Biosci. Biotechnol. Biochem., **63** (12), 2137–2143, 1999

Crystallization and Properties of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294

Osao ADACHI,# Yoshitaka ANO, Duangtip MOONMANGMEE,¶ Emiko SHINAGAWA,* Hirohide TOYAMA, Gunjana THEERAGOOL,** Napha LOTONG,** and Kazunobu MATSUSHITA

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan
**Department of Chemical and Biological Engineering, Ube National College of Technology, Tokiwadai, Ube 755-8555, Japan*
***Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

Received July 5, 1999; Accepted August 12, 1999

NADPH-Dependent L-sorbose reductase (SORD, synonimously NADP-dependent D-sorbitol dehydrogenase) was purified and crystallized for the first time from the cytosolic fraction of *Gluconobacter melanogenus* IFO 3294. The enzyme catalyzed oxidoreduction between D-sorbitol and L-sorbose in the presence of NADP or NADPH. Affinity chromatography by a Blue-dextran Sepharose 4B column was effective for purifying the enzyme giving about 770-fold purification with an overall yield of more than 50%. The crystalline enzyme showed a single sedimentation peak in analytical ultracentrifugation, giving an apparent sedimentation constant of 3.8 *s*. Gel filtration on a Sephadex G-75 column gave the molecular mass of 60 kDa to the enzyme, which dissociated into 30 kDa subunit on SDS-PAGE, indicating that the enzyme is composed of 2 identical subunits. Reduction of L-sorbose to D-sorbitol predominated in the presence of NADPH with the optimum pH of 5.0–7.0. Oxidation of D-sorbitol to L-sorbose was observed in the presence of NADP at the optimum pH of 7.0–9.0. The relative rate of L-sorbose reduction was more than seven times higher to that of D-sorbitol oxidation. NAD and NADH were inert for both reactions. D-Fructose reduction in the presence of NADPH did not occur with SORD. Since the reaction rate in L-sorbose reduction highly predominated over D-sorbitol oxidation over a wide pH range, the enzyme could be available for direct enzymatic measurement of L-sorbose. 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 L-sorbose reduced. Judging from the enzymatic properties, SORD would contribute to the intracellular assimilation of L-sorbose incorporated from outside the cells where L-sorbose is accumulated in huge amounts in the culture medium.

Key words: acetic acid bacteria; NADP-dependent D-sorbitol dehydrogenase; *Gluconobacter melanogenus*; L-sorbose reductase

Several different kinds of NAD(P)-dependent dehydrogenases in carbohydrate metabolism have been purified from the cytosolic fraction of acetic acid bacteria to characterize the membrane-bound dehydrogenases that catalyze the same reaction.[1–8] In oxidative fermentation, as we have proposed,[9] membrane-bound dehydrogenases have a practical importance to produce various oxidation products and most of the enzymes are localized on the outersurface of the cytoplasmic membrane facing to the periplasmic space. The enzyme reactions are coupled to the respiratory chain of the organism without exception and the electrons generated in substrate oxidation are transferred to the terminal oxidase in the cytoplasmic membranes yielding bioenergy. In the cytosolic fraction, on the other hand, various kinds of NAD(P)-dependent dehydrogenases predominate, most of which show the same reaction as the dehydrogenases in the cytoplasmic membranes under different reaction conditions. Oxidation of alcohol, aldehyde, D-glucose, D-fructose, D-mannitol, D-sorbitol, D-gluconate, 2-keto-D-gluconate, glycerol, and so on has been exemplified with individual enzymes from the cytoplasmic membranes as well as from the cytosolic fraction of acetic acid bacteria.[9] As has been advertised in the purification of NAD-dependent D-sorbitol dehydrogenase (NAD-SLDH) in the previous paper,[8] NADP-dependent D-sorbitol dehydrogenase (synonymously NADPH-dependent L-sorbose reductase, abbreviated as SORD in this study) has worth to compare with NAD-SLDH in many respects. NAD-SLDH catalyzes a shuttle reaction between D-sorbitol and D-fructose and does not obey the Bertrand-Hudson rule in sugar alcohol oxidation. On the other hand, SORD catalyzes a shuttle reaction between D-sorbitol and L-sorbose and fits the Bertrand-Hudson rule similar to NAD(P)-dependent D-mannitol dehydrogenases.[7] Two membrane-bound D-sorbitol dehydrogenases yielding L-sorbose catalyze irreversible one way oxidation reaction coupling to the respiratory chain of the organism.[10,11] It

