Crystalline NADP-Dependent D-Mannitol Dehydrogenase from *Glucunobacter suboxydans*†

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D-Mannitol dehydrogenase (EC 1.1.1.138) was purified and crystallized for the first time from the cell-free extract of *Glucunobacter suboxydans* IFO 12528. The enzyme was purified about 100-fold by a procedure involving ammonium sulfate fractionation, DEAE-Sephadex A-50 column chromatography, and gel filtration by a Sephadex G-75 column. The enzyme was completely separated from a similar enzyme, NAD-dependent D-mannitol dehydrogenase (EC 1.1.1.67), during enzyme purification. There being sufficient purity of the enzyme at this stage, the enzyme was crystallized, by the addition of ammonium sulfate, to fine needles. The crystalline enzyme showed a single sedimentation peak in analytical ultracentrifugation, giving an apparent sedimentation constant of 3.6 s. The molecular mass of the enzyme was estimated to be 50 kDa by SDS-PAGE and gel filtration chromatography. Oxidation of D-mannitol to D-fructose and reduction of D-fructose to D-mannitol were specifically catalyzed by NADP and NADPH, respectively. NAD and NADH were inert for the enzyme. Since the reaction equilibrium declined to D-fructose reduction over a wide pH range, the enzyme showed several advantages for direct enzymatic measurement of D-fructose. Even in the presence of a large excess of D-glucose and other substances, oxidation of NADPH to NADP was highly specific and stoichiometric to the D-fructose reduced.

**Key words:** NADP-dependent D-mannitol dehydrogenase; *Glucunobacter suboxydans*; polyol dehydrogenase; D-fructose measurement

Various kinds of unique sugar-metabolizing enzymes are known in acetic acid bacteria. Some of them are known to be specific to acetic acid bacteria. Many numbers of membrane-bound dehydrogenases, of which the actions are directly coupled to the respiratory chain of the organisms, allow a huge amount of accumulation of oxidation products in the culture medium. Quinohemoprotein alcohol dehydrogenase (EC 1.1.99.8), quinohemoprotein aldehyde dehydrogenase (EC 1.2.99.3), 5-keto-D-fructose-yielding D-fructose dehydrogenase (FDH) (EC 1.1.99.11), L-sorbose-yielding D-sorbitol dehydrogenases (EC 1.1.99.21) are typical examples of such membrane-bound dehydrogenases. In the cytoplasmic fraction of the organisms, unique NAD(P)-dependent dehydrogenases are known to exist extensively in high contents, such as 2-keto-D-glucose reductase (EC 1.1.1.215), 5-keto-D-glucose reductase (EC 1.1.1.69), 5-keto-D-fructose reductase (EC 1.1.1.124), aldehyde dehydrogenase (EC 1.2.1.4) and D-glucose dehydrogenase (EC 1.1.1.119). Recently, in D-glucose dehydrogenase (EC 1.1.99.3), we have found that 5-keto-D-glucose-yielding D-glucose dehydrogenase in acetic acid bacteria is a quinohemoprotein of which the enzyme activity depends absolutely on pyrroloquinoline quinone (PQQ), while 2-keto-D-glucose-yielding D-glucose dehydrogenase is a flavoprotein in which a covalently bound FAD functions as the primary coenzyme. It can be said that these enzymes can identify the C2 position from C5 position in D-glucose and thus D-glucose dehydrogenase may be divided into two different enzymes in the future.

