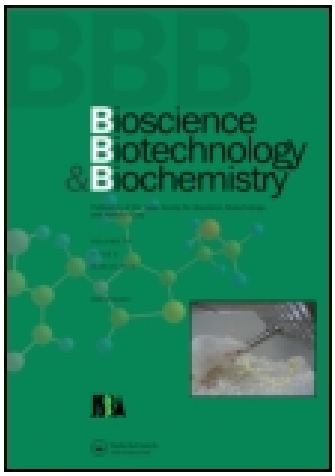

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 12 Jun 2014.

To cite this article: Kazumi Hiraga, Mitsunori Kitazawa, Norihisa Kaneko & Kohei Oda (1997) Isolation and Some Properties of Sorbitol Oxidase from Stveptomyces sp. H-7775, Bioscience, Biotechnology, and Biochemistry, 61:10, 1699-1704, DOI: 10.1271/bbb.61.1699

To link to this article: http://dx.doi.org/10.1271/bbb.61.1699

Biosci. Biotech. Biochem., **61** (10), 1699–1704, 1997

Isolation and Some Properties of Sorbitol Oxidase from *Streptomyces* sp. H-7775

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Received April 15, 1997

A sorbitol oxidase (SOX) was found in the cell-free extract of a strain isolated from soil. The strain was classified and designated as *Streptomyces* sp. H-7775. SOX is constitutively expressed in the cell. The molecular weight of SOX that purified from the cell-free extract was 45,000. The optimum pH and the K_m for sorbitol were 6.5–7.5 and 0.26 mM, respectively. The prosthetic group was a covalently bound FAD. SOX catalyzed oxidation of D-sorbitol to glucose and hydrogen peroxide without any requirements of exogenous cofactors. SOX did not react with glucose, a reaction product of D-sorbitol. This feature is useful in its application for diagnosis.

Key words: sorbitol oxidase; sorbitol; oxidase; *Streptomyces* sp.

Various pathways for the use of sorbitol have been described. Sorbitol dehydrogenase (SDH; EC 1.1.1.14) is an enzyme of the polyol pathway in a wide variety of species.[1–3] SDH acts on polyols such as D-sorbitol and D-xylitol but having no activity towards primary alcohols. SDH catalyzes the oxidation of D-sorbitol to form D-fructose with NAD^+ as a cofactor.

In the course of screening of glycerol oxidase-producing microorganisms, we found an enzyme that oxidized D-sorbitol in the cell-free extract of a strain isolated from soil. From a preliminary study using the purified enzyme, we detected an oxidase activity that catalyzed the oxidation of D-sorbitol to form glucose and hydrogen peroxide without any requirements of exogenous cofactors. Apparently, this type of sorbitol-oxidizing enzyme was thought to have different reaction mechanisms from the SDHs described above. Hence, we tentatively named the enzyme "sorbitol oxidase" and started the following experiments.

In this paper, we describe the isolation and some properties of sorbitol oxidase from *Streptomyces* sp. H-7775.

Materials and Methods

Materials. Peroxidase, catalase, 4-aminoantipyrine, and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) were purchased from Sigma Chemical Co., St. Louis, U.S.A. Hydrogen peroxide was purchased from Wako Pure Chemical Industries, Ltd. All other reagents were of the highest grade available from commercial sources.

Media. The medium used for production of sorbitol oxidase was Waksman medium, consisting of 1.0% D-glucose, 0.5% polypeptone, 0.5% meat extract, and 0.3% NaCl, pH 7.0.

Screening. 3000 microorganisms isolated from soils were inoculated into 5 ml of Waksman medium. They were cultured with shaking at 110 strokes/min for 3 days at 30°C. The cells were collected by centrifugation at $3000 \times g$ for 20 min, washed with distilled water, and suspended in 0.5 ml of 150 mM KH_2PO_4–KOH, pH 7.0, containing 1 mg/ml of lysozyme. The suspensions were incubated for 1 h at 37°C. The cell-free extracts were obtained by centrifugation at $10,000 \times g$ for 20 min. The glycerol- and sorbitol-oxidase activities in the cell-free extracts were measured under standard assay conditions using 47.3 mM glycerol and sorbitol, respectively.

