Biosci. Biotechnol. Biochem., **63** (9), 1589–1595, 1999

Crystallization and Properties of NAD-Dependent D-Sorbitol Dehydrogenase from *Gluconobacter suboxydans* IFO 3257#

Osao ADACHI,† Hirohide TOYAMA, Gunjana THEERAGOOL,* Napha LOTONG,* and Kazunobu MATSUSHITA

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan
**Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

Received April 6, 1999; Accepted April 30, 1999

NAD-dependent D-sorbitol dehydrogenase (EC 1.1.1.14) was crystallized from the cytosolic fraction of *Gluconobacter suboxydans* IFO 3257. This is the first example of the enzyme crystallized from acetic acid bacteria. The enzyme catalyzed oxidoreduction between D-sorbitol and D-fructose in the presence of NAD or NADH. The crystalline enzyme showed a single sedimentation peak in analytical ultracentrifugation, giving an apparent sedimentation constant of 5.1*s*. Gel filtration on a Sephadex G-200 column gave the molecular mass of 98 kDa for the enzyme, which dissociated into 26-kDa subunits on SDS-PAGE, indicating that the enzyme is composed of four identical subunits. Oxidation of D-sorbitol to D-fructose and xylitol to D-xylulose predominated in the presence of NAD at the optimum pH of 9.5–10.0. Reductions of D-fructose to D-sorbitol and D-xylulose to xylitol were also observed in the presence of NADH with the optimum pH around 6.0. The relative rate of D-fructose reduction was about one-fourth of that of D-sorbitol oxidation. NADP and NADPH were inert for the both reactions. Since the reation rate in D-sorbitol oxidation predominated over D-fructose reduction at some alkaline pH, the enzyme could be available for direct enzymatic measurement of D-sorbitol. Even in the presence of a large excess of D-glucose and other substances, reduction of NAD to NADH was highly specific and stoichiometric to the D-sorbitol oxidized.

Key words: acetic acid bacteria; NAD-dependent D-sorbitol dehydrogenase; fructose reductase; *Gluconobacter suboxydans*; sorbitol dehydrogenase

In relation to sugar and sugar alcohol metabolism in acetic acid bacteria, we have found many membrane-bound dehydrogenases in the cytoplasmic membranes.[1] At the same time, different kinds of NAD(P)-dependent dehydrogenases have been indicated in the cytosolic soluble fractions of the organisms.[1] Alcohol dehydrogenase is found in the cytoplasmic membranes as well as in the cytosolic fraction and the metabolic functions of individual enzymes are distinct. Quinohemoprotein alcohol dehydrogenase (EC 1.1.99.8) is localized in the cytoplasmic membranes and the enzyme activity is directly coupled to the respiratory chain of the organisms, yielding bioenergy during alcohol oxidation. On the other hand, the cytosolic alcohol dehydrogenase (EC 1.1.1.1 or EC 1.1.1.2) is common in alcohol and aldehyde metabolism in cytoplasmic functions. Aldehyde dehydrogenases,[2–4] D-glucose dehydrogenases,[5,6] D-fructose dehydrogenases[7,8] and many other enzymes[1] have been similarly characterized from two different locations in acetic acid bacteria. In our recent report, NADP-dependent D-mannitol dehydrogenase (EC 1.1.1.138) and NAD-dependent D-mannitol dehydrogenase (EC 1.1.1.67) have been identified in the cytosolic fraction of D-glucose grown cells of *Gluconobacter suboxydans* IFO 12528, both of which catalyze reversible oxidoreduction between D-mannitol and D-fructose. They were separated from each other and the NADP-dependent enzyme was finally crystallized for the first time.[9] In acetic acid bacteria, quinohemoprotein D-mannitol dehydrogenase (EC 1.1.2.2) has been purified and characterized from the membrane fraction,[10,11] of which localization is restricted to the cytoplasmic membrane and the enzyme activity is NAD(P)-independent. As has been proved,[1] only the membrane-bound dehydrogenase is responsible for oxidative fermentation to accumulate useful oxidation products in the culture medium.