Regarding D-glucose oxidizing enzymes by acetic acid bacteria, membrane-bound PQQ-dependent D-glucose dehydrogenase (EC 1.1.99.10) and NADP-dependent cytoplasmic D-glucose dehydrogenase have been known and extensively studied in our laboratory and the former enzyme is responsible for D-glucose production in oxidative fermentation. In analogy, in addition to the membrane-bound d-sorbitol dehydrogenases, cytoplasmic D-sorbitol dehydrogenase is known, of which the enzyme activity is dependent on NAD or NADP, though fermentative dehydrogenation of D mannitol by aerobic bacteria is seen in more earlier literature. The enzyme activity of D-mannitol dehydrogenase which is absolutely dependent on NADP existing in the cytoplasmic fraction of acetic acid bacteria was also found later. Recently, membrane-bound D mannitol dehydrogenase (EC 1.1.2.2) was purified from acetic acid bacteria and shown to be heme c and quinone cofactor dependent. Purification of cytoplasmic NADP-dependent D-mannitol dehydrogenase, however, has not been done so far enough to characterize it sufficiently. Recently, we found that an NADP-dependent D-mannitol dehydrogenase occurred in the cytoplasmic fraction of *Glucunobacter suboxydans* IFO 12528. In this paper, purification and characterization of the enzyme are described. Availability of the enzyme for D-fructose measurement is also discussed.

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† This article is dedicated to Dr. Jun-ichi Yamauti for his 30 years’ contributions to our laboratory for supporting our studies on acetic acid bacteria.
Materials and Methods

Chemicals. NAD, NADP, NADH, NADPH, and yeast extract were kind gifts from the Oriental Yeast Co., Tokyo. Other chemicals used were commercial sources of guaranteed grade unless otherwise stated.

Microorganisms and culture conditions. Gluconobacter suboxydans IFO 12528 was used throughout this study. The culture medium consisted of 5 g of D-glucose, 20 g of sodium D-glucanate, 3 g of glycerol, 3 g of yeast extract, and 2 g of polypeptone in 1 liter of tap water. The pH of the medium spontaneously settled to 6.5 when all these ingredients were mixed. A seed culture in 100 ml of the medium in a 500-ml Erlenmeyer flask was made overnight and transferred to 5 liters of a fresh medium in a 10-L top fermentor and cultivated for another 12 hr. Then it was transferred to 30 liters of the medium in a 50-L fermentor and cultured overnight. All cultivation was set at 30°C under shaking or vigorous aeration. About 200 g of wet cells were usually harvested from the culture described above.

Assay of enzyme activity. The enzyme activity of D-mannitol dehydrogenase was measured by a routine method used for NAD(P) enzymes by recording the rate of decrease of NADPH at 340 nm with D-fructose as the substrate in a reaction mixture at 25°C. The reaction mixture (1 ml) contained 100 µmol of D-fructose, 100 µmol of potassium phosphate, pH 6.0, 0.1 µmol of NADPH, and an appropriate amount of enzyme. D-Mannitol oxidation was measured in a reaction mixture (1 ml) containing 100 µmol of D-mannitol, 100 µmol of Tris-HCl, pH 8.0, 0.1 µmol of NADP, and the enzyme. The rate of increase in absorbance at 340 nm was recorded. One unit of the enzyme activity was defined as the amount of enzyme catalyzing 1.0 µmol of NADPH oxidation in D-fructose reduction or 1.0 µmol of NADP reduction in D-mannitol oxidation per min under these conditions. A coefficient of $E_{340,380} = 10.0$ was tentatively used for protein concentration measurement. The specific activity was defined as units of enzyme activity per milligram of protein.

Preparation of cell-free extract. A buffer solution (KPB) of potassium phosphate, pH 6.0, containing 50 mM D-fructose, and 5 mM β-mercaptoethanol was used throughout in this work. About 10 g of wet cells were suspended per 10 ml of 10 mM KPB and passed through a Rannie high pressure laboratory homogenizer (Rannie model Mini-Lab, type 8.30H, Wilmington, MA, USA) at 1,000 psi. After removal of intact cells by a conventional low speed centrifuge, the cell-free extract was further centrifuged at 68,000 × g for 90 min and the resulting supernatant was obtained as the cell-free extract.