To whom correspondence should be addressed. Osao ADACHI, Tel: +81-839-33-5857; Fax: +81-839-33-5820; E-mail: osao@agr.yamaguchi-u.ac.jp
¶ On leave from the *Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand.*

is interesting to see that one of the two membrane-bound D-sorbitol dehydrogenase contains a covalently bound FAD as the primary coenzyme,[10] while the other uses pyrroloquinoline quinone (PQQ).[11] The membrane-bound L-sorbose yielding D-sorbitol dehydrogenases obey the Bertrand-Hudson rule and is important in vitamin C manufacturing by the Reichstein method. Provided the enzyme occurs outside the organism, in the periplasmic space, the physiological roles of SORD occurring in the cytosolic fraction of the organism remain to be examined. In this study, purification of SORD was tried from the cytosolic fraction of Gluconobacter melanogenus IFO 3294. In spite of its wide distribution through the genus Gluconobacter, no reasonable purification of SORD has been done so far. Thus, the enzyme is purified and crystallized for the first time in this study. Physicochemical and catalytic properties of SORD are discussed with the crystalline enzyme. Availability of SORD for routine use to screen L-sorbose producing strain is also proposed.

Materials and Methods

Chemicals. NAD, NADP, NADH, NADPH, yeast extract, yeast NAD-dependent alcohol dehydrogenase (151 kDa), and D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (105 kDa) were kind gifts from Oriental Yeast Co., Tokyo. Blue-dextran Sepharose 4B was prepared by the method of Ryan and Vestling.[12] Other chemicals used were from commercial sources of guaranteed grade unless otherwise stated.

Microorganisms and culture conditions. G. melanogenus IFO 3294 was used throughout this study. The culture medium consisted of 10 g of D-sorbitol, 2 g of glycerol, 1 g of yeast extract, and 1 g of Polypepton 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 table 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 this kind of culture.

Assay of enzyme activity. The enzyme activity of SORD was measured by a routine method used for common NAD(P) enzymes by recording the rate of decrease of NADPH at 340 nm with L-sorbose as the substrate at 25°C. The reaction mixture (1 ml) contained 100 μmol of L-sorbose, 50 μmol of potassium phosphate (KPB), pH 6.0, 0.1 μmol of NADPH, and an appropriate amount of enzyme. D-Sorbitol oxidation was measured in a reaction mixture (1 ml) containing 100 μmol D-sorbitol, 50 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 L-sorbose reduction or 1.0 μmol of NADPH formation in D-sorbitol oxidation per min under these conditions. A spectrophotometric absorption coefficient of $E^{1\%}_{cm,280\,nm}=10.0$ was tentatively used for protein concentration measurement. The specific activity was defined as units of enzyme activity per milligram of protein.

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

Polyacrylamide gel electrophoresis (PAGE). PAGE by a disc gel in the absence of sodium dodecyl sulfate (native PAGE) was done on a 7.5% polyacrylamide and Tris-glycine buffer, pH 8.3, essentially by the method described by Davis.[13] Protein was stained by Coomassie brilliant blue (CBB R-250).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done on 12.5% (w/v) slab gel by the methods described by Laemmli.[14] Before application, samples were treated with 6% (w/v) SDS and 0.1 mM dithiothreitol at 60°C for 30 min. The following calibration proteins (Bio-Rad, Hercules, CA, U.S.A.) with the indicated molecular masses 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 SCP85H ultracentrifuge at 20°C throughout measurements. Estimations of the sedimentation coefficient was done by the methods of sedimentation velocity,[15] 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[16] 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 NAD-dependent alcohol dehydrogenase (150 kDa), NADP-dependent D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (105 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 35-drop fractions were collected and analyzed. Under these conditions, the peak fraction of the individual marker proteins used came out with the following fraction numbers: NADP-dependent D-glucose-6-phosphate dehydrogenase, 21; bovine serum albumin, 25; ovalbu-

min, 35; cytochrome *c*, 39.

Heat stability and pH stability. For examination of heat stability, a diluted enzyme solution (80 μg protein/ml in 2 mM KPB) was used. The enzyme solution (0.1 ml) in a thin glass tube was directly incubated in the presence or absence of 50 mM L-sorbose under different temperatures for 5 min, and chilled in ice water. The remaining enzyme activity was measured with 10 μl of the heat-treated enzyme solution under the standard assay conditions. For measurement of pH stability, 10 μl of the enzyme solution (800 μg protein/ml in 2 mK 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 50 mM KPB, pH 6.0, containing 300 μmol of L-sorbose and 0.1 μmol of NADPH, were added and mixed by a flash mixer. The solution was immediately transferred into a glass cuvette to measure the enzyme activity in a photometer at 25°C.