In two strains of genus *Gluconobacter*, *G. suboxydans* subsp. var. α IFO 3254 and *G. suboxydans* ATCC 621, the occurrence of two different D-sorbitol dehydrogenases (SLDH) (EC 1.1.99.21) in the membrane fraction has been indicated, one of which is a covalently bound FAD-dependent flavohemoprotein[12] and the other is a PQQ-dependent quinohemoprotein.[13] L-Sorbose fermentation is done with these membrane-bound dehydrogenases. Regarding the papers dealing with cytosolic SLDH, the enzyme sources have come from plant cells[14] and mammalian tissues,[15–17] and microorganisms.[18–22] Recently, NAD-dependent SLDH has been crystallized from *Bacillus fructosus*.[22] Since Gram-positive bacteria like bacilli would have no membrane-bound dehydrogenases as highly developed in acetic

† To whom correspondence should be addressed. Osao ADACHI, *Laboratory of Applied Microbiology, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*. Tel: +81-839-33-5857; Fax: +81-839-33-5820; E-mail: osao@agr.yamaguchi-u.ac.jp

This paper is dedicated to Prof. Hideaki Yamada for his 70th birthday. A part of this paper was presented to the annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, held in Fukuoka, March 30–April 2, 1999. The abstract paper appears in *Nippon Nogeikagaku Kaishi* (in Japanese), **73**, 39 (1999).

acid bacteria, it is interesting to check the role of SLDH in comparison with acetic acid bacteria. With respect to cytosolic NAD(P)-dependent SLDH in acetic acid bacteria, the occurrence of NAD-dependent SLDH (EC 1.1.1.14) was indicated many years ago.[23,24] However, enough purification of the enzyme remained to be done, inspite of its wide distribution through the genus *Gluconobacter*. In pseudomonads, since they would have no membrane-bound SLDH, only cytosolic SLDH is responsible for D-sorbitol metabolism. In this paper, crystallization and characterization of cytosolic NAD-dependent SLDH from *G. suboxydans* IFO 3257 were done to check the metabolic role of the enzyme in acetic acid bacteria. Comparison of catalytic and enzymatic properties of the cytosolic SLDH from acetic acid bacteria is made with those so far reported from other organisms.

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. Other chemicals used were from commercial sources of guaranteed grade unless otherwise stated.

Microorganisms and culture conditions. G. suboxydans IFO 3257 was used throughout this study. The culture medium consisted of 5 g of D-glucose, 20 g of sodium D-gluconate, 3 g of glycerol, 3 g of yeast extract, and 2 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 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 the culture described above.

Assay of enzyme activity. The enzyme activity of SLDH was measured by a routine method used for common NAD(P) enzymes by recording the rate of increase of NADH at 340 nm with D-sorbitol as the substrate in a reaction mixture at 25°C. The reaction mixture (1 ml) contained 100 μmol of D-sorbitol, 50 μmol of glycine-NaOH, pH 9.5, 0.2 μmol of NAD, and an appropriate amount of enzyme. D-Fructose reduction was measured in a reaction mixture (1 ml) containing 100 μmol D-fructose, 100 μmol of potassium phosphate, pH 6.0, 0.2 μmol of NADH, 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 NADH formation in D-sorbitol oxidation or 1.0 μmol of NADH oxidation in D-fructose reduction per min under the above conditions. An optical absorption coefficient of $E^{1\%}_{cm,280nm}=10.0$ was tentatively used for the 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 (Buffer A) of potassium phosphate, pH 6.0, containing 50 mM D-fructose and 5 mM β-mercaptoethanol was used throughout in this work. A cell suspension was made by suspending about 10 g of wet cells 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 designated 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,[25] and the protein band 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.[26] 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 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 SCP85H ultracentrifuge at 20°C throughout measurements. Estimations of the sedimentation coefficient was done by the methods of sedimentation velocity,[27] which was operated by a combination of a Hitachi UV scanner (ABS-7), an absorption scanner, and a UC processor (DA-7).

Measurement of the molecular mass. The molecular mass of the native enzyme was measured by gel filtration by the method of Andrews[28] on a Sephadex G-200 column (1 × 150 cm) which had been equilibrated with 2 mM KPB, pH 6.0, containing 5 mM β-mercaptoethanol. The following marker proteins were used as references: yeast NAD-dependent alcohol dehydrogenase (ADH) (151 kDa), D-glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PDH) (105 kDa), 5-keto-D-fructose reductase (KFR) (100 kDa),[29] and bovine serum albumin (BSA) (68 kDa). Elution was done at a flow rate of 0.5 ml/min.