Taxonomic analysis. The morphological and physiological characteristics of the isolated strain were analyzed by Bergey's Manual of Systematic Bacteriology.[4]

Measurement of sorbitol oxidase activity. The standard enzyme assay was based on the measurement of hydrogen peroxide generated during the oxidation of sorbitol. The hydrogen peroxide oxidatively couples with 4-aminoantipyrine and TOOS in the presence of peroxidase to form a quinoneimine dye, by the method of Allain *et al.*[5] The amount of quinoneimine dye ($\varepsilon_{555} = 3.92 \times 10^4\ M^{-1}\ cm^{-1}$) was measured spectrophotometrically at 555 nm. The reaction mixture in a final volume of 950 μl contained 150 mM KH_2PO_4–KOH, pH 7.0, 47.3 mM D-sorbitol, 614 μM TOOS, 158 μM 4-aminoantipyrine, 1.9 units/ml horseradish peroxidase, and a suitable amount of sorbitol oxidase. The incubation was done at 37°C for 10 min. The reaction was stopped by the addition of 2 ml of 0.5% SDS. Sorbitol oxidase activity was confirmed to be a linear function of both incubation time and protein concentration.

One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of H_2O_2 per min.

Measurement of protein concentration. Protein was measured by the method of Bradford[6] with bovine serum albumin as a standard.

Enzyme production. For a large scale fermentation, a seed culture of *Streptomyces* sp. H-7775 grown in 100 ml of Waksman medium was inoculated into a 30-liter jar fermentor containing 20 liters of the Waksman medium (2% v/v). Adecanol (0.05%) was also included in the medium as an antifoam. The cultivation was conducted at 30°C for 24 h in the fermentor at 350 rpm and 20 liters of air/min.

Purification of sorbitol oxidase.

Step 1. Preparation of cell-free extract. The mycelia from 20 liters of culture broth of *Streptomyces* sp. H-7775 were washed with distilled water and suspended in an equal volume of 25 mM sodium phosphate buffer, pH 7.0 (buffer A) containing 1 mM PMSF. The mycelial suspension was ultrasonicated (Astrason ultrasonic processor XL) for 10 min under cooling at 4°C. The mycelial debris was removed by centrifugation at $15,000 \times g$ for 15 min at 4°C.

Step 2. DEAE-Sepharose CL-6B column chromatography. The cell-free extract from step 1 was put on a DEAE-Sepharose CL-6B column (5×20 cm) previously equilibrated with buffer A. The column was washed with the same buffer. The sorbitol oxidase (SOX) was eluted with a linear 0–0.5 M NaCl gradient in buffer A and the eluate was collected in 25 ml fractions. SOX fractions were collected, then dialyzed against buffer A overnight at 4°C.

Step 3. Q-Sepharose HP column chromatography. The dialyzed sample from step 2 was put on a Q-Sepharose HP column (3.2×20 cm) previously equilibrated with buffer A. The column was washed with the same buffer.

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Abbreviations: IAA, monoiodoacetate; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline; NMR, nuclear magnetic resonance.

SOX was eluted with a linear 0–0.5 M NaCl gradient in buffer A and the eluate was collected in 15 ml fractions. SOX fractions were collected and ammonium sulfate was added at 80% saturation and the mixture was left overnight at 4°C.

Step 4. Sephadex G-75 column chromatography. After centrifugation, the precipitate was dissolved in buffer A. The enzyme solution was put on a Sephadex G-75 column (2.5 × 90 cm) previously equilibrated with buffer A and eluted with the same buffer. SOX fractions were pooled and dialyzed against buffer A overnight at 4°C.

Step 5. MonoQ FPLC. The enzyme solution was then applied to MonoQ column (0.5 × 5 cm) previously equilibrated with buffer A and eluted with a linear 0–0.5 M NaCl gradient. The enzyme fractions were pooled and dialyzed against buffer B (10 mM sodium phosphate buffer, pH 7.0) overnight at 4°C.