Results and Discussion

Purification of NADPH-dependent L-sorbose reductase

Two hundreds grams of wet cell paste of *G. melanogenus* IFO 3294 harvested from 30 liters of the culture medium was used for the starting material. The crude extract was put to a DEAE-cellulose column (2.5 × 30 cm), which had been equilibrated with 2 mM Buffer A. After the column was washed with the same buffer, elution of SORD was done stepwise with Buffer A containing 0.1 M KCl, 0.3 M KCl, and 0.5 M KCl. More than 75% of the total enzyme activity was eluted with the buffer containing 0.3 M KCl. The enzyme fractions were combined (330 ml) and fractionated with ammonium sulfate. The ammonium sulfate was added to 0.4 saturation (22.6 g/100 ml of enzyme solution) and the pH was adjusted to 6.5 with ammonia water before centrifugation at 15,000 × *g* for 20 min. To the supernatant, ammonium sulfate was further added to 0.6 saturation (12.0 g/100 ml of enzyme solution) and the precipitate emerged was collected by centrifugation at 15,000 × *g* for 20 min. About 85% of the total enzyme activity was recovered in the precipitate. The precipitate was dialyzed overnight against 2 mM Buffer A. The dialyzed enzyme was put on a DEAE-Sephadex A-50 column (1.5 × 25 cm), which had been equilibrated with 2 mM Buffer A. After the column was washed with the same buffer containing 0.075 M KCl, a linear gradient chromatography of KCl was done between KCl concentrations of 0.075 M and 0.35 M (500 ml each). The enzyme activity appeared at the KCl concentration around 0.18 M. SORD proteins were collected by ammonium sulfate precipitation at 0.75 saturation (46.5 g/100 ml) and dialyzed against 2 mM KPB containing 5 mM β-mercaptoethanol. The dialyzed enzyme solution was put on a Blue-dextran Sepharose 4B column (2.5 × 30 cm), which had been equilibrated with the same buffer used for dialysis. The enzyme activity was eluted with the buffer containing 50 mM D-sorbitol and 0.25 M KCl. By this step, contaminating similar enzymes such as NAD-dependent and NADP-dependent D-mannitol dehydrogenases,[7] which are not adsorbed by the Blue-dextran Sepharose 4B column, must be removed from SORD, though exact checking was not done. The enzyme proteins were collected by ammonium sulfate precipitation as above. The precipitate was dissolved in a small amount of the buffer and put on a Sephadex G-200 column (1 × 180 cm), which had been equilibrated with 0.1 M Tris-HCl, pH 7.4, containing 5 mM β-mercaptoethanol, 10 mM $MgSO_4$, and 10% glycerol. After a highly concentrated enzyme solution was prepared at room temperature in the presence of ammonium sulfate of 0.4 saturation, the enzyme solution was put in a refrigerator overnight. Fine needles of the enzyme crystals appeared. For the growth of enzyme crystals, saturated ammonium sulfate solution was added dropwise after several hours until the top meniscus became clear. The first crystals (crystalline fraction) were collected by a table top centrifuge and the precipitate was dissolved in a small volume of 30 mM KPB, pH 6.0, containing β-mercaptoethanol. Crystallization was repeated to give fine needles as shown in Fig. 1. SORD was purified about 770-fold from the cell-free extract with an overall yield of 55%. The summary of enzyme purification is shown in Table 1.

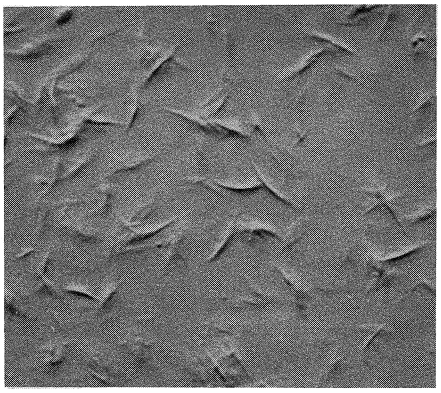

Fig. 1. Crystals of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
The photomirograph was taken under 148-fold of magnification.