Heat stability and pH stability. For examination of heat stability, a diluted crystalline enzyme (80 μg protein/ml in 2 mM KPB, pH 6.0, containing 5 mM β-mercaptoethanol) was used. The enzyme solution (0.1 ml) in a thin glass tube was directly incubated 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 mM KPB as above) 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 glycine-NaOH, pH 9.5, containing 300 μmol of D-sorbitol and 0.2 μmol of NAD, 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 D-sorbitol dehydrogenase

Cell-free extract prepared from 300 g of wet cells were put on a DEAE-cellulose column (5 × 35 cm) that had been equilibrated with 2 mM potassium phosphate buffer (KPB), pH 6.0, containing 5 mM β-mercaptoethanol and 50 mM D-sorbitol. After the non-adsorbed fraction was completely removed by washing the column with the same buffer, elution of the enzyme was done stepwise with 2 mM KPB containing 0.2 M KCl by which the majority of SLDH came out. NAD-Dependent D-mannitol dehydrogenase was eluted by increasing the KCl concentration in KPB to 0.3 M. Ammonium sulfate was added to 0.8 saturation (51.6 g/100 ml) and the precipitate was collected by conventional centrifugation. The precipitate was dialyzed against 2 mM KPB overnight. SLDH was fractionated with ammonium sulfate and SLDH precipitated between 0.4–0.6 saturation (22.6 g/100 ml to 36.1 g/100 ml) was collected and dialyzed against 2 mM KPB thoroughly. The enzyme solution containing SLDH was passed through a blue-dextran Sepharose column (3 × 20 cm), which had been equilibrated with 2 mM KPB. SLDH was not adsorbed on the column but some NAD(P)-dependent dehydrogenases such as 6-phospho-D-gluconate dehydrogenase,[30] D-glucose dehydrogenase,[6] 2-keto-D-gluconate reductase,[31] and 5-keto-D-gluconate reductase[32] were adsorbed as reported previously. Thus, SLDH was clearly separated from these contaminating proteins by this step. SLDH was further purified by DEAE-Sephadex A-50 column chromatography using a small column (2 × 10 cm), by which elution of SLDH was done under a linear gradient made by 300 ml each of KPB containing 0.125 M KCl and 0.25 M KCl. The enzyme activity appeared at the position corresponding to 0.18 M KCl. SLDH activity was concentrated by the addition of double volumes of saturation ammonium sulfate solution and the resulting precipitate was collected by a conventional centrifuge into a 2-ml Eppendorf centrifuge tube. The precipitate was dissolved in a small amount of 2 mM KPB and insoluble materials were removed by a table top flash centrigfuge. The enzyme solution was passed through a Sephadex G-200 column (1 × 150 cm) which had been equilibrated with the same KPB. SLDH fractions were collected and mixed with double volumes of saturated ammonium sulfate to precipitate the enzyme. To the concentrated SLDH solution, saturated ammonium sulfate solution was added dropwise until a faint turbidity appeared. After standing overnight in a refrigerator at 4°C, hexagonal rod of enzyme crystals appeared (Fig. 1).

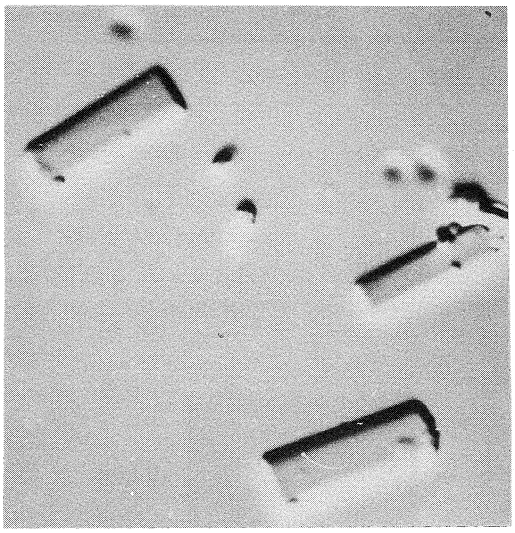

Fig. 1. Cryatalline NAD-Dependent D-Sorbitol Dehydrogenase from *G. suboxydans* IFO 3257.