Step 6. Hydroxyapatite column chromatography. The enzyme solution was then put on a hydroxyapatite column (1.0 × 5 cm) previously equilibrated with buffer B and eluted with 50 mM sodium phosphate buffer, pH 7.0. The enzyme fractions were pooled and used as a purified enzyme for further analysis.

SDS- and native-polyacrylamide gel electrophoresis. SDS-PAGE was done in a slab gel (12.5% polyacrylamide gel) by the method of Laemmli.[7] Native-PAGE was carried out in slab gel (5 to 13% gradient polyacrylamide gel) by the method of Schagger *et al.*[8]

Analysis of the amino-terminal sequence. The amino-terminal sequence of the purified SOX was identified by the method of Matsudaira,[9] using an Applied Biosystems Model 476A protein sequencing system.

Spectrophotometric measurements. Absorption spectra of SOX were recorded with a Hitachi U-2001 spectrophotometer, and fluorescence spectra were measured with a Hitachi F2000 spectrofluorometer.

Effects of pH on SOX activity. SOX activity was measured at various pH values under standard assay condition using D-sorbitol as a substrate. The buffer used was 40 mM citrate-Na_2HPO_4 (pH 4.0–7.5), 40 mM Tris-HCl (pH 7.5–8.5), or 40 mM glycine-NaOH (pH 8.5–10.0).

Heat stability of SOX. SOX was incubated at various temperatures for 15 min in 40 mM Tris-HCl (pH 7.5) and the remaining activity was measured at 37°C.

Thin-layer chromatography. A silica gel 60F-254 plate (Merck, Germany) was soaked in 30 mM borate buffer (pH 9.0) and dried at 110°C for 60 min. Then samples were spotted on the plate. Thin-layer chromatography was done with: 1st dimension, ethyl acetate-pyridine-H_2O (3:3:2); 2nd dimension, 1-butanol-methanol-H_2O (5:3:2). The chromatogram was sprayed with a color reagent: 2% (w/v) diphenyl amine–2% (v/v) aniline–15% (v/v) phosphoric acid in acetone. The plate was heated at 110°C for 10 min.

Nuclear magnetic resonance (NMR) analysis. The products of the enzymatic reaction were identified by NMR spectroscopy. The reaction mixture contained 10 mM Tris-HCl (pH 7.5), 15 mM D-sorbitol (13.7 mg), and two units of the purified SOX in a total volume of 5 ml. After 2 h of incubation at 37°C, the reaction was stopped by boiling for 5 min. The reaction product was purified by Sephadex G-15 column chromatography and preparative TLC. About 0.5 mg of the reaction product was obtained and the sample was dissolved in ^2H_2O (0.5 ml, 99.96%, CEA) for NMR analysis. The 400 MHz ^{1}H-NMR spectra were recorded on the EX-400 spectrometer (JEOL) at ambient temperature. The internal reference was DSS (sodium 4,4-dimethyl-4-silapentansulfonate). To obtain the NMR data for the authentic sample, α-D-glucose was dissolved in ^2H_2O at 80°C and then left overnight for establishing the equilibrium between α- and β-anomers.

Results

Isolation and identification of a sorbitol oxidase-producing microorganism

In the course of screening for glycerol oxidase-producing strain, four positive strains were obtained. Among these strains, strong SOX activity was detected reproducibly in the cell-free extract of the isolated strain H-7775. Table I summarizes taxonomic studies of the strain H-7775. H-7775 had wall chemotype I and showed typical morphological and physiological properties of the genus *Streptomyces* according to Bergey's Manual of Systematic Bacteriology.[4] Accordingly, this strain was designated *Streptomyces* sp. H-7775.

Table I. Characteristics of H-7775 Strain

Morphological characteristics		
Fragmentation of substrate mycelium		−
Aerial hyphae with chains of arthrospores		+
Arthrospores in verticils		−
Spore surface		Smooth
Motility of spores		Immotile
Wall chemotype		Type I (LL-DAP* and Glycine)
Physiological characteristics		
Growth temperature		10–42°C
Melanin pigment production		−
Carbon source utilization	Positives:	L-Arabinose
		D-Glucose
		Raffinose
		D-Xylose
	Negatives:	Inositol
		D-Mannitol
		L-Rhamnose
		Sucrose

* LL-DAP, LL-diaminopimelic acid.
+, positive; −, negative.