Physicochemical properties of crystalline enzyme

When analyzed in analytical ultracentrifugation, SORD showed a single sedimentation peak with an apparent sedimentation coefficient of 3.8 *s* (Fig. 2). The photograph was taken every 25 min due to its small molecular mass giving a diffused meniscus when centrifuged over 80 min. Molecular mass measurement by SDS-PAGE gave an apparent molecular mass of 30 kDa, which was almost the same position to that of carbonic anhydrase (Fig. 3-A). The crystalline enzyme was homogeneous in disc gel electrophoresis, showing a single protein band (Fig. 3-B). When a mixture of SORD and standard marker proteins was put on a Sephadex G-75 column, SORD appeared at the fraction number of 28, which corresponded to an apparent molecular mass of 60 kDa. In an alternative molecular mass measurement by the same method, SORD was mixed with NADP-dependent D-mannitol dehydrogenase of which

Table 1. Purification of NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294

Step	Total protein (mg)	Total activity* (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	35350	3248	0.1	100	1
DEAE-cellulose	4050	2460	0.6	76	6
DEAE-Sephadex A-50	2205	2438	1.1	75	11
Bluedextran Sepharose	320	2825	8.8	87	88
Sephadex G-200	143	2550	17.8	78	178
DEAE-Sephadex A-50	55	2390	43.4	73	434
Crystalline fraction	24	1860	77.0	57	770

* Enzyme activity was assayed in 50 mM KPB, pH 6.0, by measuring the decrease of absorbance of NADPH using L-sorbose as the substrate.

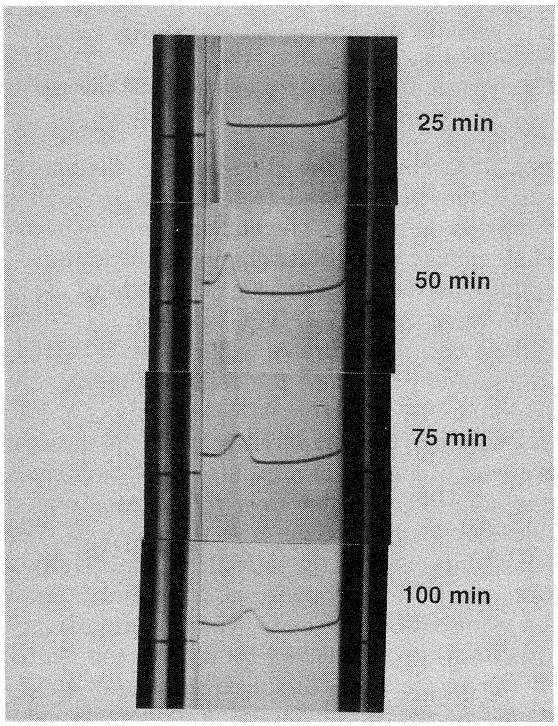

Fig. 2. Sedimentation Patterns of Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
Photographs were taken every 25 min as indicated after reaching 60,000 rpm. The enzyme solution containing 11.8 mg protein/ml in 2 mM KPB, pH 6.0, was used.

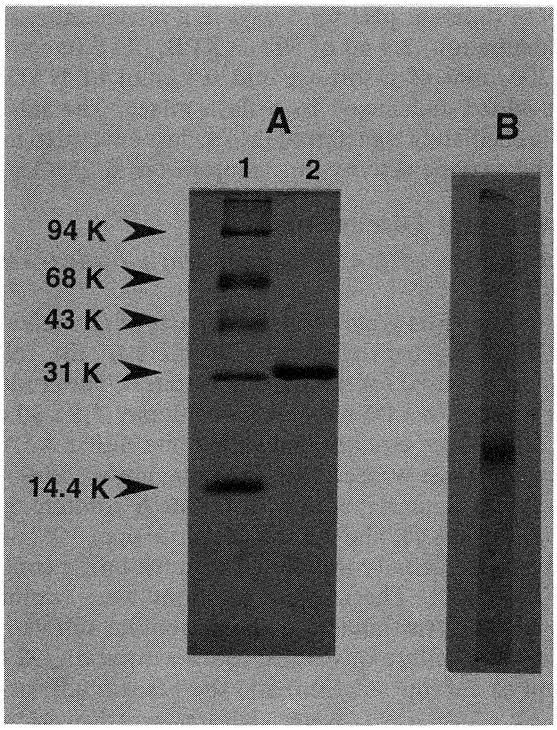

Fig. 3. Gel Electrophoresis of Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.
A diluted crystalline enzyme was used. (A) SDS-PAGE. Lane 1, marker proteins; lane 2, SLDH (10 μg protein). (B) Native gel electrophoresis. Protein (25 μg) was loaded.

the molecular mass had been measured to be 50 kDa[7] and analyzed under the same conditions as above. When compared the elution profiles of SORD with those of NADP-dependent D-mannitol dehydrogenase, SORD always came out immediately before NADP-dependent D-mannitol dehydrogenase. Thus, the apparent molecular mass of 60 kDa must be probable to SORD. It is also acceptable to conclude that SORD is composed of two identical subunits of 30 kDa.

