitol dehydrogenase with a yield of 25% and the best recovery was obtained with detergents used in the experiment for solubilization. The recovery of solubilization with Span 20, Tween 80 or Brij 35 was about 10% and prolonged treatment could not bring about higher recovery of solubilization. Non-ionic detergents did not influence the enzyme activity but ionic detergents such as cetylpyridinium chloride, cetyltrimethyl ammonium bromide and sodium stearate inactivated the enzyme. The effects of KCl and D-sorbitol on enzyme solubilization with Triton X-100 were also investigated. As shown in Table I, the addition of KCl increased both the enzyme activity and the amount of protein solubilized. An increase of the concentration of KCl had no remarkable effect on enzyme solubilization. The addition of D-sorbitol increased the enzyme activity but did not increase the amounts of protein solubilized. D-Sorbitol was thought to have a stabilizing effect on the enzyme in solubilization. The treatment of membrane fraction with 1% Triton X-100 in the presence of 0.1 m KCl and 0.1 m D-sorbitol could solubilize D-sorbitol dehydrogenase near
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Table I. Effect of Potassium Chloride and d-Sorbitol on Enzyme Solubilization

The membrane suspension (10 mg/ml protein) was treated with Triton X-100 in the presence or absence of 0.1 M KCl and 0.1 M d-sorbitol. Each mixture was stirred for 2 hr in an ice-bath and centrifuged at 80,000 x g for 90 min. Resulting supernatants were subjected to the enzyme assay. Enzyme activity is expressed relative to that of the original membrane suspension.

<table>
<thead>
<tr>
<th>Solubilizing system</th>
<th>Recovery (%)</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>25</td>
<td>7.2</td>
</tr>
<tr>
<td>Triton X-100 + d-sorbitol</td>
<td>55</td>
<td>12.8</td>
</tr>
<tr>
<td>Triton X-100 + KCl</td>
<td>70</td>
<td>7.1</td>
</tr>
<tr>
<td>Triton X-100 + KCl + d-sorbitol</td>
<td>130</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatography of d-Sorbitol Dehydrogenase on DEAE-Cellulose.

Enzyme solution obtained by polyethylene glycol fractionation was applied to a DEAE-cellulose column (3.5 x 20 cm) equilibrated with 0.01 M sodium acetate buffer (pH 5.0) containing 0.1% Triton X-100. The column was washed with the same buffer. The enzyme activity was eluted backwards far from major impurities during the washing of the column (Fig. 1). Fractions having enzyme activity were combined.

A summary of the enzyme purification is presented in Table II.

Ultracentrifugal analysis

Homogeneity of the purified enzyme was investigated with an analytical ultracentrifuge and the sedimentation pattern of the enzyme showed a single symmetrical peak (Fig. 2). In the figure, the area from the sedimenting peak to the bottom is dark because of the red color of cytochrome contained in the enzyme.
Table II. Purification of d-Sorbitol Dehydrogenase from Membranes of Gluconobacter suboxydans var. x IFO 3254

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane fraction</td>
<td>9120</td>
<td>4940</td>
<td>1.8</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized fraction</td>
<td>10350</td>
<td>900</td>
<td>12</td>
<td>113</td>
</tr>
<tr>
<td>PEG* fraction</td>
<td>6800</td>
<td>180</td>
<td>38</td>
<td>75</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>5320</td>
<td>22</td>
<td>241</td>
<td>58</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>1690</td>
<td>3.9</td>
<td>433</td>
<td>19</td>
</tr>
</tbody>
</table>

* PEG, polyethylene glycol 6000.

apparent sedimentation constant of the enzyme was calculated to be 5.9 S in the presence of 1% Triton X-100.

Electrophoretic analyses

When the purified enzyme having a specific activity of 430 was subjected to conventional polyacrylamide gel electrophoresis, the enzyme exhibited one major protein band ($Rm = 0.6$) having enzyme activity and one minor protein band having no enzyme activity. And upon gel electrophoresis with sodium dodecyl sulfate, the purified enzyme dissociated into three major protein bands with molecular weights of 63,000, 51,000 and 17,000 (Fig. 3). Of the three components of d-sorbitol dehydrogenase, the largest component having a molecular weight of 63,000 was a flavoprotein, since exposure of unstained gel to ultraviolet rays showed intense fluorescence. The second component with a molecular weight of 51,000 which showed a red protein band on unstained gel
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was a cytochrome. The sum of each molecular weight of the three subunits gave 131,000 as the total molecular weight of D-sorbitol dehydrogenase.

Absorption spectra

The absorption spectrum of the purified enzyme reduced with sodium dithionite showed absorption maxima at 551, 522 and 417 nm in the visible region, suggesting the presence of a cytochrome component in the purified enzyme preparation (Fig. 4). The cytochrome was determined to be a c-type cytochrome judging from the pyridine hemochrome. The cytochrome was not reduced so rapidly by the addition of D-sorbitol, until coenzyme Q₁ was added to the enzyme solution. When the buffer solution containing 10 mM D-sorbitol was used during the purification, the cytochrome of the purified enzyme was reduced by about 70% of the reduction by sodium dithionite.

Storage of the purified enzyme solution at 5°C for several months changed the absorption spectrum, namely, the purified enzyme preparation after storage did not show the characteristic absorption spectrum for cytochrome. By pyridine hemochrome, also, the presence of cytochrome was not detected. The reduced minus oxidized difference spectrum of the preparation after storage showed absorption maxima at 460 and 360 nm, suggesting a flavin but not a cytochrome. Because decomposition of the heme structure proceeded and flavin which was bound to the dehydrogenase became apparent.