Purification of SOX

A summary of a typical purification of sorbitol oxidase is shown in Table II. From the mycelia of 20 liters of culture broth, 1.5 mg of SOX was obtained with a yield of 12.1%. The purified enzyme had a yellow color and a sorbitol oxidase activity with a weak glycerol oxidase activity. SOX activity in the eluate of hydroxyapatite column chromatography showed the same chromatographic profile with glycerol oxidase activity (data not shown). In this step, the specific activity of SOX was reduced for some unknown reason.

SDS- and native-PAGE of SOX

The purified SOX had a single band in SDS- and native-PAGE (Fig. 1, (A) lane 2, and (B)). No other extra bands were visible in the gels. The molecular weight of the purified SOX was estimated to be 45,000 by both SDS-PAGE and Sephadex G-75 column chromatography (data not shown). The results showed that the enzyme was pure and a monomer. Before staining, the gels were soaked in 1% (v/v) acetate for 3 min, then a yellowish fluorescence was clearly observed under UV light at the position of the protein band in SDS- and native-PAGE.

Amino-terminal sequence of SOX

The amino-terminal sequence of the purified SOX was identified as MTPAEKNWAGNITF–. Screening of the DDBJ data base found no proteins with similar sequences.

Spectral properties of SOX

As shown in Figs. 2(A) and (B), the absorption spectrum of SOX purified from *Streptomyces* sp. H-7775 had a typical flavoprotein spectrum with the absorption maxima at 276,

Table II. Summary of Purification

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Cell free extract	6,790	260,000	200	0.0008	100
DEAE-Sepharose CL-6B	525	1,000	145	0.145	72.5
Q-Sepharose HP	141	160	77.8	0.486	38.9
Sephadex G-75	160	11.2	59.0	5.30	29.5
Mono Q FPLC	25.6	2.2	48.2	22.0	24.1
Hydroxyapatite	4.3	1.5	24.1	16.0	12.1

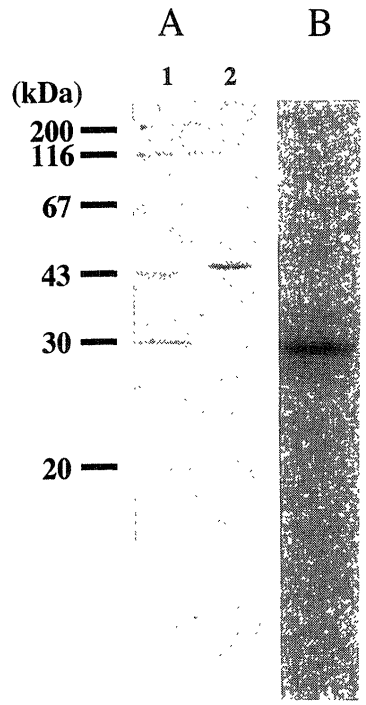

Fig. 1. SDS- and Native-PAGE of the Purified Sorbitol Oxidase.

(A) SDS-PAGE was done as follows: Lane 1: the marker proteins are, from top to bottom, myosin (M.W.: 200,000), phosphorylase *b* (116,000), bovine serum albumin (67,000), aldolase (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000). Purified sorbitol oxidase (0.5 μg) was applied on lane 2. (B) Native-PAGE was done as follows: The purified sorbitol oxidase (1.5 μg) was put on native-PAGE, and electrophoresis was done at 4°C overnight at 100 V. In both cases, protein was detected by staining with Coomassie brilliant blue R250 followed by destaining of the gel in 7.5% acetic acid.