Catalytic properties of crystalline enzyme

L-Sorbose was most rapidly reduced to D-sorbitol at pH 5.0–7.0 in the presence of NADPH, but NADH was inert as the electron donor. Potassium phosphate seemed to be a favorable buffer. McIlvaine buffer also showed the highest enzyme rate at a similar level as shown by potassium phosphate buffer. However, the enzyme activity was repressed at about 60% with acetate buffer compared to those observed with potassium phosphate buffer. D-Sorbitol was oxidized by the enzyme but the relative rate of D-sorbitol oxidation was less than 15% of that of L-sorbose reduction, when assayed with Tris-HCl buffer. It is very much like the case of 5-keto-D-fructose reductase,[6] by which D-fructose is oxidized at lower levels, though keto-D-gluconate reductases[2,3] show a reasonable oxidation for D-gluconate. The effects of buffer species on the enzyme activity of SORD are shown in Fig. 4. Other substrates including various sugars and sugar alcohols were used as substrates for SORD. The enzyme was concluded to be highly specific to L-sorbose reduction, thus the terminology as NADPH-dependent L-sorbose reductase would be better than NADP-dependent D-sorbitol dehydrogenase, as judged from its substrate specificity as shown in Table 2.

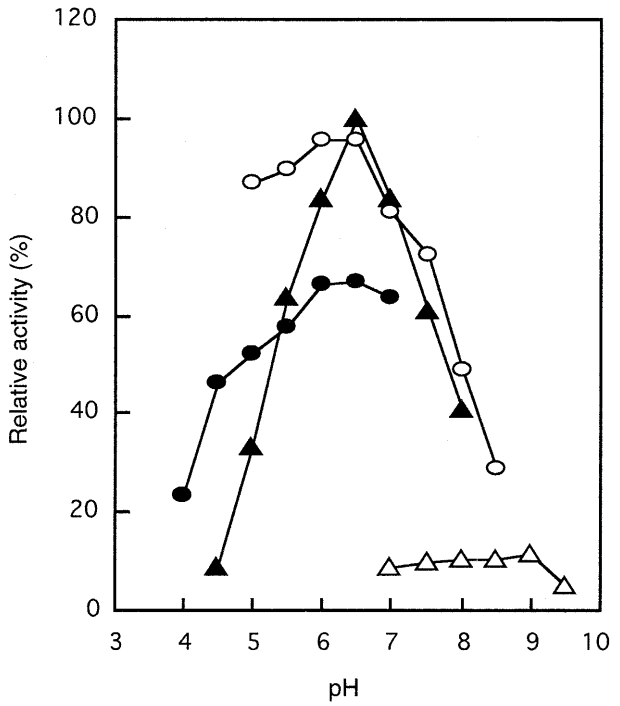

Fig. 4. Effects of pH on Crystalline NADPH-Dependent L-Sorbose Reductase from *Gluconobacter melanogenus* IFO 3294.

Enzyme activity (0.4 μg of protein for one assay) was measured with different pHs of buffer solution as indicated. (▲), McIlvaine buffer; (●), acetate buffer; (○), potassium phosphate buffer; (△), Tris-HCl buffer. D-Sorbitol oxidation was done with Tris-HCl buffer.

Table 2. Substrate Specificity of NADPH-Dependent L-Sorbose Reductase

Substrate	Polyol dehydrogenase (units/mg protein)	Substrate	Ketose reductase (units/mg protein)
D-Glucose	0	D-Fructose	0
D-Mannose	0	L-Sorbose	77
D-Galactose	0	D-Xylulose*	0
D-Tagatose	0	D-Ribulose*	0
D-Sorbitol	11	Dihydroxyacetone	0
D-Mannitol	0		
L-Iditol	0		
Dulcitol	0		
D-Xylose	0		
D-Arabinose	0		
D-Arabitol	0		
L-Arabitol	0		
Xylitol	0		
Ribitol	0		
Erythritol	0		
D-Erythrose	0		
Glycerol	0		

Enzyme activity was measured with the crystalline preparation of SORD under the standard assay conditions as described in the Materials and Methods.