Flavin determination

Extraction of flavin, the prosthetic group of D-sorbitol dehydrogenase, was performed by usual methods such as acid precipitation by trichloroacetic acid, but no flavin release was observed. After gel electrophoresis in the presence of sodium dodecyl sulfate, it was observed that a flavin component still remained in the enzyme protein. From these results, it was suggested that a flavin was bound covalently to the enzyme protein. When the enzyme was digested with pronase and trypsin, a significant amount of flavin was released. Although complete proteolytic digestion was not achieved, the content of covalently bound flavin thus obtained was estimated to be about 3 nmol per mg protein and this value corresponded to about 0.4 mol flavin per mol of the enzyme.

Catalytic properties

D-Sorbitol dehydrogenase could be assayed in vitro in the presence of any one of the following dyes as an electron acceptor: potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol, nitroblue tetrazolium, and Wurster's blue. NAD, NADP and oxygen were completely inactive as electron acceptors.

Sugars, sugar alcohols and organic acids, and many kinds of substances were tested as substrates for the purified enzyme. Sugar alcohols tested are listed in Table III. Among tested substances, D-sorbitol was specifically oxidized and D-mannitol was also oxidized, 5% of the reaction rate with D-sorbitol.

The optimum temperature of D-sorbitol dehydrogenase was found to be 25°C. Oxidation of D-sorbitol proceeded most rapid-
Table III. Substrate Specificity of D-Sorbitol Dehydrogenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
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<tbody>
<tr>
<td>D-Sorbitol</td>
<td>100</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>5</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>0</td>
</tr>
<tr>
<td>L-Iditol</td>
<td>0</td>
</tr>
<tr>
<td>meso-Erythritol</td>
<td>0</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>0</td>
</tr>
<tr>
<td>Ribitol</td>
<td>0</td>
</tr>
<tr>
<td>Xylitol</td>
<td>0</td>
</tr>
</tbody>
</table>

ly at pH 4.5. The apparent Michaelis constant for D-sorbitol was determined to be 30 mM at pH 4.5.

DISCUSSION

To study enzymatically sorbose fermentation as one oxidative fermentation, we solubilized and purified D-sorbitol dehydrogenase from membranes of *G. suboxydans* var. α IFO 3254. As to the enzyme which catalyzes the oxidation of D-sorbitol to L-sorbose, NADP-dependent D-sorbitol dehydrogenase was reported by Cummins et al. Judging from the rapid accumulation of L-sorbose in the culture medium, the enzyme that participated in sorbose fermentation is reasonably thought to be a membrane-bound enzyme and not an NADP-dependent enzyme occurring in the cytosol. And it was thought that the membrane-bound D-sorbitol dehydrogenase is localized on the outer surface of the cytoplasmic membrane and the oxidative conversion of D-sorbitol to L-sorbose is linked to the respiratory chain of the organism. The same model has been proposed by Dawes et al. for the pathway of glucose metabolism in *Pseudomonas aeruginosa* and by us for many kinds of membrane-bound dehydrogenases of acetic acid bacteria.

The membrane-bound D-sorbitol dehydrogenase could be solubilized with non-ionic detergent Triton X-100. However, to obtain a good yield of solubilization with Triton X-100, the addition of KCl and D-sorbitol was necessary. D-Sorbitol played the role of a stabilizing agent for the enzyme during solubilization. The enzyme was purified by using the conventional techniques in the presence of Triton X-100. The final enzyme preparation purified about 240-fold from the membrane fraction was at least 90% pure judging from the densitometric scanning of sodium dodecyl sulfate-disc gel stained with Coomassie brilliant blue.

The purified enzyme was found to be bound with a cytochrome, which is similar to some other membrane-bound dehydrogenases reported previously. However, the cytochrome of D-sorbitol dehydrogenase was not reduced quickly on the addition of D-sorbitol unless quinone was added to the enzyme solution, differing from cytochrome of D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase which were reduced rapidly on the addition of substrate. When the buffer containing D-sorbitol was used during the purification, the cytochrome of the purified enzyme stayed in the reduced form. It was thought that this cytochrome was one component of the D-sorbitol oxidizing system, but could not be reduced directly by D-sorbitol, because the purified enzyme probably lacked in the quinone.

The D-sorbitol dehydrogenase preparation was composed of three subunits, a flavoprotein, a c-type cytochrome and an unknown polypeptide, and these seemed to be components of the D-sorbitol oxidizing system in vivo. The primary dehydrogenase in the D-sorbitol oxidizing system was found to be a flavoprotein, of which the flavin moiety was covalently bound and could not be extracted by the usual methods unless the enzyme was digested with protease. D-Sorbitol dehydrogenase resembled D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase in subunit composition and in possessing covalently bound flavin.

The purified enzyme was labile and activity of the enzyme decreased by half when the enzyme solution was left standing overnight at 5°C. D-Sorbitol was effective as a stabilizing agent on solubilization of the enzyme but had
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no effect during the purification. Storage of the purified enzyme led to not only a decrease of the enzyme activity but also decomposition of the cytochrome resulting in the loss of the characteristic absorption spectrum and pyridine hemochrome. It is not yet known how cytochrome of the purified enzyme is decomposed or how decomposition of cytochrome is related the decrease in enzyme activity.

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REFERENCES