When the first enzyme crystallization appeared to have finished, the crystals were collected by a conventional centrifuge to separate the enzyme crystals from the mother liquor. The precipitate was dissolved in a small amount of KPB designated as the crystalline fraction. The overall purification of SLDH is summarized in Table 1. Crystalline SLDH was obtained with an overall yield of 40% with 500-fold purification from the cell-free extract. It can be roughly estimated that about 0.2% of soluble protein is SLDH in the cell-free extract. The occurrence of SLDH was comparable to that in *Pseudomonas* sp.[21] and more abundant than that in *B. fructosus*.[22]

Physicochemical properties of crystalline enzyme

When analyzed in an analytical ultracentrifugation, the enzyme showed a single sedimentation peak as shown in Fig. 2. An apparent sedimentation coefficient was measured to be 5.1*s*. Judging from the data obtained with the enzyme from sheep liver,[15] the sedimentation coefficient of SLDH largely depends on the protein concentrations examined. The $S^0_{20,w}$ of sheep enzyme has been estimated to be 7.7*s*, while it gave an observed value of 6.9 when measured at about 20 mg/ml.[15] The crystalline SLDH from *G. suboxydans* was homogeneous in disc gel electrophoresis, showing a single protein band (Fig. 3). Molecular mass measurement by SDS-PAGE gave an apparent molecular mass of 26 kDa (Fig. 3). A mixture of SLDH and standard marker proteins was analyzed by gel filtration on a Sephadex G-200 column (1 × 150 cm) and 15-drop fractions (about 0.5 ml) were collected. SLDH was eluted from the column at the position far less than yeast ADH (151 kDa) and clearly bigger than BSA (68 kDa). The molecular mass of SLDH was a little smaller than G6PDH (105 kDa) and almost the same as KFR (100 kDa). When compared the elution profiles of SLDH with those of

Table 1. Summary of Enzyme Purification of NAD-Dependent D-Sorbitol Dehydrogenase

Step	Total protein (mg)	Total activity* (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	28,214	8,150	0.3	100	1
DEAE-Sephadex A-50	3,780	8,870	2.4	108	8
$AmSO_4$ fraction	2,138	7,270	3.4	90	12
Sephadex A-50	180	6,100	34.0	75	121
Sephadex G-200	43	4,400	102.7	55	366
Crystalline fraction	24	3,276	136.5	40	488

* Enzyme activity was assayed in 50 mM glycine-NaOH, pH 9.5, by measuring the increase of absorbance of NADH using D-sorbitol as the substrate.

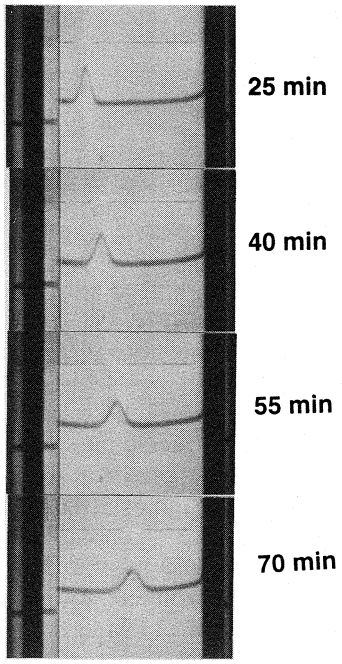

Fig. 2. Sedimentation Patterns of Cryatalline SLDH from *G. suboxydans* IFO 3257.

Photographs were taken every 15 min as indicated after reaching 60,000 rpm. The enzyme solution containing 8.5 mg protein/ml was used.

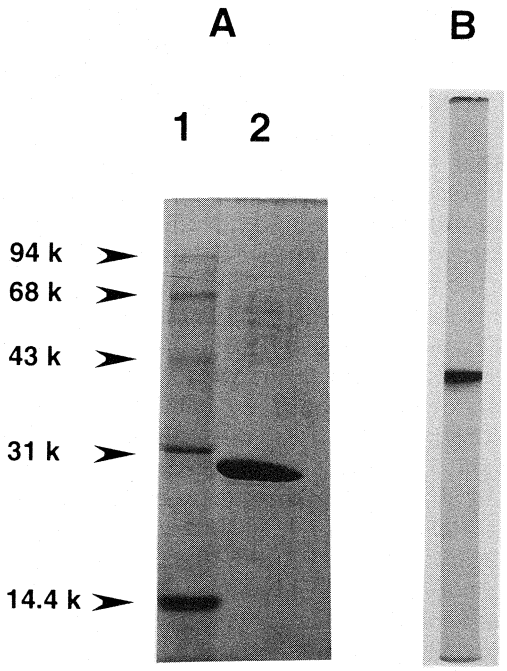

Fig. 3. Gel Electrophoresis of SLDH from *G. suboxydans* IFO 3257.

