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Purification and Properties of Membrane-bound D-Sorbitol Dehydrogenase from *Gluconobacter suboxydans* IFO 3255*

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D-Sorbitol dehydrogenase was solubilized from the membrane fraction of *Gluconobacter suboxydans* IFO 3255 with Triton X-100 in the presence of D-sorbitol. Purification of the enzyme was done by fractionation with column chromatographies of DEAE-Cellulose, DEAE-Sepharose, hydroxylapatite, and Sephacryl HR300 in the presence of Triton X-100.

The molecular mass of the enzyme was 800 kDa, consisting of homologous subunits of 80 kDa. The optimum pH of the enzyme activity was 6.0, and the optimum temperature was 30°C.

Western blot analysis suggested the occurrence of the enzyme in all the *Gluconobacter* strains tested.

Key words: acetic acid bacteria; *Gluconobacter* strains; D-sorbitol dehydrogenase

Related to studies on the enzymes participating in the formation of L-sorbose from D-sorbitol, Widmer[1] and Cummins *et al.*[2] have reported that the cell-free extract of *Acetobacter suboxydans* (syn. *Gluconobacter suboxydans*) contains three kinds of enzymes participating in D-sorbitol oxidation. In addition to a dehydrogenase in the membrane fraction, there are two enzymes in the soluble fraction; one catalyzes D-fructose formation in the presence of NAD and another catalyzes L-sorbose formation in the presence of NADP. The NAD-dependent dehydrogenase has been partially purified,[3] and the NADP-dependent dehydrogenase has been isolated as an NADPH-linked L-sorbose reductase.[4] The membrane-bound D-sorbitol dehydrogenase of *G. suboxydans var.* α IFO 3254 has been purified and characterized by Shinagawa *et al.*[5] and had an optimum pH of 4.5. However, its product is not mentioned in the report. It is known that acetic acid bacteria produce L-sorbose from D-sorbitol at a low pH range of 3.5 to 5.0. Therefore, it was considered that the enzyme isolated by Shinagawa *et al.*[5] was involved in L-sorbose production from D-sorbitol.

However, D-sorbitol was converted to L-sorbose efficiently by *Gluconobacter suboxydans* IFO 3255 at pH 7.0, suggesting occurrence of another type of membrane-bound D-sorbitol dehydrogenase functioning at a pH of around 7.0 in the membrane fraction of the organism.

Here, we describe the purification and characterization of a new type of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255, and discuss distribution of the enzyme in *Gluconobacter* strains.

Materials and Methods

Chemicals. D-Sorbitol and 2,6-dichlorophenolindophenol (DCIP) were purchased from Nakarai Tesque. Inc. and E. Merck, respectively. Triton X-100 and hydroxylapatite (Bio-Gel HTP) were purchased from Bio-Rad Laboratories. DEAE-cellulose DE-52 was from Whatman, Ltd. DEAE-Sepharose CL-6B and Sephacryl S300 were from Pharmacia Fine Chemicals. All other chemicals were reagent grade.

Microorganism and cultivation. G. suboxydans IFO 3255, and other *Gluconobacter*, *Acetobacter*, and *Frateuria* strains were purchased from the Institute for Fermentation, Osaka (IFO). The medium consisted of 20 g of D-sorbitol, 3 g of yeast extract, 3 g of beef extract, 3 g of corn steep liquor, 10 g of polypeptone, 1 g of urea, 1 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 g of $CaCO_3$ in 1 liter of deionized water. The pH was adjusted to 7.0 with sodium hydroxide before sterilization. The cultivation in a flask was done aerobically with rotary shaking for one day; the cultivation in a 30-liter jar fermentor

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was done with an aeration rate of 15 L/min and an agitation speed of 500 rpm at 30°C for 21.5 hours. The broth was centrifuged at $400 \times g$ for 10 minutes to remove calcium carbonate, and then at $10,000 \times g$ for pelleting the cells. The cell cake was washed once with physiological saline solution. The cells (200 g wet weight from 20 liters of the culture) were frozen at −20°C until use.

Preparation of membrane fraction. The cells (100 g wet weight) were suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) and passed through a French pressure cell press at 20,000 *psi*. After centrifugation to remove intact cells, the supernatant (cell free extract) was centrifuged at $80,000 \times g$ for one hour. We designated this precipitate as the membrane fraction, which was resuspended into the above buffer (100 ml), and the protein concentration was 22.8 mg/ml.

