Purification and Properties of NADPH-Linked L-Sorbose Reductase from *Gluconobacter melanogenus* N44-1

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NADPH-Linked L-sorbose reductase from the cytosol fraction of *Gluconobacter melanogenus* N44-1, which is a 2-keto-L-gulonic acid producer from L-sorbose or D-sorbitol, was purified about 500-fold by DEAE-Cellulose, Blue Sepharose, and DEAE-Sepharose column chromatographies. The purified enzyme was entirely homogeneous on polyacrylamide gel and SDS gel electrophoresis. NADPH and NADP were specifically required for the reduction and oxidation of the substrate, respectively. The apparent molecular weight was 60,000 by Sephadex G-200 column chromatography, and that of its subunit was 60,000 by SDS-gel electrophoresis. The pH optimum for the reduction of L-sorbose and D-fructose was 7.0. The pH optimum for the oxidation of D-sorbitol to L-sorbose and D-mannitol to D-fructose was 10.0–10.5.

As previously reported,¹⁾ we have developed a fermentation process that produces 2-keto-L-gulonic acid (2KGA) from L-sorbose and D-sorbitol via L-sorbose by a mutant derived from *G. melanogenus* IFO 3293, and isolated the two key enzymes of membrane-bound L-sorbose dehydrogenase²⁾ and NAD(P)-dependent L-sorbose dehydrogenase³⁾ found through the study of the metabolic pathway for 2KGA formation.⁴⁾

When *G. melanogenus* UV10 and its mutants¹⁾ were used in 2KGA-fermentation process, a substantial amount of L-sorbose fed to the medium was consumed to yield carbon dioxide without contributing to 2KGA formation. The CO₂ evolved from L-sorbose by the 2KGA high producer was mainly via the pentose phosphate pathway.⁵⁾ This shows that L-sorbose is metabolized to D-sorbitol besides the oxidation of L-sorbose to L-sorbose for the main pathway to produce 2KGA. In addition, we showed⁴⁾ D-sorbitol was produced from L-sorbose by the enzyme contained in the cytosol fraction of 2KGA producers. If we could obtain a mutant such that the reducing activity of L-sorbose to D-sorbitol was

decreased, further improvement of 2KGA could be expected.

The enzyme reducing L-sorbose (we say L-sorbose reductase) is found in various *Gluconobacters* as an NADP-linked enzyme catalyzing the L-sorbose-D-sorbitol conversion, but the enzyme has not been isolated.

As the characterization of the enzyme reducing L-sorbose in a high 2KGA producer (*G. melanogenus* N44-1) was thought to be advantageous to the further improvement of 2KGA yield, we tried to isolate the L-sorbose reductase.

This report deals with the purification and characterization of NADPH-linked L-sorbose reductase isolated from the cytosol fraction of *G. melanogenus* N44-1 during study of the mechanism of 2KGA formation from L-sorbose and D-sorbitol (Fig. 1).⁴⁾

Materials and Methods

Chemicals. NADP and reduced NADP were purchased from Oriental Yeast Ind., Co.; D-sorbitol, D-fructose, and D-mannitol, from Nakarai Chemicals, Ltd. 5-Keto-fructose was purchased from Chemical Dynamics Co.; L-Sorbose,

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section. Products were identified by gas chromatography, high performance liquid chromatography, and thin layer chromatography.¹⁾

Results

1. Enzyme purification

All operations were done at 4°C to 8°C, unless otherwise stated. The buffer mentioned in all steps is 10 mM potassium phosphate (pH 7.0).

Step 1: Preparation of cytosol fraction. The frozen cells (20 g) stored at -20°C were thawed and suspended in 80 ml of buffer. The cells were disrupted in the presence of glass beads (0.1 mm diameter) with a Dyno Mill homogenizer (Willy, A Bachofen Co., Basle) at 2,000 rpm for 4 min. The cells, debris, and glass beads were removed by centrifugation at $1,800 \times g$ for 10 min. The supernatant was then centrifuged at $100,000 \times g$ for 60 min. The resulting supernatant was dialyzed against 3 liters of the same buffer (changed twice) for 18 hr and is referred to here as the cytosol fraction (65 ml).