A diluted crystalline fraction was used. (A) SDS-PAGE. Lane 1, marker proteins; lane 2, SLDH (10 μg protein). (A) Native gel electrophoresis. Protein (25 μg) was loaded.

KFR, KFR always came faster than SLDH. In one experiment, peak fractions of the two enzyme activities appeared in the same fraction and only one fraction was different in another experiment between the two enzymes. Measurement of the molecular mass of SLDH based on the specific elution volume allowed us to guess it to be 98 kDa as the most probable one, which is comparable to SLDHs from rat liver (96 kDa)[16] and *B. fructosus* (102–110 kDa).[20] The molecular weight of sheep SLDH, 115,000,[15] was reported with an observed sedimentation constant, diffusion constant (6.1×10^{-7} cm^2/sec), and an assumed partial specific volume (0.745). SLDH from *G. suboxydans* differs from two SLDHs from *Pseudomonas* sp. in molecular size as well as subunit composition.[20,21] The two *Pseudomonas* SLDHs have a molecular mass of 65 kDa composed of two identical subunits. The isoelectric point of SLDH was measured to be 4.9, which is comparable to SLDH from *Bacillus* enzyme.[22]

Catalytic properties of crystalline enzyme

As shown in Fig. 4, D-sorbitol was rapidly oxidized to D-fructose under alkaline pHs such as 9.0–10.5 in the presence of NAD, but NADP was inert as the coenzyme. The oxidation product of D-sorbitol, D-fructose, was identified and measured stoichiometrically by NADP-dependent D-mannitol dehydrogenase.[9] Xylitol was oxidized by the enzyme at 68% of the relative rate to D-sorbitol oxidation. Oxidation of D-mannitol was less than 1% of that of D-sorbitol. Other polyols, such as dulcitol, ribitol, and D-arabitol, did not act as substrates. On the other hand, D-fructose was reduced to D-sorbitol in the presence of NADH with a pH optimum around 6.0 (Fig. 4). The relative rate of D-fructose reduction was about one fourth of that of D-sorbitol oxidation. D-Xylulose was reduced to xylitol by SLDH and the relative reaction rate was more than two times of that of D-fructose reduction. L-Sorbose was also available as the substrate but the reaction rate was less than

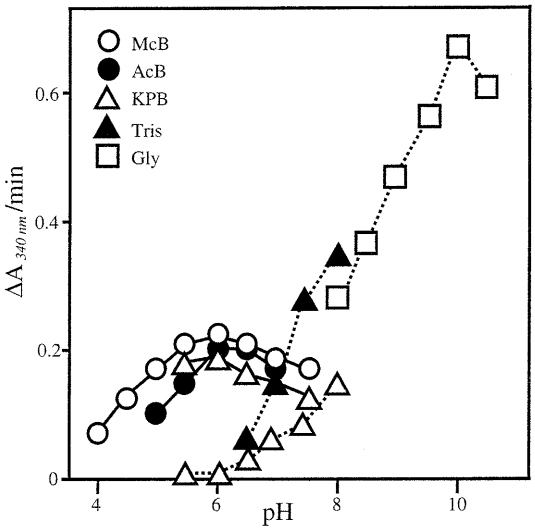

Fig. 4. Effects of pH on SLDH from *G. suboxydans* IFO 3257.
Enzyme activity (0.4 μg of protein for one assay) was measured with different pHs of buffer solution as indicated. McB, McIlvaine buffer; AcB, acetate buffer; KPB, potassium phosphate buffer; Gly, glycine-NaOH buffer; Tris, Tris-HCl buffer. The enzyme reaction in D-sorbitol oxidation is expressed by dotted lines and D-fructose reduction is by solid lines.

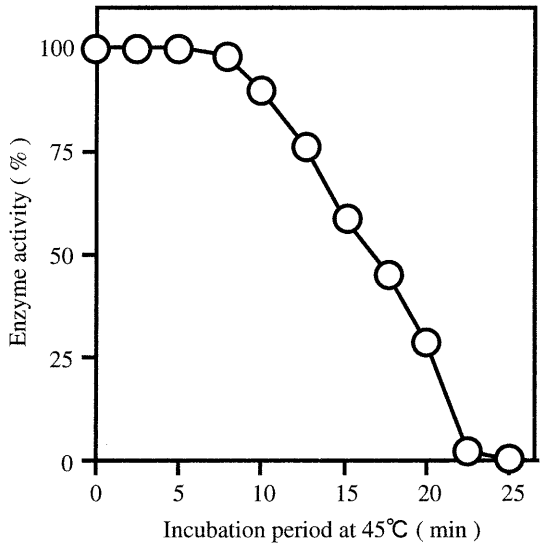

Fig. 5. Thermal Denaturation of SLDH from *G. suboxydans* IFO 3257.
A diluted SLDH (0.8 μg protein in 10 μl) was heated at 45°C for different periods as indicated. The remaining enzyme activity was measured under the standar assay conditions.