Enzyme assay. The basal reaction mixture for assaying D-sorbitol dehydrogenase activity consisted of 50 mM potassium phosphate buffer (pH 6.0), 0.25 mM DCIP, and 0.325 mM phenazine methosulfate (PMS), which was prepared just before the assay. A cuvette had a 1-cm light path and contained 0.4 ml of the basal reaction mixture, 0.1 ml of 0.4 M D-sorbitol and the enzyme solution, and water with a total volume of 0.51 ml. The reference cuvette contained all components without the substrate. The reaction was started at 25°C with D-sorbitol, and the enzyme activity was measured at the initial reduction rate of DCIP at 600 nm. One enzyme unit is defined as the amount of the enzyme that catalyzes the reduction of 1 μmol of DCIP per min. The extinction coefficient of DCIP at pH 5.5, 6.0, 6.5, and 7.0 to 10.0 was 9.45, 10.8, 13.0, and 15.0 mM^{-1}, respectively.

Measurement of protein concentration. BCA protein assay reagent (Pierce, Rockford, IL) was used for the measurement of protein concentrations with bovine serum albumin as the standard.

Results

Conversion of D-sorbitol to L-sorbose by G. suboxydans IFO 3255

G. suboxydans IFO 3255 was cultivated in glass jar fermentor, type MB-C2000 (Iwashiya, Tokyo, Japan) which had a total volume of 3 l with a top drive system and temperature, pH, D.O., and exhaust gas monitors, containing the medium (weight/volume; 10% D-sorbitol, 0.5% yeast extract, 2.5% corn steep liquor, 0.0086% $MgSO_4 \cdot 7H_2O$, 0.086% urea, 0.086% KH_2PO_4, and 0.15% antifoam). Its working volume was 2 liters and the fermentation was done at 30°C, 700 rpm, 0.5 vvm for temperature, agitation speed, and aeration speed, respectively. During the fermentation, pH was controlled at 7.0 with Na_2CO_3. As a result, D-sorbitol was converted to L-sorbose within 18.5 hours cultivation and 89.3 g/l of L-sorbose from the initial D-sorbitol concentration of 98.9 g/l were produced. The average productivity of L-sorbose was 4.83 g/l/h.

Solubilization

We tried to solubilize the membrane-bound D-sorbitol dehydrogenase of *G. suboxydans* IFO 3255. At first, we tried to apply the method reported by Shinagawa *et al.*,[5] in which the membrane-bound D-sorbitol dehydrogenase of *G. suboxydans var.* α IFO 3254 was solubilized. However, the D-sorbitol dehydrogenase activity of *G. suboxydans* IFO 3255 was lost in the supernatant and residual membrane fractions after the solubilization of the membrane in 0.01 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100, 0.1 M KCl, 0.1 M D-sorbitol and about 10 mg/ml of the membrane protein for 2 h at 5°C. It was suggested that the membrane-bound D-sorbitol dehydrogenase of *G. suboxydans* IFO 3255 has different properties from that of *G. suboxydans var.* α IFO 3254.

Therefore, we modified this method for enzyme solubilization. The effects of pH and the concentrations of buffers, detergents, and KCl on the solubilization were studied. When the membrane fraction was mixed in 0.05 M potassium phosphate buffer (pH 7.0) containing 1% Triton X-100 and 0.04 M D-sorbitol for 2 h at 5°C, 74% of D-sorbitol dehydrogenase activity was recovered into the solubilized supernatant from the membrane fraction as shown in Table 1. The enzyme was not solubilized with *n*-octyl-β-D-glucopyranoside and the activity was lost by the addition of 0.1 M KCl. D-Sorbitol had a stabilizing effect on the enzyme solubilization.

Purification

All steps were done between 4 and 8°C with the buffer 0.01 M potassium phosphate unless otherwise stated.

Step 1. Solubilization. The membrane fraction was suspended in the buffer (pH 7.0) to give about 10 mg/ml of protein, and then 1% Triton X-100 and 0.1 M D-sorbitol were added. The suspension was shaken at 180 rpm for 2 h and centrifuged at $80,000 \times g$ for 60 min for the removal of the precipitate. The D-sorbitol dehydrogenase activity was recovered in the supernatant (200 ml).