Polyacrylamide gel electrophoresis (PAGE). PAGE in the absence of sodium dodecylsulfate (native PAGE) was done on a 7.5% polyacrylamide disc gel and Tris-glycine buffer, pH 8.3, essentially by the same method described by Davis, and the protein band was stained by Coomassie brilliant blue.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done on 12.5% (w/v) slab gel by the methods described by Laemmli. Before application, samples were treated with 6% (w/v) SDS and 0.1 mM diithiothreitol at 60 for 30 min. The following calibration proteins (Bio-Rad, Hercules, CA, U.S.A.) with the indicated molecular mass were used as references: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa).

Analytical ultracentrifugation. Analytical ultracentrifugation was done by a Hitachi model SCP58H ultracentrifuge at 20°C throughout measurements. Estimations of the sedimentation coefficient was done by the methods of sedimentation coefficient, which was operated by a combination of a Hitachi UV scanner (ABS-7), an absorption scanner, and a UC processor (DA-7).

Measurement of molecular mass. Molecular mass of the native enzyme was measured by gel filtration by the method of Andrews on a Sephadex G-75 column (1 × 120 cm) that had been equilibrated with 2 mM KPB. The following marker proteins were used as references: yeast NADP-dependent 6-phospho-D-gluconate dehydrogenase (100 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and cytochrome c (12.4 kDa). Elution was done at a flow rate of 0.5 ml/min with 2 mM KPB and every 35 drops were collected and analyzed. Under these conditions, the peak fraction of the individual marker proteins used came out with the following fraction numbers: yeast NADP-dependent 6-phospho-D-gluconate dehydrogenase, 21; bovine serum albumin, 27; ovalbumin, 36; cytochrome c, 44.

Heat stability and pH stability. For both examinations, a highly diluted enzyme solution (10 µg/ml in 2 mM KPB) was used. The enzyme solution in a thin glass tube was directly incubated for the necessary period as indicated in the text and chilled in ice water. The remaining enzyme activity was measured with 10 µl of the enzyme solution under the standard assay conditions. For measurement of pH stability, 10 µl of the same diluted enzyme solution (10 µg/ml in 2 mM KPB) was incubated with 90 µl of various buffer solutions of different pHs for 5 days in a refrigerator. After the incubation, 2.9 ml of 100 mM KPB, pH 6.0, containing 300 µmol of D-fructose and 0.3 µmol of NADPH, were added. The solution was immediately transferred into a glass cuvette to measure the enzyme activity in a photometer.

Results and Discussion

Purification of D-mannitol dehydrogenase

To the cell-free extract, ammonium sulfate was added to 0.8 saturation (50 g/100 ml) and pH was adjusted to 6.0 with ammonia water. The precipitate was dissolved in a least volume of 2 mM KPB allowing the solution to become a heavy suspension, and the enzyme activity was collected in the supernatant after centrifugation at
68,000 × g for 60 min. The supernatant solution was dialyzed thoroughly against the same buffer overnight at 4°C. The dialyzed enzyme solution was put on a DEAE-Sephadex A-50 column (2 × 50 cm), which had been equilibrated with the same buffer. After the column was washed with the same buffer containing 40 mM KCl, elution of the enzyme was done with the buffer containing 60 mM KCl. Colorless fractions containing the majority of the enzyme activity was clearly separated from contaminating yellowish impurities in this step. The enzyme precipitate made by ammonium sulfate from 0.55 saturation (32.6 g ammonium sulfate/100 ml at 4°C) to 0.85 saturation (56 g ammonium sulfate/100 ml at 4°C) was dissolved in a small volume of 50 mM KPB. After insoluble materials were removed by ultracentrifugation at 68,000 × g for 60 min, the enzyme solution was divided into three portions. Each portion (about 1.5 ml) was put on a Sephadex G-75 column (1 × 150 cm), which had been equilibrated with the same buffer. When fractionated by every 35 drops (about 1 ml), a sharp elution of the enzyme was observed at the fraction number 55, clearly separated from major impurities. The fractions showing the specific activity higher than 180 units/mg protein were collected together and concentrated by dialyzing it overnight against 50 mM KPB containing excess ammonium sulfate, which was allowed to dissolve during dialysis. To the precipitate collected by a conventional table top centrifuge in an Eppendorf plastic centrifuge tube, a least volume of 10 mM KPB was added to make a heavy suspension. After standing for a while in an ice bath, the enzyme solution was spun down. To the supernatant, saturated ammonium sulfate in 50 mM KPB was added dropwise until a faint turbidity appeared. The enzyme was crystallized soon and fine needle crystals appeared after leaving the enzyme solution in a refrigerator (first crystals). The recrystallization was done by repeating almost the same procedure and the crystal formation occurred in a few hours (Fig. 1). In summary of enzyme purification, crystalline D-mannitol dehydrogenase was obtained after 100-fold purification from the cell free extract with 53% recovery (Table).