3% of that of D-fructose. A strong L-sorbose reductase that catalyzes D-sorbitol formation in the presence of NADPH occurs in the same cytosolic fraction and it is now under purification (O. Adachi *et al.*, unpublished observations). Since L-sorbose reduction to D-sorbitol by L-sorbose reductase is highly specific to L-sorbose reductase, reduction of L-sorbose to D-sorbitol by SLDH observed here may be observed only *in vitro*. No ketohexose, aldohexose, ketopentose, or aldopentose was found to be the substrate when checked with various substances such as D-psicose, D-tagatose, D-galactose, D-mannose, D-ribulose, D-ribose, and D-xylose. Apparent K_m values for D-sorbitol, NAD, D-fructose, and NADH were 5 mM, 2.1×10^{-4} M, 20 mM, and 2.8×10^{-4} M, respectively. The effects of other hexoses or pentoses on the reaction rate of D-sorbitol oxidation were examined with the crystalline enzyme to see whether any disturbance in D-sorbitol oxidation by such the compounds occurs. When D-glucose, D-fructose, D-mannitol, and D-xylose were present more than 100 times higher than the concentration of D-sorbitol in the reaction mixture, no great disturbance in D-sorbitol oxidation was observed. These data strongly support the idea that SLDH prepared in this study can be available for the enzymatic measurement of D-sorbitol even in the presence of other sugars or sugar alcohols. In fact, D-sorbitol in commercial apple juices and apple vinegars was measured satisfactorily by SLDH. However, the accuracy by SLDH assay was unclear, because an absolute content of D-sorbitol in most samples is usually not disclosed. Microbial preparation of D-fructose from D-sorbitol would be closed up to be a new topic in biotechnology in the future to produce a high fructose syrup by a one-step enzymatic method as reported by Ueda *et al.*,[33] which cannot be done by the glucose isomerase method already established. SLDH prepared in this study would be the most suitable enzyme to assay both the product, D-fructose, and the substrate, D-sorbitol, remaining in the reaction mixture, because the enzyme activity of SLDH crystallized in this study was more active than that crystallized from *B. fructosus*.[22]

SLDH from *G. suboxydans* was stable for standing at 40°C for 30 min, as used for a criterion of thermal stability of SLDH from *Pseudomonas* sp.[21] The enzyme became labile when heated at 45°C for more than 10 min as shown in Fig. 5 and completely denatured after 20 min. No protective effect on SLDH by substrates or substrate analogs against heat denaturation was observed and heating SLDH for 5 min at 50°C denatured it. It is quite interesting to compare the thermal stability with a similar enzyme from *G. suboxydans*. As reported previously,[9] the NADP-dependent D-mannitol dehydrogenase from *G. suboxydans*, that is common as D-fructose reductase to SLDH, was stable against heating at 50°C for 30 min. SLDH was stable for several days in a refrigerator on standing in buffer solutions of pH 4.5–11. When D-sorbitol oxidation was compared in 50 mM solution of different buffers at pH 8.0, the reaction rate declined by the order of Tris-HCl, glycine-NaOH, and potassium phosphate. Potassium phosphate was three times as repressive in D-sorbitol oxidation than Tris-HCl (Fig. 4).

It is interesting to see that the substrate specificity of SLDH differs enzyme by enzyme from different sources. SLDH from human brain shows an almost equal reaction rate in D-sorbitol oxidation and D-fructose reduction.[17] D-Sorbitol reduction around pH 6.0 is much higher than D-sorbitol oxidation around pH 9.0 with the enzyme from rat liver.[16] Judging from the kinetic constants, the enzyme from apple callus contributes to D-

Table 2. Comparison of Properties among Sugar Alcohol Dehydrogenases from *Gluconobacter suboxydans*