Step 2. DEAE-Cellulose column chromatography. The supernatant (200 ml) was put on a column of DEAE-cellulose (ϕ2.5 by 30 cm) equilibrated with the buffer (pH 7.0) containing 0.05 M D-sorbitol and 0.1% Triton X-100. After it was washed with the same buffer, elution of the en-

Table 1. Solubilization of Membrane-bound D-Sorbitol Dehydrogenase

	Conditions (1) to (4) for solubilization	Relative activity of D-sorbitol dehydrogenase after solubilization (%)	
		Activity in supernatant	Activity in membrane fraction
(1)	Membrane fraction before solubilization	—	100
(2)	Solubilization in 0.01 M sodium acetate buffer (pH 5.0) containing 0.1 M KCl and 0.1 M D-sorbitol with:		
	0.5% Triton X-100	0.0	0.0
	1.0% Triton X-100	0.0	0.0
	0.2% *n*-octyl-*β*-glucopyranoside	6.0	0.0
	0.3% *n*-octyl-*β*-glucopyranoside	8.0	0.0
(3)	Solubilization in various concentrations of potassium phosphate buffer (pH 7.0) containing 1% Triton X-100 without KCl:		
	0.01 M buffer + 0.04 M D-sorbitol	23.0	2.5
	0.05 M buffer + 0.04 M D-sorbitol	74.0	6.0
	0.1 M buffer + 0.04 M D-sorbitol	38.5	3.5
	0.05 M buffer without D-sorbitol	65.5	5.0
(4)	Solubilization in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.04 M D-sorbitol with 0.5% *n*-octyl-*β*-glucopyranoside	2.0	119

Table 2. Purification of Membrane-bound D-Sorbitol Dehydrogenase from *Gluconobacter suboxydans* IFO 3255

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg-protein)
Cell-free extract	320	9,939	9,024	1.10
Membrane fraction	100	6,894	2,280	3.02
Solubilized fraction	200	2,878	1,480	1.94
DEAE-cellulose (DE52)	125	1,812	250	7.25
DEAE-Sepharose (CL6B)	40.0	924.0	56.5	16.4
Hydroxylapatite (Bio-Gel HTP)	52.0	415.4	13.5	30.9
Sephacryl HR300	13.0	173.5	3.83	45.3

zyme was done with the same buffer containing 0.1 M NaCl. The fractions having enzyme activity were collected.

Step 3. DEAE-Sepharose column chromatography. The pooled enzyme fractions (125 ml) from the previous step were dialyzed against two batches, each of one liter of the buffer containing 0.05 M D-sorbitol and 0.1% Triton X-100. The dialyzed enzyme fractions were put on a DEAE-Sepharose column (ϕ1.5 by 50 cm) equilibrated with the same buffer. After it was washed with the same buffer, D-sorbitol dehydrogenase was eluted with a linear gradient of NaCl (0 to 0.2 M). The major enzyme activity was eluted at the NaCl concentration of 0.16 to 0.18 M.

Step 4. Hydroxylapatite column chromatography. The pooled enzyme fractions (40 ml) were dialyzed against two batches, each of 500 ml of the buffer containing 0.05 M D-sorbitol and 0.1% Triton X-100. A portion of the enzyme sample (5 ml) was put on a hydroxylapatite column (ϕ2.5 by 20 cm) and equilibrated with the same buffer. The enzyme activity was eluted during the washing of the column. The above fractionation step was repeated and the fractions having the enzyme activity were combined. The total volume was 52 ml after the active fraction was dialyzed against the buffer containing 0.05 M D-sorbitol and 0.1% Triton X-100. Then, the fraction was concentrated to 10 ml by ultrafiltration (PM 10, Amicon).

Step 5. Sephacryl HR300 column chromatography. A portion of the enzyme fraction (2 ml) was put on Sephacryl HR300 column (ϕ1.0 by 120 cm) equilibrated with the buffer (pH 7.0) containing 0.05 M NaCl, 0.05 M D-sorbitol and 0.1% Triton X-100, and then developed. This fractionation step was repeated and the active fractions were combined. The active fraction (13 ml) dialyzed against the buffer containing 0.05 M D-sorbitol and 0.1% Triton X-100 was pooled and stored at $-80°C$.

Purification of the enzyme is summarized in Table 2.

