Detection and Characterization of Sorbitol Dehydrogenase from Apple Callus Tissue[1, 2]

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ABSTRACT

Sorbitol dehydrogenase (L-iditol:NAD$^+$ oxidoreductase, EC 1.1.1.14) has been detected and characterized from apple (*Malus domestica* cv. Granny Smith) mesocarp tissue cultures. The enzyme oxidized sorbitol, xylitol, L-arabitol, ribitol, and L-threitol in the presence of NAD. NADP could not replace NAD. Mannitol was slightly oxidized (8% of sorbitol). Other polyols that did not serve as substrate were galactitol, *myo*-inositol, D-arabitol, erythritol, and glycerol. The dehydrogenase oxidized NADH in the presence of D-fructose or L-sorbose. No detectable activity was observed with D-tagatose. NADPH could partially substitute for NADH.

Maximum rate of NAD reduction in the presence of sorbitol occurred in tris(hydroxymethyl)aminomethane-HCl buffer (pH 9), or in 2-amino-2-methyl-1,3-propanediol buffer (pH 9.5). Maximum rates of NADH oxidation in the presence of fructose were observed between pH 5.7 and 7.0 with phosphate buffer. Reaction rates increased with increasing temperature up to 60 C. The K_m for sorbitol and xylitol oxidation were 86 millimolar and 37 millimolar, respectively. The K_m for fructose reduction was 1.5 molar.

Sorbitol oxidation was completely inhibited by heavy metal ions, iodoacetate, p-chloromercuribenzoate, and cysteine. ZnSO$_4$ (0.25 millimolar) reversed the cysteine inhibition. It is suggested that apple sorbitol dehydrogenase contains sulfhydryl groups and requires a metal ion for full activity.

Sorbitol (D-glucitol) is an alditol distributed widely in plants (14, 24). It is found mainly in the Rosaceae where it occurs in all genera of the tribes Spiraeoideae, Pomoideae, and Prunoideae (19), and it is common in many fruits (26). Information on sorbitol metabolism in plants is limited even though in apple and related species sorbitol is the major product of photosynthesis (5) and the principal transport material (2, 25). Sorbitol has also been implicated in many other roles in these plants (6).

Sorbitol dehydrogenase (L-iditol:NAD$^+$ oxidoreductase, EC 1.1.1.14) catalyzes the reversible reaction D-sorbitol + NAD$^+$ $\rightleftarrows$ D-fructose + NADH + H$^+$. The enzyme was first partially purified by Blakley in 1951 from rat liver (4). Since then the enzyme has been found in the liver of a variety of mammalian species and it has been purified from other animal tissues (12, 21, 23, 27).

There are very few reports on the isolation of polyol dehydrogenases from higher plants (8, 16), and only one report has

provided evidence for the presence of a polyol dehydrogenase (13). Data from labeling studies suggest that sorbitol is synthesized in the leaf by way of fructose-6-P which is reduced to sorbitol-6-P and subsequently dephosphorylated (3, 20). Others, however, have reported the conversion of D-glucose to sorbitol in apple (11) and plum leaves (1) when [^{14}C]glucose was used as a substrate. Studies of breakdown of [^{14}C]sorbitol in fruit and other tissues indicate that the polyol may be converted to a hexose (1), primarily fructose (9).

Chong and Taper (6) were able to grow apple callus tissue using sorbitol as a carbon source. We have developed similar cultures grown on sorbitol on the assumption that these should provide a model system with high activity of an enzyme or enzymes involved in sorbitol metabolism.

The following paper is a description of some characteristics of an enzyme preparation from *Malus* tissue cultures which is capable of reducing NAD in the presence of sorbitol and oxidizing NADH in the presence of fructose.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical Co.

Plant Material. *Malus domestica* cv. Granny Smith mesocarp tissues were excised from 10-week-old fruits in July, 1976. Intact fruits were washed in water and detergent (Alconox), surface-sterilized for 3 min in 70% ethanol followed by 15 min in 10% Clorox containing 3 drops of Tween 20, rinsed three times in sterile H$_2$O, and the skin removed. Sections of mesocarp tissue were placed on a medium consisting of Murashige and Skoog inorganics (18) plus thiamine (0.5 mg/l), pyridoxin (0.1 mg/l), niacin (0.1 mg/l), inositol (100 mg/l), sucrose (3%), 2,4-D (2.5 mg/l), BA (5.0 mg/l), and Difco Bacto-agar (0.8%). The pH of the medium was adjusted to 5.7 and all components were autoclaved for 15 min at 121 C. The resulting callus was subsequently subcultured monthly on the same medium containing 1 mg/l 2,4-D and 1 mg/l BA. One month prior to enzyme extraction, the callus was transferred to a medium containing D-sorbitol (2.5%) instead of sucrose.