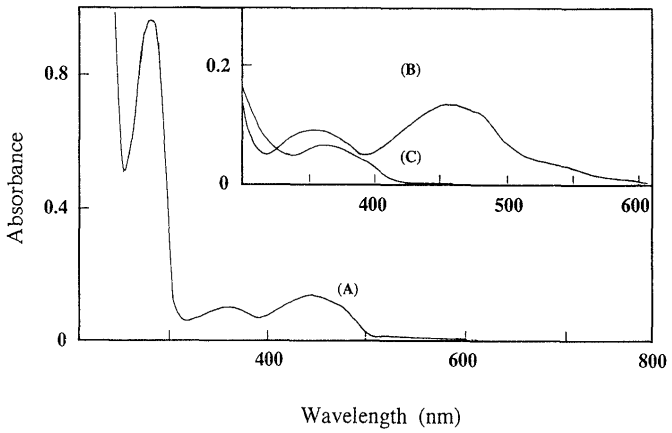

Fig. 2. Absorption Spectra of the Purified SOX.

Absorption spectrum of the purified SOX (A) in 20 mM potassium phosphate buffer, pH 6.0, at the final concentration of 0.64 mg/ml. The insert (B) shows the enlarged spectrum before and after addition of 50 mM D-sorbitol (C).

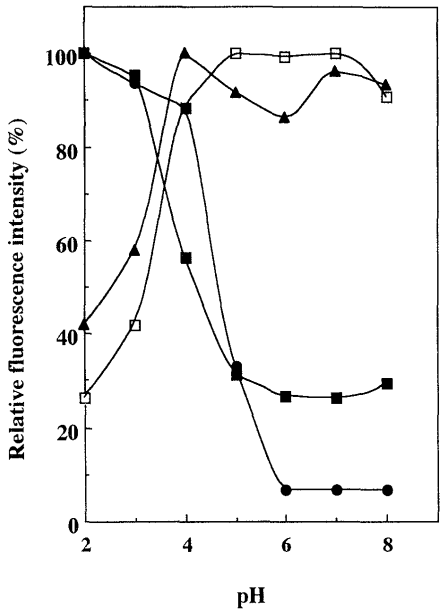

Fig. 3. pH–Fluorescence Profiles of the Purified SOX.

Closed triangles (—▲—), open squares (– □—), closed squares (—■—), and closed circles (—●—), represent riboflavin (100 μM), FMN (100 μM), FAD (100 μM), and SOX (1 mg/ml), respectively. The excitation and emission wavelengths were 450 nm and 526 nm, respectively. The experiments were done in 20 mM glycine–HCl buffer for pH 2.0–3.0, 20 mM acetate buffer for pH 3.0–5.0, and 20 mM potassium phosphate buffer for pH 5.0–8.0, respectively.

358, and 455 nm and a shoulder around 480 nm. A hypsochromic shift of the second absorption band to 358 nm relative to that of riboflavin at 372 nm was also observed. The spectrum was similar to those of flavoproteins with covalently bound flavin.[10-13] By the addition of D-sorbitol, the peaks at 358 and 455 nm were decreased due to the reduction of flavin (Fig. 2(C)). The results indicate that the flavin component was functionally involved in the oxidation of D-sorbitol. The correlation between pH and the fluorescence of the purified SOX was determined and compared with that of FAD, FMN, or riboflavin as shown in Fig. 3. The profile of fluorescence intensity of the purified SOX was pH-dependent, and was similar to that of FAD, but different from that of FMN and riboflavin as in the case for choline oxidase,[13] which has covalently bound FAD.

Properties of flavin coenzyme

The flavin prosthetic group could not be liberated from the purified SOX protein by (1) acidification with 5% trichloroacetic acid, (2) boiling for 5 min, (3) treatment with 1% SDS, and (4) dialysis *versus* 3 M KBr, 1 mM EDTA for 2 days at 4°C[14] (data not shown). It was calculated that 0.9 mol of FAD is bound to one mol of SOX, assuming the molecular absorption coefficient for FAD at 460 nm to be 11,300 $M^{-1} cm^{-1}$.[15]