Molecular structure of purified membrane-bound D-sorbitol dehydrogenase by HPLC and SDS-gel electrophoresis

The purified enzyme with a specific activity of 45.45 units per mg of protein (0.2 mg/ml) that was used for the analysis was dissolved in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.05 M D-sorbitol and 0.1% Triton X-100.

The molecular weight of the native D-sorbitol dehydrogenase was measured by HPLC using a size exclusion gel column (TSK gel G3000 SWXL column, ϕ7.8 by 300 mm) equilibrated with a 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl. The molecular standards were cyanocobalamin (1.35 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), γ-globulin (158 kDa), and thyroglobulin (670 kDa). The purified enzyme showed a single peak and the molecular mass was estimated to be 800 kDa, however its peak was out of the range of molecular standards of 1.35 kDa to 670 kDa.

In the SDS-PAGE, the enzyme showed a single band with a molecular mass of 80 kDa $\pm$ 1.0 kDa on (Fig. 1, lanes 2 and 3). It was therefore estimated the purified D-sorbitol dehydrogenase consists of ten homologous subunits.

Catalytic properties of membrane-bound D-sorbitol dehydrogenase

Substrate specificity. Substrate specificities of the purified enzyme for sugars, sugar alcohols, and organic acid were surveyed as the catalytic activity and the product from each sugar alcohol was identified (Table 3). Among the substances tested, D-sorbitol, D-arabitol, erythritol, and glycerol were highly oxidized, and D-mannitol and D-adonitol were also oxidized at 49.9 and 66.6% of the reaction rate with D-sorbitol, respectively. L-Sorbose, D-fructose, D-xylulose, erythrulose, D-ribulose, and dihydroxyacetone were produced from D-sorbitol, D-mannitol, D-arabitol, erythritol, D-adonitol, and glycerol, respectively. The purified membrane-bound enzyme is thought to be a sugar alcohol dehydrogenase. The enzyme had the activity on D-gluconate, however, the product analysis was not done because of its low activity (6.65%).

Effects of substrate concentration of reaction rate. The velocity of the oxidizing reaction using varying the concentrations of D-sorbitol from 0.5 to 80 mM was measured to calculate the *Km* value for D-sorbitol. It was calculated to be 18 mM when DCIP was used as the electron acceptor for the reaction.

Effects of pH on the activity and the stability. The correlation between the reaction rates of D-sorbitol dehydrogenase and pH values of the reaction mixture was measured, with all other assay conditions being kept the same. The enzyme showed the highest activity at pH 6.0 (Fig. 2A). As shown in Fig. 2B, more than 60% of the initial activity remained when the reaction was done in the pH range between 7.0 and 9.0.

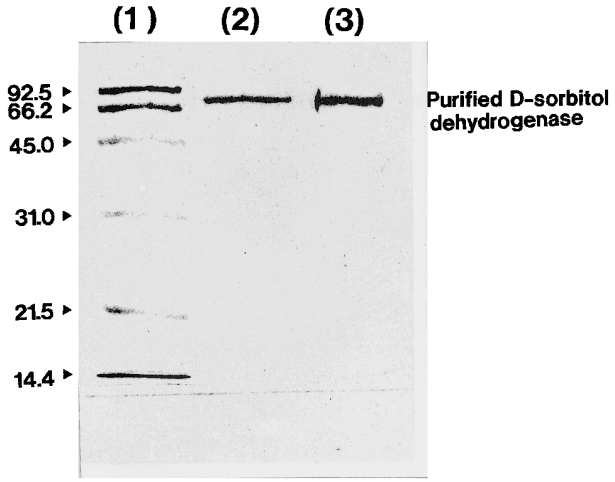

Fig. 1. SDS-Gel Electropholesis of Purified D-Sorbitol Dehydrogenase.

Purified D-sorbitol dehydrogenase with a specific activity of 45.3 was used; 3 or 5 μg of protein was added. After the protein staining with Coomassie brilliant blue R-250, the molecular mass of D-sorbitol dehydrogenase was measured. Lane (1), Standard marker proteins (kDa); Lane (2), 3 μg of purified D-sorbitol dehydrogenase; Lane (3), 5 μg of purified D-sorbitol dehydrogenase.