Besides the NADP-specific D-mannitol dehydrogenase, NAD-dependent D-mannitol dehydrogenase was assayed to be 0.51 units/mg with the cell-free extract. The ratio of the enzyme activity with NADH in D-fructose reduction with the cell-free extract was estimated to be 24% of that with NADPH. Since the crystalline D-mannitol dehydrogenase was NADP-specific, it suggested the existence of another NAD-dependent enzyme like the NAD-dependent D-mannitol dehydrogenase (EC 1.1.1.67) from lactric acid bacteria.21,22) The NAD-dependent D-mannitol dehydrogenase found with G. suboxydans IFO 12528 in this study came out from the DEAE-Sephadex A-50 column with KPB containing 100 mM KCl, giving a complete separation from the NAD-dependent one. The NAD-dependent enzyme was further removed from the NADP-dependent one at the step of gel filtration by a Sephadex G-75 column, from which the NAD-dependent enzyme eluted at almost the void volume of the column, while the NAD-dependent one went through the Sephadex beads and came out almost at the same position as ovalbumin did, as described below.

### Physicochemical properties of crystalline enzyme

When analyzed in analytical ultracentrifugation, the enzyme showed a single sedimentation peak with an apparent sedimentation coefficient of 3.6 s (Fig. 2). The crystalline enzyme was homogeneous in disc gel electrophoresis, showing a single protein band, even when nearly 100 μg of the protein was put on (Fig. 3-A). Molecular mass measurement by SDS-PAGE gave an apparent molecular mass of 50 kDa, which was smaller than bovine serum albumin (68 kDa) and larger than ovalbumin (43 kDa) (Fig. 3-B). When a mixture of the enzyme and standard marker proteins was put on a

### Table. Summary of Enzyme Purification of NADP-Dependent D-Mannitol Dehydrogenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity* (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>11,660</td>
<td>24,500</td>
<td>2.1</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>952</td>
<td>22,000</td>
<td>23.1</td>
<td>89.8</td>
<td>11</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>110</td>
<td>18,000</td>
<td>163.7</td>
<td>73.5</td>
<td>78</td>
</tr>
<tr>
<td>First crystals</td>
<td>70</td>
<td>14,500</td>
<td>206.5</td>
<td>59.3</td>
<td>98</td>
</tr>
<tr>
<td>Recrystallization</td>
<td>56</td>
<td>13,000</td>
<td>235.0</td>
<td>53.0</td>
<td>112</td>
</tr>
</tbody>
</table>

* Enzyme activity was assayed by measuring the decrease of absorbance of NADPH using D-fructose as the substrate.
Fig. 2. Sedimentation Pattern of NADP-Dependent d-Mannitol Dehydrogenase from *G. suboxydans*.

The crystalline enzyme (14.8 mg/ml in 2 mM KPB, pH 6.0) was centrifuged at 60,000 rpm at 20°C. Photographs were taken at 30, 60, and 90 min after reaching the maximum speed.

Fig. 3. Electrophoresis of NADP-Dependent d-Mannitol Dehydrogenase from *G. suboxydans*.