Step 2: DEAE-Cellulose column chromatography. The dialyzed enzyme solution (60 ml) was put on a DEAE-Cellulose DE-52 column (2.5×20 cm), which had been equilibrated with the same buffer. An NaCl linear gradient (0 to 0.2 M) was added and the enzyme activity was found in the fractions eluted with about 0.075 M NaCl.

The combined active fractions (about 60 ml) were concentrated to 18 ml with a membrane filter (Amicon PM-10) and dialyzed against the buffer for 5 hr.

Step 3: Blue-Sepharose column chromatography. The dialyzed enzyme solution (20 ml) was passed through a Blue-Sepharose CL-6B column (1.5×15 cm), which had been equilibrated with the buffer.

Step 4: DEAE-Sepharose column chromatography. The active fractions from Step 3 were combined and put on a DEAE-Sepharose CL-6B column (1.0×50 cm), which had been equilibrated and washed with the buffer. A linear gradient of NaCl (0–0.15 M) was added

to the buffer and the enzyme was eluted at around 0.1 M NaCl. The specific activity of peak fractions (No. 76–78) were almost the same and the fractions were stored separately at -20°C.

Purification of the enzyme, summarized in Table I, was approximately 520-fold with a yield of 23.9% if the crude enzyme was taken to be 100%.

2. Properties of purified enzyme

To investigate the catalytic properties of L-sorbose reductase, the purified enzyme, with a specific activity of 140.8, was used under the

Table I. PURIFICATION OF L-SORBOSE REDUCTASE

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Yield (%)
Crude enzyme (Cytosol fraction)	282	1,032	0.27	100
DEAE-Cellulose	150.5	31.2	4.83	53.4
Blue-Sepharose	114.8	4.1	28.0	40.7
DEAE-Sepharose	67.6	0.48	140.8	23.9

Fig. 2. Polyacrylamide Gel Electrophoresis of L-Sorbose Reductase.

standard assay conditions unless otherwise stated.

Acrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis of the purified enzyme obtained from Step 4 is shown in Fig. 2. A single protein band was found, indicating the electrophoretic homogeneity of the preparation.

Molecular weight. The purified L-sorbose reductase was put on a Sephadex G-200 column (10 × 500 mm) chromatography and developed with 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The molecular weight of the enzyme was estimated to be $58,000 \pm 1,000$, but about 22% of the activity

was lost in Sephadex G-200 column chromatography.

The molecular weight of the subunit was estimated by 10% (w/v) SDS-polyacrylamide gel electrophoresis. The enzyme preparation gave a single protein band with a molecular weight of $60,000 \pm 1,000$ (Fig. 3). This indicates that L-sorbose reductase from *G. melanogenus* is monomeric.

Effects of pH on activity and stability of the enzyme. The enzyme activity for oxidation and reduction reactions was measured at various pHs. The pH optimum of the reduction of L-sorbose was about 7.0 and that of the oxidation of D-sorbitol was about 10 (Fig. 4).

The enzyme was stable at pH 6.5–7.5 on incubation at 30°C for 30 min and was stored with little loss of activity at –20°C for a month or more in 0.01 M potassium phosphate buffer, pH 7.0.

Effects of temperature on stability and activity of the enzyme. The effects of temperature on the stability and activity of the enzyme were investigated. Thermostability of the enzyme was tested by a 10-min incubation of the enzyme at various temperatures and then its activity to reduce L-sorbose was tested. The enzyme was stable at up to 50°C–55°C, but

Fig. 3. SDS-Gel Electrophoresis of the Purified NADPH-Linked L-Sorbose Reductase.