Table 3. Substrate Specificity of Purified Enzyme and Identification of its Product

Substrate	Relative activity[a] (%)	Product
D-Sorbitol	100	L-Sorbose
D-Mannitol	49.9	D-Fructose
D-Arabitol	175	D-Xylulose
meso-Erythritol	172	Erythrulose
D-Adonitol	66.6	D-Ribulose
Glycerol	117	Dihydroxyacetone
D-Glucose	0	—[b]
D-Fructose	0	—
L-Sorbose	0	—
Methanol	0	—
Ethanol	0	—
Sucrose	0	—
D-Gluconate	6.65	not done

To identify the product from each substrate, 2 units of purified enzyme were incubated in 1.0 ml of reaction mixture (8 mM PMS and 0.2 potassium phosphate buffer pH 7.0) with 0.04 M D-sorbitol, D-mannitol, D-arabitol, erythritol, D-adonitol, or glycerol for 4 h at 30°C. The reaction product was analyzed by HPLC and thin layer chromatography.[6]

[a] Relative activity is expressed as percentage of the reaction rate obtained with the substrate D-sorbitol.

[b] not tested.

Effects of temperature on the activity and the stability. As shown in Fig. 3A, the enzyme showed the highest activity at 30°C. Decreases of 60 and 70% in its activity were observed at 45 and 50°C, respectively, and no activity was detected at 60°C.

The enzyme was stable up to 35°C, and lost about

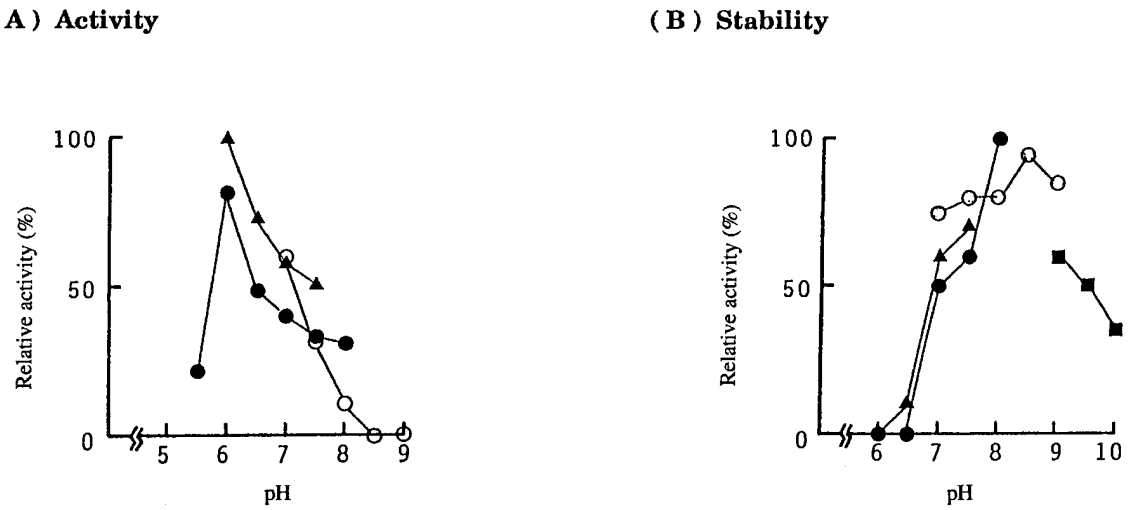

Fig. 2. Effects of pH on the Activity and the Stability of Purified D-Sorbitol Dehydrogenase.
The activity was measured under standard assay conditions with the following buffers: 0.1 M McIlvaine buffer (pH 5.5~8, —●—); 0.1 M Phosphate buffer (pH 6~7.5, —▲—); 0.2 M Tris buffer (pH 7~9, —○—); and 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9~10, —■—). The enzyme was left standing in buffers containing 0.05 M D-sorbitol and 0.1% Triton X-100 of different pH for 16 h at 4°C, and then the residual activity was measured.