* The final substrate concentration was adjusted to 0.1 M in the reaction mixture (total volume 1 ml), except that D-xylulose and D-ribulose were done with the final concentration of 0.01 M.

There were no data about reduction of L-ribulose and L-erythrulose with SORD, due to unavailability of the substrates from commercial sources. Apparent Km values for L-sorbose and NADPH were measured to be 35 mM and 32 μM, respectively. As judged from the lower reaction rates for D-sorbitol oxidation by SORD as above, measurements of Km for D-sorbitol and NADP were not done, because there would have no significance. SORD was stable to heating at 45°C for 5 min, however enzyme activity was completely lost when the enzyme was heated for 5 min over 55°C. The thermal stability was not improved in the presence of L-sorbose. The enzyme activity of SORD was stable in solution of pH 5–7.5 for several days when a diluted enzyme solution was stored in a refrigerator (data not shown).

As judged from the catalytic properties examined with SORD, the role of the physiological function of SORD can be assigned to L-sorbose reduction yielding D-sorbitol in the cytoplasm. The following discussion would be accepted reasonably. Acetic acid bacteria produce a huge amount of various oxidation products in the culture medium, as 2-keto-D-gluconate and/or 5-keto-D-gluconate when grown on D-glucose, and as 5-keto-D-fructose when grown on D-fructose.[9] Such novel oxidation products can be thought to be a temporary stock substance which can be used thereafter only by acetic acid bacteria, because acetic acid bacteria have a novel enzyme, 2-keto-D-gluconate reductase[1,2] and/or 5-keto-D-gluconate reductase[3] catalyzing D-gluconate formation which can be used via pentose phosphate pathway of the organisms after incorporated and phosphorylated. 5-Keto-D-fructose is reduced to D-fructose by 5-keto-D-fructose reductase[6] and then further metabolized by the organisms. The enzyme reaction of 5-keto-D-fructose reductase is much intense in the reduction of 5-keto-D-fructose yielding D-fructose, and D-fructose oxidation occurs at very low level even though the assay is made under optimum condition. Likewise, L-sorbose accumulated outside the cells is incorporated into the cells when other usable carbon and energy sources come to be exhausted. L-Sorbose would be converted to D-sorbitol by SORD which can be used as carbon and energy sources.

The effects of other hexoses or pentoses on the reaction rate of L-sorbose reduction were examined with the crystalline enzyme to see whether any disturbance in L-sorbose reduction by such the compounds occurs. When D-glucose, D-fructose, D-mannitol, and D-xylose were present more than 10 times higher than the concentration of L-sorbose in the reaction mixture, no inhibition on L-sorbose reduction was observed (data not shown). This strongly supports an idea that SORD prepared in this study is available for the measurement of L-sorbose in the presence of other sugars or sugar alcohols. Due to lack of good enzymes for enzymatic L-sorbose measurement, HPLC is the only technique at this moment. The only example is the membrane-bound L-sorbose dehydrogenase from *G. melanogenus* UV10 characterized by Sugisawa *et al.*[17] and SORD in this study. If the membrane-bound enzyme were available for the purpose, the enzyme would be useful for the end point measurement, by which a trace of L-sorbose could be assayed with high accuracy. Membrane-bound dehydrogenases can be available for the measurement of individual substrates

Table 3. Comparison of Properties among NAD(P)-Dependent Sugar Alcohol Dehydrogenases from *Gluconobacter* strains

	NADP-MDH[a)]	NAD-MDH[b)]	NAD-SLDH[c)]	NADP-SLDH[d)]
Molecular mass	50 kDa	130 kDa	98 kDa	60 kDa
Subunit	50 kDa	ND*	26 kDa	30 kDa
Numbers of subunits	1	ND	4	2
Svedverg unit	3.6 s	ND	5.1 s	3.8 s
Substrate				
	D-mannitol	D-mannitol	D-sorbitol	D-sorbitol
	D-fructose	D-fructose	D-fructose	L-sorbose
Km for				
D-Mannitol	10 mM	20 mM	—	—
D-Sorbitol	—	—	5 mM	ND
D-Fructose	12 mM	33 mM	20 mM	—
L-Sorbose	—	—	—	35 mM
NAD	—	2.5×10^{-4} M	2.1×10^{-4} M	—
NADH	—	1.0×10^{-5} M	2.8×10^{-4} M	—
NADP	2.5×10^{-5} M	—	—	ND
NADPH	1.9×10^{-5} M	—	—	3.2×10^{-5} M
Vmax for				
D-Mannitol	221 μmol/mg	150 μmol/mg	—	—
D-Sorbitol	—	—	136 μmol/mg	11 μmol/mg
D-Fructose	235 μmol/mg	188 μmol/mg	34 μmol/mg	—
L-Sorbose	—	—	—	77 μmol/mg
Optimum pH				
D-Mannitol	9.0	9.0	—	—
D-Sorbitol	—	—	9.0–11.0	7.0–9.0
D-Fructose	6.5–7.5	6.0	5.0–6.0	—
L-Sorbose	—	—	—	5.0–7.0