Purified L-sorbose reductase with a specific activity of 140.8 was used and 3.2 μ g of protein was put on. The direction of electrophoresis was left to right. After the protein staining with Coomassie brilliant blue R-250 in 7% acetic acid, the molecular weight of L-sorbose reductase was measured by the plotting mobility versus molecular weight of the standard marker proteins. The position of L-sorbose reductase is marked by a closed circle. The standard marker proteins are indicated alphabetically. a, phosphorylase, mol. wt. 92,500; b, bovine serum albumin, mol. wt. 66,200; c, ovalbumin, mol. wt. 45,000; d, carbonic anhydrase, mol. wt. 31,000; e, soybean trypsin inhibitor, mol. wt. 21,500; f, lysozyme, mol. wt. 14,400.

Fig. 4. Effects of pH on Enzyme Activity.

The purified enzyme was used and the activity was measured under the standard assay conditions using the following buffers: Reduction of L-sorbose: —●—, 0.1 M McIlvaine buffer; —▲—, 0.1 M phosphate buffer; —■—, 0.2 M Tris buffer. Oxidation of D-sorbitol: —○—, 0.1 M McIlvaine buffer; —□—, 0.2 M Tris buffer; —▽—, 0.1 M $\text{NH}_4\text{OH-NH}_4\text{Cl}$ buffer; —☆—, 0.2 M $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer.

rapid inactivation occurred when the enzyme was treated at over 60°C. The highest activity was found at 30°C.

Inhibitors. The effects of various metals and reagents on L-sorbose reductase activity were studied. The activity was inhibited by Cu^{2+} , Fe^{2+} , and Ni^{2+} and completely inhibited by

Table II. EFFECTS OF INHIBITORS ON THE ACTIVITY OF L-SORBOSE REDUCTASE

Inhibitor	Concentration (mM)	Relative activity ^a (%)
EDTA	0.97	98
Quinine	0.49	0
	0.97	0
N-Ethylmaleimide	0.97	81
Sodium azide	0.97	90
Monoiodoacetate	0.97	96
PCMB ^b	0.19	43
$\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$	0.97	90
Sodium fluoroacetate	0.97	103
Sodium fluoride	0.97	103
KCN	0.19	101
	0.38	102

^a Relative activity is expressed relative to that without the inhibitor.

^b PCMB, *p*-chloromercuribenzoic acid.

Table III. SUBSTRATE SPECIFICITY OF PURIFIED L-SORBOSE REDUCTASE

Substrate	Relative activity ^a (%)	
	NADPH (pH 7.0)	NADP (pH 10.0)
Sorbose	(100)	0
Glucose	0	0
Mannitol	0	134
Sorbitol	0	134
Fructose	142	0
Glycerol	0	0
Adonitol	0	0
Erythritol	0	65.3
Na-Gluconate	0	— ^b
Ca-Idonate	0	—
Xylose	0	—
Xylulose	25.7	—
Ribulose	2.4	—
Tagatose	0.8	—
5-Keto-D-fructose	86.8	0

^a Each reaction rate with substrate is expressed as per cent of sorbose.

^b Not tested.

0.49 mM quinine (Table II).

Substrate specificity. The relative activities of the purified enzyme for various substrates were surveyed and are listed in Table III. L-Sorbose reductase from *G. melanogenus* N44-1 had a high rate of activity to reduce L-sorbose to D-sorbitol and to oxidize D-sorbitol to L-sorbose in the presence of NADPH, but NADH did not work as a cofactor for this reaction. In addition, D-fructose, 5-keto-D-fructose, D-xylulose, D-ribulose, and D-tagatose were found to be substrates for L-sorbose reductase. This enzyme also oxidized D-mannitol and erythritol.