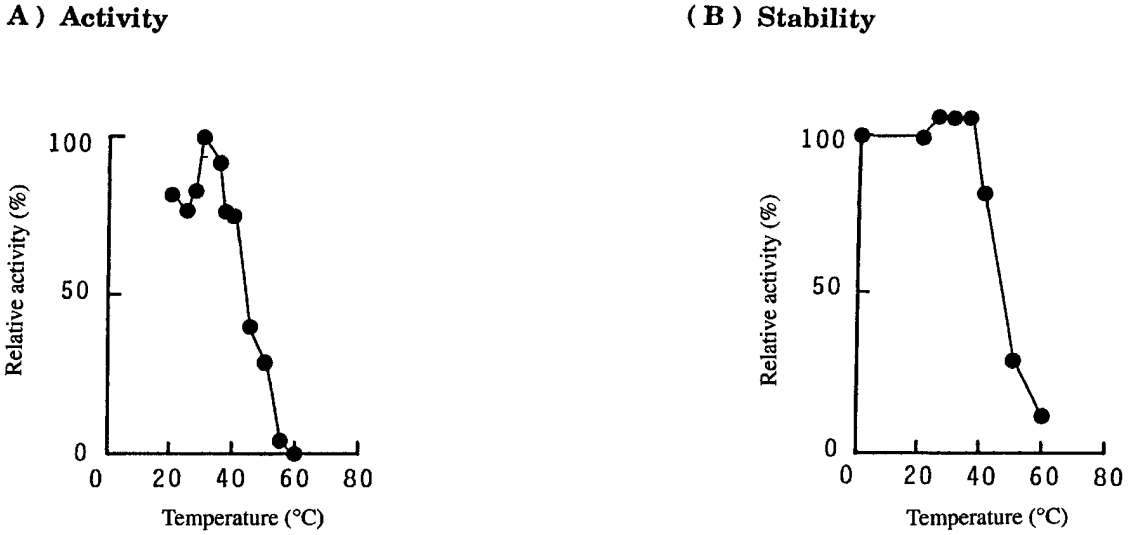

Fig. 3. Effects of temperature on the Activity and the Stability of Purified D-Sorbitol Dehydrogenase.
Data are expressed as a percentage of the activity at 30°C for activity (A) and and 25°C for stability (B). Thermostability was tested by incubating the enzyme for 5 min at various temperatures in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.05 M D-sorbitol and 0.1% Triton X-100. The residual activity was measured after the treated enzyme was immediately cooled in iced water.

20, 70, or 90% of its activity after by the incubation at 40, 50, or 60°C, respectively (Fig. 3B).

Effects of metal ions and inhibitors. As shown in Table 4, 17% of the activity was stimulated by the addition of 0.91 mM Co^{2+}. However, the addition of either 0.91 mM of Cu^{2+} or 0.91 mM of Fe^{2+} was strongly inhibitory, and the addition of 1.79 mM of Zn^{2+} was inhibitory by 68%. The effects of various inhibitors on the activity were investigated. Quinine and monoiodoacetate was inhibitory by 25 and 75%, respectively.

Distribution of membrane-bound D-sorbitol dehydrogenase in other acetic acid bacteria

Distribution of membrane-bound D-sorbitol dehydrogenase in various acetic acid bacteria was surveyed with Western blot analysis using the antibody for the enzyme. Cell homogenates containing 3 to 5 μg of protein treated with 2% sodium dodecyl sulfate (SDS) were prepared, and then the SDS-PAGE was done. As shown in Table 5, all of the tested *Gluconobacter* strains showed a positive band at the position of molecular mass of 80 kDa, indicating the D-sorbitol dehydrogenase is present in these microorganisms. On the other hand, the critical positive band

Table 4. Effects of Metal Ions and Inhibitors on D-Sorbitol Dehydrogenase

Compounds added	Relative activity (%) Concentration in reaction mixture	
〈Metals〉	0.91 mM	1.79 mM
$CaCl_2$	96	96
$CoCl_2 \cdot 6H_2O$	117	108
$CuCl_2 \cdot 2H_2O$	0	0
$Fe(SO_4) \cdot xH_2O$	0	0
$MgCl_2 \cdot 6H_2O$	88	92
$MnCl_2 \cdot 4H_2O$	80	88
$ZnCl_2$	56	32
$ZnSO_4 \cdot 7H_2O$	44	40
〈Inhibitors〉	0.96 mM	1.89 mM
EDTA	96.7	100
Quinine·HCl	79.2	75
NaN_3	91.7	113
Monoiodoacetate	75.0	25

Relative activity is expressed as percentage of the reaction rate obtained without metals and inhibitors. The effects of metal ions and inhibitors on D-sorbitol dehydrogenase activity were investigated by measuring the activity with a standard assay. After the addition of the enzyme solution to the basal reaction mixture as described in **Materials and Methods**, a solution of each metal ion source was stirred in and the reaction started with the addition of D-sorbitol.