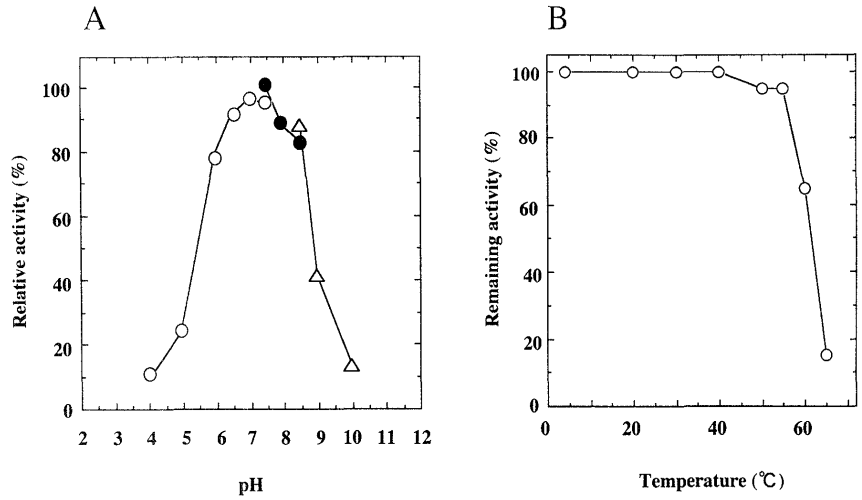

Fig. 4. Optimum pH (A) and Heat Stability (B) of SOX.

(A): SOX activity was measured at various pHs for 10 min at 37°C. Buffers used were 40 mM citrate–Na_2HPO_4 (pH 4.0–7.5, open circles (—○—)), 40 mM Tris–HCl (pH 7.5–8.5 closed circles (- ● -)), 40 mM glycine–NaOH (pH 8.5–10.0, open triangles (—△—)).
(B): SOX was incubated at various temperatures for 15 min in 40 mM Tris–HCl buffer, pH 7.5, and the remaining activity was measured at 37°C.

Table III. Substrate Specificity of Sorbitol Oxidase

Compound	Relative activity (%)	Compound	Relative activity (%)
D-Sorbitol	100 (K_m, 0.26 mM)	Saccharose	0.30
D-Xylitol	93.5 (K_m, 0.38 mM)	Maltose	1.30
D-Mannitol	55.0 (K_m, 7.38 mM)	Lactose	0.10
D-Arabitol	39.0 (K_m, 7.73 mM)	Methanol	0
Ribitol	4.10	Ethanol	0
meso-Erythritol	3.80	1-Propanol	0
D-Threitol	0	1-Butanol	0
Inositol	3.30	1-Propanol	0
Glycerol	3.70	2-Propanol	0
D-Glyceraldehyde	0	1,2-Propanediol	0.80
Dihydroxyacetone	0	1,3-Propanediol	2.70
Dihydroxyacetone phosphate	0	1,2-Butanediol	0.80
D-Arabinose	0	1,3-Butanediol	3.20
D-Glucose	0	2,3-Butanediol	0
D-Galactose	3.50	1,4-Butanediol	7.40
D-Mannose	1.00	Ethylene glycol	0.20
L-Sorbose	0.70	PVA*	0.10
D-Fructos	0		

The enzyme assay was carried out at 50 mM concentration of each compound.
* PVA, polyvinyl alcohol 2000.

Table IV. Comparison of Sorbitol Oxidases

Strain	*Streptomyces* sp. H-7775	*Xanthomonas maltophilia* E-3539[13]
Localization	Intracellular	Intracellular
Inducible enzyme	No	Yes
Molecular mass		
SDS–PAGE	45 kDa	43 kDa
Gel filtration	45 kDa	54 kDa
Optimum pH	6.5–7.5	6.5–7.5
pH stability (after 24 h at 30°C)	7.5–10.0	5–11
Optimum temp. (pH 7.5, 10 min)	50°C	55°C
Heat stability (after 15 min at each temp., pH 7.5)	below 55°C	below 50°C
Substrate specificity[a]		
D-Sorbitol	100 (K_m=0.26 mM)	100 (K_m=0.60 mM)
D-Xylitol	93.5 (K_m=0.38 mM)	89.0
D-Mannitol	55.0 (K_m=7.38 mM)	86.6
D-Arabitol	39.0 (K_m=7.73 mM)	23.2
Glycerol	3.70	3.50
D-Glucose	0	0.54
D-Fructose	0	2.88
D-Mannose	1.00	1.01
Inhibitor[b]		
Zn^{2+}	96.8	N.D.[c]
Hg^{2+}	90.7	N.D.[c]
IAA	53.8	N.D.[c]
NEM	54.8	N.D.[c]
Cofactor	FAD	N.I.[d]
Reaction product	Glucose	Glucose→?