A) Polyacrylamide disc gel electrophoresis (native-PAGE) was developed with 100 µg of crystalline enzyme. B) SDS-PAGE: lane 1, crystalline enzyme (5 µg); lane 2, marker proteins. 94 k, phosphorylase b; 68 k, bovine serum albumin; 43 k, ovalbumin; 31 k, carbonic anhydrase; 14.4 k, lysozyme.

Sephadex G-75 column, the enzyme appeared at the fraction number of 31. There was some delay from the position where bovine serum albumin (68 kDa) came out, suggesting that the apparent molecular size of 50 kDa must be probable to the enzyme (data not shown). It is clearly different from a similar enzyme, NAD-dependent d-mannitol dehydrogenase from lactic acid bacteria, which has a molecular mass of 137 ± 1 kDa with 7.37 s for the sedimentation coefficient.²³,²⁴ Thus the enzyme from lactic acid bacteria is composed of four identical subunits of 38 kDa.²⁵ On the other hand, the NAD-dependent enzyme from *G. suboxydans* was a monomer and looked to be always monodispersed. NAD-dependent d-mannitol dehydrogenase from *G. suboxydans* was separated from the NAD-dependent one by DEAE-Sephadex A-50 chromatography as described above, and it came out at the void volume while the latter went through the gel beads when a mixture of both enzymes was put on the same Sephadex G-75 column. Further purification of the NAD-dependent enzyme from *G. suboxydans* and comparison of the enzyme from lactic acid bacteria are in progress. It will be interesting to check the physiological roles of the two enzymes and find why *G. suboxydans* has two similar enzymes in the soluble fraction.

Catalytic properties of crystalline enzyme

As shown in Fig. 4, d-mannitol was most rapidly oxidized to d-fructose under alkaline pHs such as 9.0–10.0 in the presence of NADP, but NAD was inert as the coenzyme. The relative rate of NAD reduction was less than 1% of that of NADP. d-Sorbitol was oxidized by the enzyme but the relative rate of d-sorbitol oxidation was less than 4% of that of d-mannitol. Other polyols, such as d-dulcitol, d-riboitol, and d-arabitol, did not act as substrates. On the other hand, d-fructose was reduced to d-mannitol in the presence of NADPH within the pH range of 5.0–9.5 with the optimum pH 6.0–8.0 (Fig. 4). L-Sorbose was also available as the substrate but the reaction rate was less than 5% of that of...
d-fructose. No ketohexose, aldohexose, ketopenose, or aldopenose was found to be the substrate when checked with various substances such as d-psicose, d-tagatose, d-galactose, d-mannose, d-ribulose, d-xylulose, d-ribose, and d-xylene. Apparent Km values for d-mannitol, NADP, d-fructose, and NADPH were 10 mm, 2.5 × 10⁻⁵ M, 12 mm, and 1.9 × 10⁻⁵ M, respectively. Judging from the data obtained, these are very much like the properties of NAD-dependent d-mannitol dehydrogenase from lactic acid bacteria. However, as described in enzyme purification, a similar NAD-dependent enzyme to the enzyme from lactic acid bacteria existed in G. suboxydans used in this study and was clearly separated from the NADP-dependent enzyme.

The effects of other hexoses or pentoses to the reaction rate of d-fructose reduction were examined with the crystalline enzyme to see whether any disturbance in d-fructose reduction by such the compounds occurs. When d-glucose, d-sorbitol, d-mannitol, and d-xylene were present more than 100 times higher than the concentration of d-fructose in the reaction mixture, no great delay in d-fructose reduction was observed. These data strongly support the idea that NADP-dependent d-mannitol dehydrogenase prepared in this study can be available for the determination of d-fructose in the presence of other sugars or sugar alcohols. Due to lack of good enzymes for enzymatic d-fructose measurement, a coupling enzyme reaction system using hexokinase, 6-phosphohexose isomerase, and d-glucose-6-phosphate dehydrogenase in the presence of a large excess of ATP and NADP are still valid in our routine method. The weakest point of this method is the disturbance of assayed data by the presence of a trace of d-glucose in the samples. There has not been developed a highly qualified d-fructokinase which is stable enough for the purpose and insensitive to d-glucose and other sugars, to replace the hexokinase in the above coupling system. Therefore, NADP-dependent d-mannitol dehydrogenase from G. suboxydans must be available for d-fructose measurement instead of such a coupling enzyme system, as similarly indicated the availability of NAD-dependent enzyme from lactic acid bacteria.