Table 5. Distribution of the D-Sorbitol Dehydrogenase in Other Acetic Acid Bacteria

Strain tested	Immunological blotting
Gluconobacter albidus IFO 3250	+
G. albidus IFO 3251	+
G. albidus IFO 3253	+
G. capsulatus IFO 3462	+
G. cerinus IFO 3263	+
G. cerinus IFO 3264	+
G. cerinus IFO 3265	+
G. cerinus IFO 3267	+
G. cerinus IFO 3270	+
G. dioxyacetonicus IFO 3271	+
G. dioxyacetonicus IFO 3274	+
G. gluconicus IFO 3171	+
G. gluconicus IFO 3285	+
G. gluconicus IFO 3286	+
G. industrius IFO 3260	+
G. melanogenus IFO 3292	+
G. melanogenus IFO 3293	+
G. melanogenus IFO 3294	+
G. nonoxygluconicus IFO 3276	+
G. oxydans IFO 3189	+
G. oxydans subsp. sphaericus IFO 12467	+
G. roseus IFO 3990	+
G. rubiginosus IFO 3244	+
G. suboxydans IFO 3130	+
G. suboxydans IFO 3172	+
G. suboxydans IFO 3254	+
G. suboxydans IFO 3255	+
G. suboxydans IFO 3256	+
G. suboxydans IFO 3257	+
G. suboxydans IFO 3258	+
G. suboxydans IFO 3289	+
G. suboxydans IFO 3290	+
G. suboxydans IFO 3291	+
Acetobacter aceti subsp. aceti IFO 3281	weakly positive
A. aceti subsp. aceti IFO 3284	d
A. aceti subsp. orleansis IFO 3259	+
A. aceti subsp. xylinum IFO 3288	+
A. aceti subsp. xylinum IFO 13772	+
A. acetigenus IFO 3277	d
A. acetigenus IFO 3280	d
A. acetosus IFO 3129	d
A. ascendens IFO 3188	d
A. liquefaciens IFO 12257	d
A. liquefaciens IFO 12388	weakly positive
A. pasteurianus subsp. lovaniensis IFO 13753	—
A. pasterianus subsp. paradoxus IFO 13754	d
A. pasterianus subsp. pasteurianus IFO 3222	—
A. peroxydans IFO 13755	—
A.rancens IFO 3298	d
A. sp. IFO 3248	—
Frateuria aurantia IFO 3245	weakly positive
F. aurantia IFO 13328	—

+: The positive band was strongly detected at the 80 kDa position on SDS-PAGE.
−: A crossing band was not detected at any position on SDS-PAGE.
d: A crossing band was detected at a position different from the 80 kDa position on SDS-PAGE.

was not observed in *Acetobacter* strains, with the exception of *A. aceti subsp. orleansis* IFO 3259, *A. aceti subsp. xylinum* IFO 3288 and *A. aceti xylinum* IFO 13722. The cell homogenate of *A. aceti subsp. aceti* IFO 3281, *A. liquefaciens* IFO 12388 and *F. aurantia* IFO 3245 showed a slight positive band at the position of molecular mass 80 kDa.

Discussion

Shinagawa *et al.*[5] solubilized and purified a D-sorbitol dehydrogenase from the membrane fraction of *G. suboxydans var.* α IFO 3254 which had significantly high activity at a low pH of 4.5, and more than 90% of its activity was lost at a pH of 7.0. They suggested that the enzyme participating in L-sorbose fermentation[7] is a membrane-bound enzyme and not an NADP-dependent enzyme since L-sorbose rapidly accumulates in the culture medium. Baker and Claus[8] studied the solubilization of NAD(P)-independent D-sorbitol dehydrogenase of *G. oxydans subsp. suboxydans* ATCC 621 using octyl-glucoside and reported that D-sorbitol dehydrogenase from the strain ATCC 621 was substantially different from the enzyme obtained by Shinagawa *et al.*[5] However, the enzyme was not isolated nor characterized except for its solubilization study. Recently, Choi *et al.*[9] isolated a PQQ-dependent membrane-bound D-sorbitol dehydrogenase from *G. suboxydans* KCTC 2111 (equivalent to ATCC 621), which consisted of three subunits having molecular masses of 75, 50, and 14 kDa. This enzyme is thought to be the same enzyme as that described by Baker and Claus.[8] The prosthetic group of the Shinagawa's enzyme is FAD and heme *c*, whereas that of the Choi's one is PQQ and heme *c*. Both membrane-bound enzymes catalyz-