a) NADP-Dependent D-mannitol dehydrogenase from *G. suboxydans* IFO 12528.[7)]
b) NAD-Dependent D-mannitol dehydrogenase from *G. suboxydans* IFO 12528.[7)]
c) NAD-Dependent D-sorbitol dehydrogenase from *G. suboxydans* IFO 3257.[8)]
d) NADP-Dependent D-sorbitol dehydrogenase(=NADPH-dependent L-sorbose reductase, SORD) from *G. melanogenus* IFO 3294 (this study).
ND: not determined.

by the end point measurement.[18)] These enzymes can measure traces of the substrates by which an absolute measurement of the substrate becomes possible, because the membrane-bound enzymes catalyze a one way oxidation reaction. On the other hand, the rate assay system using NAD(P)-dependent enzymes has still a merit by its simplicity for handling the enzyme in the routine assays. Though they are unable to make an absolute measurement of the substrate, unlike membrane-bound dehydrogenases, they can outdo HPLC by their simplicity and rather higher performance. It has become urgently important to establish an enzymatic measurement for D-fructose, D-sorbitol, D-mannitol, and L-sorbose. The NADP-dependent D-mannitol dehydrogenase was discussed as useful enzyme for D-fructose measurement in our previous paper.[7)] It is important for microbial screening to search for a useful strain producing D-fructose. Similarly, SORD must be useful for the screening of L-sorbose producing microorganisms.

Development and application of thermotolerant acetic acid bacteria for oxidative fermentation has attracted strong interest in many respects when compared with nonthermotolerant strains.[19)] Screening of thermotolerant acetic acid bacteria that produce L-sorbose and D-fructose has been done successfully using SORD and NADP-dependent D-mannitol dehydrogenase (D. Moonmangmee *et al.*, manuscript in preparation). Occurrence of many different novel dehydrogenases in carbohydrate metabolism is known, as has been reviewed.[9,20)] Though Kersters *et al.* have reviewed a wide variety of NAD(P)-linked dehydrogenases in acetic acid bacteria,[21)] purification of an enzyme corresponding to SORD has not been done. Hoshino *et al.* reported a novel enzyme, NAD(P)-dependent L-sorbosone dehydrogenase from *G. melanogenus* UV10,[22)] however the enzyme is absolutely different from the SORD reported in this paper. Thus, SORD in this paper would be the first successful example in which an enzyme catalyzing L-sorbose reduction to D-sorbitol with high specificity is crystallized. The physicochemical and catalytic properties of SORD were compared with other similar enzymes from *Gluconobacter* strains, since they are useful in enzymatic measurement for D-fructose, D-sorbitol, D-mannitol, and L-sorbose (Table 3). All of these enzymes must be useful for monitoring ketohexoses, ketopentoses, and other related substances.

Acknowledgments

We wish to express our sincere thanks to Dr. M. Ameyama, Prof. Emeritus of Yamaguchi University, for his encouragement throughout this work. Photo picture processing of crystalline SORD were kindly contributed by Dr. S. Tanaka of our faculty. A part of this work was done by collaboration in a Core University Program between Yamaguchi University and Kasetsart University supported by the Scientific Cooperation Program agreed by the Japan Society for Promotion of Science (JSPS) and the National Research Council of

Thailand (NRCT) and by a grant for Promotion of Education and Research from Yamaguchi University (1998).