[a] Relative activity, %.
[b] Inhibition, %.
[c] Not determined.
[d] Flavoprotein, but cofactor is not identified.

Optimum pH and heat stability of SOX

The optimum pH of purified SOX is between 6.5 to 7.5. SOX was stable below 55°C in 15 min incubation at pH 7.5 (Fig. 4).

Substrate specificity of SOX

Table III shows the substrate specificity of the purified SOX. Assays were done with 50 mM of each substrate. D-Sorbitol and D-xylitol were oxidized most rapidly. D-Xylitol, D-mannitol, and D-arabitol were oxidized at a rate of 93.5% (K_m, 0.38 mM), 55.0% (K_m, 7.38 mM), and 39.0% (K_m, 7.73 mM) to that for sorbitol (K_m, 0.26 mM), respectively. Glycerol, 1,3-propanediol, 1,3-butanediol, and 1,4-butanediol were oxidized at low rates, while D-glyceraldehyde, dihydroxyacetone, methanol, ethanol, 1-propanol, and 1-butanol were not attacked at all. D-Glucose and D-fructose were not attacked at all at various substrate concentrations from 1 to 100 mM.

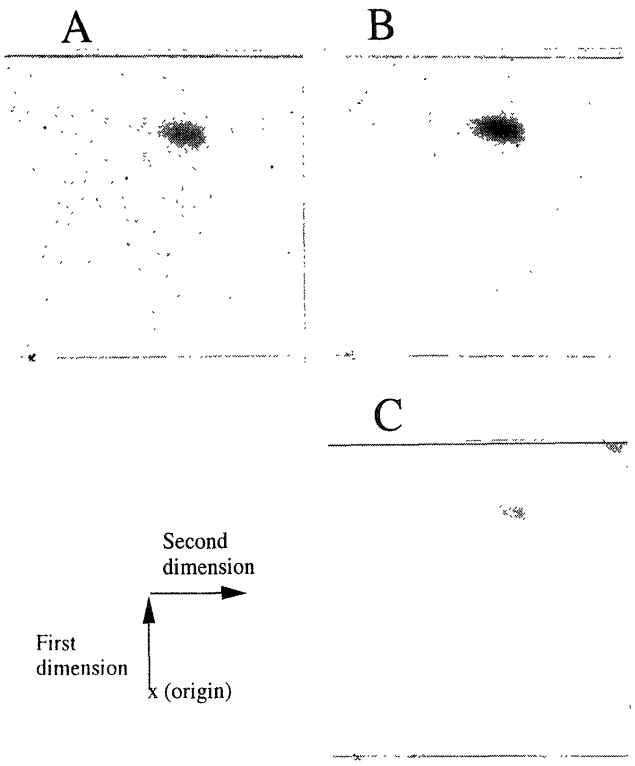

Fig. 5. Two-dimensional Thin Layer Chromatography of the Reaction Product.

Analyzed samples were: Panel A, reaction product when sorbitol was used as a substrate; Panel B, mixture of the reaction products as used in panel A and D-glucose; Panel C, D-glucose.

Effects of inhibitors on activity of SOX

One milimolar Zn^{2+} and Hg^{2+} strongly inhibited SOX activity (Table IV). One milimolar monoiodoacetate (IAA) and *N*-ethylmaleimide (NEM) also inhibited SOX activity. Thiol-groups might be involved in the SOX activity.