Table 6. Properties of Related Membrane-bound Enzymes

Enzyme	D-Sorbitol dehydrogenase			Glycerol dehydrogenase
Reference	This study	Shinagawa *et al.*[5]	Choi *et al.*[7]	Adachi *et al.*[10]
Microorganism	*G. suboxydans* IFO 3255	*G. suboxydans* IFO 3254	*G. suboxydans* ATCC 621	*G. industrius* IFO 3260
Molecular weight (Subunit)	800 kDa (80 kDa × 10)	131 kDa (63, 51, 17 kDa)	(139 kDa?) (75, 50, 14 kDa)	Huge aggregation (80 kDa)[11]
Optimum pH	6.0–7.0	4.5	(5.0?)	7.5–8.0
pH stability	7.5–8.5	4.5–5.5	no data	8.5–10.0
Substrate specificity				
D-Sorbitol	100	100	100	45
D-Mannitol	49.9	5	68	26
D-Arabitol	175	0	no data	136
meso-Erythritol	172	0	no data	111
Glycerol	117	no data	no data	100
D-Xylitol	0	0	no data	0
D-Adonitol	66.6	no data	70	42
Ethanol	0	no data	20	0
Km with D-sorbitol	18 mM at pH 6.0	30 mM at pH 4.5	no data	34 mM with glycerol
Solubilization	1% Triton X-100 with 0.04 M D-sorbitol	1% Triton X-100 with 0.1 M D-sorbitol and 0.1 M KCl	1.5% *n*-octyl-glucoside	0.5% dimethyldodecyl-amineoxide
Prosthetic group	not determine	FAD and heme *c*	PQQ and heme *c*	PQQ

ing the oxidation of D-sorbitol were purified and their properties were studied, and their results seemed to give the product L-sorbose from D-sorbitol. However, the product is not mentioned in the above reports.[5,9]

The membrane-bound D-sorbitol dehydrogenase from *G. suboxydans* IFO 3255 in this paper has quite different properties from those of the enzymes described above. The present membrane-bound D-sorbitol dehydrogenase had an optimal pH range around neutral. We tried to measure absorption spectra of the purified enzyme to investigate the prosthetic group of the enzyme; however, peaks corresponding to cytochrome *c* and PQQ were not observed (data not shown). Generally, the activity of PQQ enzyme is stimulated by the addition of Ca^{2+} and PQQ when these co-factors of the enzyme are free, and inhibited by the addition of EDTA. The isolated enzyme, however, did not show the properties mentioned above and the enzyme activity was not stimulated by the addition of PQQ or Ca^{2+} and was not inhibited by the addition of EDTA. The study of the prosthetic group of this enzyme is insufficient at this time.

Adachi *et al.*[10] summarized the properties of various membrane-bound sugar alcohol dehydrogenases in acetic acid bacteria and quoted that the membrane-bound glycerol dehydrogenase[11] of *G. indusrius* IFO 3260 consists of one subunit having the molecular mass of 80 kDa and its prosthetic group is PQQ. Although the optimum pH is different between those of *G. suboxydans* IFO3255 and *G. indusrius* IFO 3260, we think the D-sorbitol dehydrogenase of *G. suboxydans* IFO3255 is similar to the glycerol dehydrogenase[11] because of the similarities in molecular mass and substrate specificity. In addition, it is described how the glycerol dehydrogenase[11] formed huge aggregation in the purification step. Our enzyme seemed to be an aggregation form because the non-denaturing D-sorbitol dehydrogenase had a relatively high molecular mass of 800 kDa consisting of 10 homologous subunits (80 kDa). The comparison of the properties of membrane-bound sugar alcohol dehydrogenases discussed here is summarized in Table 6.

Interestingly, it was suggested by Western blot analysis that our enzyme is located in all of *Gluconobacter* strains tested (33 strains). The enzyme isolated here is thought to have an important role relating to the first reaction of sugar alcohol oxidation for *Gluconobacter* strains in a neutral pH range.

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