	NADP-MDH[a)]	NAD-MDH[b)]	SLDH[c)]
Molecular mass	50 kDa	130 kDa	98 kDa
Subunit	50 kDa	nd*	26 kDa
Numbers of subunits	1	nd	4
Svedverg units	3.6*s*	nd	5.1*s*
Substrate			
	D-mannitol	D-mannitol	D-sorbitol
	D-fructose	D-fructose	D-fructose
K_m for			
D-Mannitol	10 mM	20 mM	—
D-Sorbitol	—	—	5 mM
D-Fructose	12 mM	33 mM	20 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	—	—
NADPH	1.9×10^{-5} M	—	—
V_{max} for			
D-Mannitol	221 μmol/mg	150 μmol/mg	—
D-Sorbitol	—	—	136 μmol/mg
D-Fructose	235 μmol/mg	188 μmol/mg	34 μmol/mg
Optimum pH			
D-Mannitol	9.0	9.0	—
D-Sorbitol	—	—	9.0–11.0
D-Fructose	6.5–7.5	6.0	5.0–6.0

a) NADP-Dependent D-mannitol dehydrogenase from *G. suboxydans*.[9)]
b) NAD-Dependent D-mannitol dehydrogenase from *G. suboxydans*.[9)]
c) NAD-Dependent D-sorbitol dehydrogenase from *G. suboxydans* (this study).
nd*: not determined.

sorbitol oxidation to yield D-fructose.[14)] The enzymes from *Pseudomonas* sp.[21)] and *B. fructosus*[22)] oxidize D-sorbitol to D-fructose. Different from the cases of NAD(P)-dependent D-mannitol dehydrogenase,[9)] it is easier to deduce the physiological role for SLDH from *G. suboxydans* in this study as an oxidant of D-sorbitol to D-fructose in the cytoplasmic fraction. On the other hand, the organism produces L-sorbose from D-sorbitol outside the cell. A metabolic route, in which L-sorbose can be reduced to D-sorbitol by NADP-dependent L-sorbose reductase and then to D-fructose by SLDH can be assumed in the organism. It is said that TCA cycle is incomplete or lacking in the genus *Gluconobacter*, especially in the *Suboxydans* group, while the genus *Acetobacter* has a complete TCA cycle.[34)] Therefore, a strong ketogenic activity must support the growth of the genus *Gluconobacter*, in which the enzymes in the pentose phosphate pathway predominate in the cytoplasmic carbohydrate metabolism.

Finally, the physicochemical and catalytic properties of SLDH were compared with other similar enzymes from *G. suboxydans*, in which D-fructose reductase activity assayed *in vitro* is common to all of them (Table 2). The two crystalline enzymes in Table 2 must be a strong clue to disclose the enzymatic basis by X-ray crystallographic criteria of the Bertrand-Hudson rule in sugar alcohol oxidation. NADP-Dependent D-mannitol dehydrogenase fits the rule, while SLDH does not.

Acknowledgments

We wish to express our sincere thanks to Dr. M. Ameyama, Prof. Emeritus of Yamaguchi University, for his encouragement throughout this work. Photopicture processings of crystalline SLDH were kindly contributed by Dr. S. Tanaka of our faculty. Many valuable suggestions given by Dr. T. Uwajima, Novo Nordisk Bioindustry, Co. Ltd., should be mentioned. 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) 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).
2) Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., and Ameyama, M., Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **44**, 503–515 (1980).
3) Ameyama, M., Osada, K., Shinagawa, E., Matsushita, K., and Adachi, O., Purification and characterization of aldehyde dehydrogenase of *Acetobacter aceti*. *Agric. Biol. Chem.*, **45**, 1889–1890 (1981).
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) Ameyama, M., Shinagawa, E., Matsushita, K., and Adachi, O., D-Glucose dehydrogenase of *Gluconobacter suboxydans*. Solubilization, purification and characterization. *Agric. Biol. Chem.*, **45**, 851–861 (1981).
6) Adachi, O., Matsushita, K., Shinagawa, E., and Ameyama, M.,