Identification of the reaction product by two-dimensional thin layer chromatography and NMR

The identification of the reaction product from D-sorbitol was done by thin-layer chromatography. The reaction mixture in a final volume of 1 ml contained 50 μmol of D-sorbitol, 1 unit of purified sorbitol oxidase, and 100 units of catalase in buffer A. After incubation at 37°C for 24 h, the reaction was stopped by boiling for 5 min, then the solution was separated by two-dimensional thin-layer chromatography. The reaction product was detected by a color reagent as described in Materials and Methods. Figure 5 shows that the R_f was identical with that of D-glucose. The color of the reaction product was dark gray; this is the same as D-glucose. D-Fructose and D-sorbose had different R_f values and different colors (data not shown). These results strongly suggest that the reaction product is glucose.

The ^{1}H NMR spectrum of the purified reaction product from D-sorbitol was compared with the authentic sample of D-glucose. Because these spectra were identical (data not shown), the product of the enzymatic oxidation was confirmed as glucose.

Discussion

In this study, a sorbitol oxidase (SOX) was purified and characterized from *Streptomyces* sp. H-7775 that was found initially as a glycerol-oxidase producing strain (unpublished data). This is the first report from *Streptomyces* sp. SOX from another genus, *Xanthomonas maltophilia* TE-3539, is only found in Japan Kokai Tokkyo Koho (No. 94169764).[16]

The SOX from *Streptomyces* sp. H-7775 was not induced when this strain was cultivated in Waksman medium containing any of the following sugars as a carbon source instead of D-glucose: D-sorbitol, D-xylitol, D-arabitol, or glycerol. About 0.029 unit of SOX activity was equally detected in 1 ml of the cell-free extracts. This suggests that SOX of *Streptomyces* sp. H-7775 is not inducible, while that of *X. maltophilia* TE-3539 is inducible.[16]

As shown in Table III, sugar alcohols that have the *R* configuration on C3, such as D-sorbitol, D-xylitol, D-mannitol (C2 epimer of D-sorbitol), and D-arabitol (C4 epimer of D-xylitol) can be good substrates of SOX. However, ribitol (C3 epimer of D-xylitol) and D-threitol, which have *S* configuration at C3 are poor substrates of SOX. The results may suggest that the stereochemical requirement at position C3 of sugar alcohol is important for SOX activity.

Table IV summarizes the characteristics of these two enzymes. SOX of *Streptomyces* sp. H-7775 is stable up to 55°C. SOX of *Streptomyces* sp. H-7775 acts on D-sorbitol (K_m, 0.26 mM), D-xylitol (K_m, 0.38 mM), D-mannitol (K_m, 7.38 mM), D-arabitol (K_m, 7.73 mM), but does not act on the reaction product, glucose. However, SOX of *X. maltophilia* acts on both D-sorbitol (K_m, 0.60 mM) and the reaction product, glucose. Moreover, SOX activity from *Streptomyces* sp. H-7775 is not affected by exogenous cofactors. Based on these properties, SOX from *Streptomyces* sp. H-7775 might be useful for enzymatic analysis of D-sorbitol and other sugar alcohols. The sorbitol concentration in blood might be related to diabetic complications such as cataracts and neuro-, retino-, and nephropathies.[17] Perturbation of the polyol pathway will cause the increase of D-sorbitol and cofactors such as NADP or NAD in the cell. This will make it more difficult to measure correct sorbitol concentration by sorbitol dehydrogenase. Based on the properties of SOX from *Streptomyces* sp. H-7775 as described above, this enzyme might be useful for simple measurement of D-sorbitol in a clinical field.

Recently, by using the oligonucleotide probe designed from the fourteen *N*-terminal sequence (MTPAEKNWAG-NITF–) of SOX from *Streptomyces* sp. H-7775, we successfully cloned the genomic DNA encoding the enzyme. There are not any nucleotide-binding motifs (*e.g.*, FAD-binding, NADH-binding) in the deduced amino acid sequence. Location of the site of covalently bound FAD of SOX and stoichiometric analysis of the oxidase reaction will be done in near future after construction of high-expression systems.

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