Identification of reduction and oxidation products. The reaction mixture (1 ml) containing 50 mM each of L-sorbose, D-fructose and 5-keto-D-fructose, and 0.28 mM of NADPH was incubated for 30 min in pH 7.0 buffer (100 mM potassium phosphate) at 30°C with about 4.4 units of the purified enzyme. On the other hand, the reaction mixture (1 ml) consisting of 40 mM each of D-sorbitol and D-mannitol and 0.28 mM of NADP was incubated for 30 min in pH 10.0 buffer (100 mM Na_2CO_3 – NaHCO_3) at 30°C with about 4.4 units of the purified enzyme. After the reaction, as described in Materials and Methods, the products were identified TLC and GLC (data not shown). L-Sorbose, D-fructose, and 5-keto-D-fructose were reduced to D-sorbitol, D-mannitol, and D-fructose, respectively, in the presence of NADPH. On the other hand, D-sorbitol and D-mannitol were oxidized to

Table IV. APPARENT MICHAELIS CONSTANTS FOR VARIOUS SUBSTRATES

Substrate	pH	K_m (M)
L-Sorbose ^a	7.0	3.28×10^{-1}
D-Fructose ^a	7.0	1.60×10^{-1}
D-Sorbitol ^a	10.0	1.67×10^{-1}
D-Mannitol ^a	10.0	7.30×10^{-3}
NADPH ^b	7.0	1.11×10^{-4}

^a NADPH was used as cofactor for L-sorbose and D-fructose, and NADP for D-sorbitol and D-mannitol.

^b The assay was done in the presence of 490 mM L-sorbose.

L-sorbose and D-fructose respectively in the presence of NADP. However, the oxidation of D-fructose was not observed in the presence of NADP.

Effects of substrate concentration on reaction rate. The effects of substrate concentration on the enzyme activity were tested. Apparent Michaelis constants for various substrates were calculated⁹⁾ and are summarized in Table IV. It was found that the affinity of the enzyme in the presence of NADP was highest for D-mannitol among all the carbohydrates tested.

Discussion

We purified and characterized the NADPH-linked L-sorbose reductase of *G. melanogenus* N44-1, which was one of the reductases functioning in the metabolic pathway of 2KGA production from D-sorbitol or L-sorbose (Fig. 1). This enzyme was apparently different from other reductases such as L-sorbose and 2KGA reductase because it was not active in the reduction of L-sorbose and 2KGA. The L-sorbose reductase with regards to its function can also be called an oxidoreductase because it catalyzes not only the reduction of L-sorbose and D-fructose in the presence of NADPH but also the oxidation of D-sorbitol and D-mannitol in the presence of NADP as illustrated below:

Optimum pHs were around 10.0 for the forward reaction and 7.0 for the reverse reaction.

Here we report a purification scheme of NADP-linked L-sorbose reductase from *G. melanogenus*, an enzyme active in the catalysis of D-sorbitol and D-mannitol. Tsukada and Perlman¹⁰⁾ have reported that the cell-free extract of *G. melanogenus* IFO 3293 have activity to convert L-sorbose to D-sorbitol and *vice versa* in the presence of NADP(H); they postulated that the enzyme is a coenzyme-dependent dehydrogenase and that the reaction favors the conversion from L-sorbose to D-sorbitol. According to Widmer¹¹⁾ and

Cummins *et al.*,^{12,13)} three enzymes for the oxidation of D-sorbitol were found in the cell-free extract of *A. suboxydans* (syn. *G. suboxydans*). In addition to a dehydrogenase in the membrane fraction,^{14,15)} there were 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. However, the NADP-linked D-sorbitol dehydrogenase has not been purified because of its instability. Shaw¹⁶⁾ and Kersters *et al.*,¹⁷⁾ have reported on NADP-linked D-mannitol dehydrogenase activity in cell extracts of *A. suboxydans* TACC621 (syn. *G. suboxydans*) and *G. suboxydans* strain SU, respectively. Yamada *et al.*,¹⁸⁾ reported the purification and properties of NADP-linked 5-keto-D-fructose reductase of *G. albidus* IFO 3250, but the substrate specificity of the enzyme was significantly different from ours, namely 5-keto-D-fructose reductase is not active in the catalysis of L-sorbose.