It is important to show another characteristics of the enzyme for the purpose of d-fructose measurement. When a highly diluted enzyme solution (10 µg/ml in 2 mM KPB) was heated, the loss of enzyme activity was not observed after heating the enzyme for 30 min at 50°C irrespective to the presence and absence of d-fructose or d-mannitol in the enzyme solution. Then the enzyme became heat labile after prolonged incubation for 50 min at 50°C in the absence of substrate. In the presence of d-fructose, d-mannitol, and even d-glucose, the enzyme activity was protected against heat denaturation after incubation for 50 min at 50°C. The critical point of heat inactivation of the enzyme appeared at 53°C. About 20% and 50% of the original enzyme activities remained after incubating the enzyme at 53°C for 20 min in the absence and presence of the substrate, 50 mM d-fructose, respectively as shown in Fig. 5. These properties would be useful information for the enzyme when it is used for a rapid enzymatic d-fructose measurement at higher temperatures. No information is available about the heat stability of the coupling enzyme system composed of hexokinase, phosphohexose isomerase, and d-glucose-6-phosphate dehydrogenase, indicating how the system is heat stable. In general, all or one of the enzymes concerned in the coupling system may be heat-sensitive entities. Moreover, the coupling enzyme system is rather expensive due to the essential requirement for excess ATP and NADP, in addition to requirement for highly purified enzymes. The pH stability of NADP-dependent d-mannitol dehydrogenase from G. suboxydans was shown to be stable without concomitant loss of enzyme activity over the pH range of 3.5-9.5, when checked the enzyme activity after standing for several days in solutions at 4°C. All the data indicated above allow the enzyme to be a good candidate for enzymatic diagnosis of d-fructose and replacement by it in the conventional coupling enzyme system may be emphasized.

As a preliminary experiment, the NADP-dependent d-mannitol dehydrogenase from G. suboxydans crystallized in this study was examined for enzymatic d-fructose measurement. A sample of a reaction mixture in which d-mannitol had been reacted with membrane-bound quinoprotein d-mannitol dehydrogenase from G. suboxydans, a similar enzyme purified by Okawa et al., was incubated with NADP-dependent d-mannitol dehydrogenase. The reaction product by the membrane-bound dehydrogenase was confirmed to be d-fructose stoichiometrically (data not shown). In our earlier study on the membrane-bound quinohemoprotein d-fructose dehydrogenase (FDH) from G. industrius, we proposed that the enzyme can measure a trace of d-fructose such as 1 µg/ml of the substrate. Different from reversible

Fig. 5. Effect of Heating on NADP-Dependent d-Mannitol Dehydrogenase from G. suboxydans.

A diluted enzyme solution of NADP-dependent d-mannitol dehydrogenase (10 µg/ml in 10 mM KPB) was heated at 50°C in the presence (→) or absence (○) of d-fructose (50 mM). Similar experiment was done at 53°C in the presence (△) or absence (●) of d-fructose (50 mM). Immediately after the enzyme was heated for the period as indicated, it was chilled in an ice bath. The remaining enzyme activity was measured under the standard assay conditions.
NAD(P)-dependent enzymes, FDH catalyzes an irreversible oxidation reaction of d-fructose without reaction equilibrium until d-fructose is completely exhausted. Thus, based on the end point measurement, FDH may be the best enzyme for enzymatic measurement of d-fructose so far established. However, based on the rate assay measurement, the NADP-dependent d-mannitol dehydrogenase developed in this study can offer a routine and simple enzymatic assay system for d-fructose like similar NAD(P)-dependent enzymes, though it is based on a different mechanism from FDH.

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