Crystallization and characterization of NADP-dependent D-glucose dehydrogenase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **44**, 301–308 (1980).
7) Ameyama, M., Shinagawa, E., Matsushita, K., and Adachi, O., D-Fructose dehydrogenase of *Gluconobacter industrius*: Purification, characterization, and application to enzymatic microdetermination of D-fructose. *J. Bacteriol.*, **145**, 814–823 (1981).
8) 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).
9) 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).
10) Cho, N. C., Kim, K., and Jhon, D-Y., Purification and characterization of polyol dehydrogenase from *Gluconobacter melanogenus*. *Korean Biochem. J.*, **23**, 172–178 (1990).
11) Oikawa, T., Nakai, J., Tsukagawa, Y., and Soda, K., A novel type of D-mannitol dehydrogenase from *Acetobacter xylinum*: Occurrence, purification, and basic properties. *Biosci. Biotechnol. Biochem.*, **61**, 1778–1782 (1997).
12) 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).
13) 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).
14) Negm, F. B. and Loescher, W. H., Detection and characterization of sorbitol dehydrogenase from apple callus tissue. *Plant Physiol.*, **64**, 69–73 (1979).
15) Smith, M. G., Polyol dehydrogenases. 4. Crystallization of the L-iditol dehydrogenase of sheep liver. *Biochem. J.*, **83**, 135–144 (1962).
16) Leissing, N. and McGuinness, E. T., Rapid affinity purification and properties of rat liver sorbitol dehydrogenase. *Biochim. Biophys. Acta*, **524**, 254–261 (1978).
17) O'Brien, M. M., Schofield, P. J., and Edwards, M. R., Polyol-pathway enzymes of human brain. Partial purification and properties of sorbitol dehydrogenase. *Biochem. J.*, **211**, 81–90 (1983).
18) Horwitz, S. B. and Kaplan, N. O., Hexitol dehydrogenases of *Bacillus subtilis*. *J. Biol. Chem.*, **239**, 830–838 (1964).
19) Ng, K., Ye, R., Wu, X.-C., and Wong, S.-L., Sorbitol dehydrogenase from *Bacillus subtilis*. *J. Biol. Chem.*, **267**, 24989–24994 (1992).
20) Schneider, K. and Giffhorn, F., Sorbitol dehydrogenase from *Pseudomonas* sp.: Purification, characterization and application to quantitative determination of sorbitol. *Enzyme Microb. Technol.*, **13**, 332–337 (1991).
21) Minamihara, T. and Suzuki, M., Properties of sorbitol dehydrogenase from *Pseudomonas* sp. KS-E1806 and comparison with other sorbitol dehydrogenases. *J. Ferment. Bioeng.*, **84**, 254–256 (1997).
22) Uwajima, T., Purification and properties of NAD-dependent sorbitol dehydrogenase from *Bacillus fructosus*. *Biosci. Biotechnol. Biochem.*, **63**, 573–574 (1999).
23) Arcus, A. C. and Edson, N. L., Polyol dehydrogenase. 2. The polyol dehydrogenases of *Acetobacter suboxydans* and *Candida utilis*. *Biochem. J.*, **64**, 385–394 (1956).
24) Kersters, K., Wood, W. A., and De Ley, J., Polyol dehydrogenases of *Gluconobacter oxydans*. *J. Biol. Chem.*, **240**, 965–974 (1965).
25) Davis, B. J., Disc electrophoresis. II. Methods of application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**, 404–427 (1964).
26) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London), **227**, 680–685 (1970).
27) 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).
28) 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).
29) Ameyama, M., Matsushita, K., Shinagawa, E., and Adachi, O., 5-Keto-D-fructose reductase of *Gluconobacter industrius*. *Agric. Biol. Chem.*, **45**, 863–869 (1981).
30) Adachi, O., Osada, K., Matsushita, K., Shinagawa, E., and Ameyama, M., Purification, crystallization and properties of 6-phospho-D-gluconate dehydrogenase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **46**, 391–398 (1982).
31) Adachi, O., Chiyonobu, T., Shinagawa, E., Matsushita, K., and Ameyama, M., Crystalline 2-keto-gluconate reductase from *Acetobacter ascendens*, the second instance of crystalline enzyme in genus *Acetobacter*. *Agric. Biol. Chem.*, **42**, 2057–2062 (1978).
32) Adachi, O., Shinagawa, E., Matsushita, K., and Ameyama, M., Crystallization and properties of 5-keto-D-gluconate reductase from *Gluconobacter suboxydans*. *Agric. Biol. Chem.*, **43**, 75–83 (1979).
33) Ueda, K., Higashi, S., and Origuchi, K., Studies on production of D-fructose from D-sorbitol by fermentation. (Part II) Isolation of fructose-producing bacteria. *J. Ferment. Tech.*, **45**, 541–549 (1967).
34) Asai, T., Biochemical activities of acetic acid bacteria. In "Acetic Acid Bacteria", ed. Asai, T., Univ. of Tokyo Press, Tokyo, pp. 9–102, and pp. 105–314 (1968).