References

1) Chiyonobu, T., Shinagawa, E., Adachi, O., and Ameyama, M., Crystallization of 2-ketogluconate reductase from *Gluconobacter liquefaciens*. *Agric. Biol. Chem.*, **39**, 2263–2264 (1975).
2) Adachi, O., Chiyonobu, T., Shinagawa, E., Matsushita, K., and Ameyama, M., Crystalline 2-ketogluconate reductase from *Acetobacter ascendens*, the second instance of crystalline enzyme in genus *Acetobacter*. *Agric. Biol. Chem.*, **42**, 2057–2062 (1978).
3) Adachi, O., Shinagawa, E., Matsushita, K., and Ameyama, M., Crystallization and properties of 5-ketogluconate reductase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **43**, 75–83 (1979).
4) Adachi, O., Matsushita, K., Shinagawa, E., and Ameyama, M., Crystallization and properties of NADP-dependent aldehyde dehydrogenase from *Gluconobacter melanogenus*. *Agric. Biol. Chem.*, **44**, 155–164 (1980).
5) Adachi, O., Matsushita, K., Shinagawa, E., and Ameyama, M., Crystallization and properties of NADP-dependent D-glucose dehydrogenase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **44**, 301–308 (1980).
6) Ameyama, M., Matsushita, K., Shinagawa, E., and Adachi, O., 5-Keto-D-fructose reductase of *Gluconobacter industrius*. Purification, crystallization and properties. *Agric. Biol. Chem.*, **45**, 863–869 (1981).
7) Adachi, O., Toyama, H., and Matsushita, K., Crystalline NADP-dependent D-mannitol dehydrogenase from *Gluconobacter suboxydans* IFO 12528. *Biosci. Biotechnol. Biochem.*, **63**, 402–407 (1999).
8) Adachi, O., Toyama, H., and Matsushita, K., Three different D-fructose reductases occur in the genus *Gluconobacter*. *Proc. Annual Meeting of Japn. Soc. Biosci. Biotechnol. Agrochem.*, **73**, 39 (1999).
9) Matsushita, K., Toyama, H., and Adachi, O., Respiratory chains and bioenergetics of acetic acid bacteria. in "*Advances in Microbial Physiology*," Vol. **36**, ed. Rose, A. H. and Tempest, D. W., Academic Press, Ltd., London, pp. 247–301 (1994).
10) Shinagawa, E., Matsushita, K., Adachi, O. and Ameyama, A., Purification and characterization of D-sorbitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. α. *Agric. Biol. Chem.*, **46**, 135–141 (1982).
11) Choi, E-S., Lee, E-H., and Rhee, S-K., Purification of a membrane-bound sorbitol dehydrogenase from *Gluconobacter suboxydans*. *FEMS Microbiol. Lett.*, **125**, 45–50 (1995).
12) Ryan, L. D. and Vestling, C. S., Rapid purification of lactate dehydrogenase from rat liver and hepatoma: a new approach. *Arch. Biochem. Biophys.*, **160**, 279–284 (1974).
13) Davis, B. J., Disc electrophoresis. II. Methods of application to human serum proteins. *Ann. N. Y. Acad. Sci.*, **121**, 404–427 (1964).
14) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London), **227**, 680–685 (1970).
15) Chervenka, C. H., Determination of sedimentation rates. In "*A Manual of Methods for the Analytical Ultracentrifuge*," ed. Chervenka, C. H., Spinco Division of Beckman Instruments Inc., Palo Alto, California, pp. 23–37 (1970).
16) Andrews, P., Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.*, **91**, 222–233 (1964); Andrews, P., The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.*, **96**, 595–606 (1965).
17) Sugisawa, T., Hoshino, T., Nomura, S., and Fujiwara, A., Isolation and characterization of membrane-bound L-sorbose dehydrogenase from *Gluconobacter melanogenus* UV10. *Agric. Biol. Chem.*, **55**, 363–370 (1991).
18) Ameyama, M., Enzymic microdetermination of D-glucose, D-fructose, D-gluconate, 2-keto-D-gluconate, aldehyde, and alcohol with membrane-bound dehydrogenases. In "Methods in Enzymology," Vol. **89**, ed. by W. A. Wood, Academic Press, Inc., New York, 1982, pp. 20–29.
19) Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N., and Adachi, O., Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. *Biosci. Biotechnol. Biochem.*, **61**, 138–145 (1997).
20) Asai, T., Biochemical activities of acetic acid bacteria. In "*Acetic Acid Bacteria*," ed. Asai, T., Univ. of Tokyo Press, Tokyo, pp. 124–327 (1968).
21) Kersters, K., Wood, W. A., and De Ley, J., Polyol dehydrogenases of *Gluconobacter oxydans*. *J. Biol. Chem.*, **240**, 965–974 (1965).
22) Hoshino, T., Sugisawa, T., and Fujiwara, A., Isolation and characterization of NAD(P)-dependent L-sorbosone dehydrogenase from *Gluconobacter melanogenus* UV10. *Agric. Biol. Chem.*, **55**, 665–670 (1991).