Comparing the catalytic properties of the enzymes described above with ours, we suggest that our L-sorbose reductase is in fact the same as NADP-linked D-sorbitol dehydrogenase and D-mannitol dehydrogenase as described by Widmer, Cummins, Shaw, and Kersters *et al.*

In the metabolic pathway for the production of 2KGA, our enzyme was thought to function mainly as a reductase *in vivo* during the fermentation of 2KGA, which means that the conversion of L-sorbose to D-sorbitol is the main function of the enzyme as described by Tsukada and Perlman¹⁰⁾ because the reductive activity of the enzyme was higher than the oxidative activity at the pH between 5 and 7 as shown in Fig. 4, which was the pH range suitable for cell growth and 2KGA production as described in a previous paper,¹⁾ and in addition, the intracellular pH of the acetic acid bacteria is thought to be around 7.0. The products formed from L-sorbose by the reductive function of the enzyme are D-sorbitol and NADP.

In the 2KGA fermentation from L-sorbose or D-sorbitol, our mutants of *G. melanogenus*, as previously reported,¹⁾ about 40% of both

substrates added was wasted without contributing to 2KGA formation. L-Sorbose was considered to be dissimilated finally to CO₂ via D-sorbitol and then via the pentose cycle. In fact, a blocked mutant of L-sorbose reductase could not grow on L-sorbose as a sole carbon source. In this context, the L-sorbose reductase seems to be the first-step enzyme for the dissimilation of L-sorbose in our microorganisms.

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References

- 1) T. Sugisawa, T. Hoshino, S. Masuda, S. Nomura, Y. Setoguchi, M. Tazoe, M. Shinjoh, S. Someha and A. Fujiwara, *Agric. Biol. Chem.*, **54**, 1201 (1990).
- 2) T. Sugisawa, T. Hoshino and A. Fujiwara, *Agric. Biol. Chem.*, **55**, 363 (1991).
- 3) T. Hoshino, T. Sugisawa and A. Fujiwara, *Agric. Biol. Chem.*, **55**, 665 (1991).
- 4) T. Hoshino, T. Sugisawa, M. Tazoe, M. Shinjoh and A. Fujiwara, *Agric. Biol. Chem.*, **54**, 1211 (1990).
- 5) M. Shinjoh, Y. Setoguchi, T. Hoshino and A. Fujiwara, *Agric. Biol. Chem.*, **54**, 2257 (1990).
- 6) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
- 7) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
- 8) K. Weber and D. J. Kuter, *J. Biol. Chem.*, **246**, 4504 (1971).
- 9) H. Lineweaver and D. Bruk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
- 10) Y. Tsukada and D. Perlman, *Biotech. and Bioeng.*, **14**, 799 (1972).
- 11) C. Widmer, T. E. King and V. H. Cheldelin, *J. Bacteriol.*, **71**, 737 (1956).
- 12) J. T. Cummins, T. E. King and V. H. Cheldelin, *J. Biol. Chem.*, **224**, 323 (1957).
- 13) J. T. Cummins, V. H. Cheldelin and T. E. King, *J. Biol. Chem.*, **226**, 301 (1957).
- 14) E. Shinagawa, K. Matsushita, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **46**, 135 (1982).
- 15) C. A. Baker and G. W. Claus, *FEMS Microbiol. Lett.*, **18**, 123 (1983).
- 16) D. R. D. Shaw, *Biochim. Biophys. Acta*, **113**, 608 (1966).
- 17) K. Kersters, W. A. Wood and J. De Ley, *J. Biol. Chem.*, **240**, 965 (1965).
- 18) Y. Yamada, K. Aida and T. Uemura, *J. Biochem.*, **61**, 803 (1967).