Enzyme Extraction. It was necessary to prepare extracts of fresh tissue prior to each experiment, since enzyme preparations were found to lose substantial activity with storage at 0 C overnight or with freezing and thawing. Homogenization and all subsequent steps were carried out at 0 to 2 C. In a typical preparation, approximately 60 g callus tissue were ground in a mortar and pestle with 120 ml 0.1 M Tris-HCl buffer (pH 8) containing 1 mM DTT and 6 g insoluble PVP. The homogenate was transferred to a Sorval Omni-Mixer and homogenized for four 15-s bursts at full speed. The homogenate was squeezed through a polypropylene cloth and the filtrate was centrifuged at 1,100g for 10 min. The 1,100g supernatant was centrifuged at 20,000g for 20 min, and the pellet discarded. Solid (NH$_4$)$_2$SO$_4$ was added slowly with constant

stirring to the 20,000g supernatant to reach 30% saturation (17.6 g/100 ml). The suspension was stirred slowly for 30 min, centrifuged at 12,000g for 10 min, and the precipitate discarded. The 12,000g supernatant was then brought to 70% saturation by further addition of solid $(NH_4)_2SO_4$ (27.3 g/100 ml). The suspension was stirred for 1 h and centrifuged at 20,000g for 20 min. The 20,000g pellet was dissolved in a minimum volume of the extracting buffer and dialyzed overnight against 2 liters of 10 mM Tris-HCl buffer (pH 8) containing 0.1 mM DTT and 1 mM 2-mercaptoethanol. The dialyzed 30 to 70% fraction was used for enzyme assays.

Protein Measurement. The method of Lowry et al. (15) was used with BSA fraction V as a standard.

Enzyme Assays. All experiments reported here were repeated at least twice. Several buffer systems were used with each assay in determining the pH optimum. NAD and NADP were dissolved in glass-redistilled H_2O, and NADH and NADPH were dissolved in 1% $NaHCO_3$ and kept on ice.

Sorbitol dehydrogenase activity was assayed either by following the reduction of NAD in the presence of D-sorbitol or the oxidation of NADH in the presence of D-fructose at 340 nm using a Beckman DB-GT spectrophotometer equipped with a constant temperature cuvette compartment.

Routine assays for sorbitol dehydrogenase were performed at 25 C in a reaction mixture (3 ml) containing 1 mM NAD, 93 mM Tris-HCl (pH 9), 0.1 to 0.2 ml enzyme (30–70% fraction), and 500 mM D-sorbitol. For the reverse reaction, 0.1 mM NADH, 93 mM Na-phosphate (pH 6.5), 0.1 to 0.2 ml enzyme, and 500 mM D-fructose were used in a total volume of 3 ml. In all assays, reference cuvettes contained the same components without substrates, reactions were initiated by the addition of substrates 2.5 min after incubating the enzyme with NAD or NADH unless otherwise specified, and sugars and polyols were dissolved in the corresponding buffer used in the assay.

RESULTS

Cofactor Requirements. NAD was reduced when sorbitol was used as a substrate. NADP was completely inactive, and it was not inhibitory since subsequent addition of NAD initiated the reaction. NADH was oxidized in the presence of fructose or L-sorbose. NADPH could partially substitute for NADH. NADPH oxidation in the presence of fructose or L-sorbose was 17% or 31%, respectively, of the rates of NADH oxidation in Na-phosphate (pH 6.5). The inability of NADP to replace NAD and a partial substitution of NADPH for NADH have been reported for sorbitol dehydrogenase in some mammalian systems (7, 21).

Effect of pH and Temperature. Maximum rate of NAD reduction in the presence of sorbitol occurred at pH 9 in Tris-HCl, at pH 9.5 in Ammediol[3] buffer, and at pH 10 in glycine buffer (Fig. 1). For the oxidation of NADH in the presence of fructose, a broad range of optimal activity was observed between pH 5.7 and pH 7 in phosphate buffer (Fig. 2). At lower pH, ≤4.5, denaturation of protein occurred and very low activity was observed. The reaction rate increased with increasing temperature up to 60 C (Fig. 3).

Kinetic Properties. The kinetic constants for the oxidation of sorbitol and xylitol in the presence of NAD were determined in 0.1 M Tris-HCl (pH 9) at 25 C. The K_m values for sorbitol and xylitol oxidation were 86 mM and 37 mM, respectively (Fig. 4). High concentrations of either sorbitol or xylitol tended to inhibit enzyme activity. The V_{max} for xylitol was 78% that of sorbitol.

For fructose reduction in the presence of NADH, a much higher concentration was required than for polyol oxidation (Fig. 5). The K_m value was 1.5 M.

Substrate Specificity. The relative rates of oxidation of several

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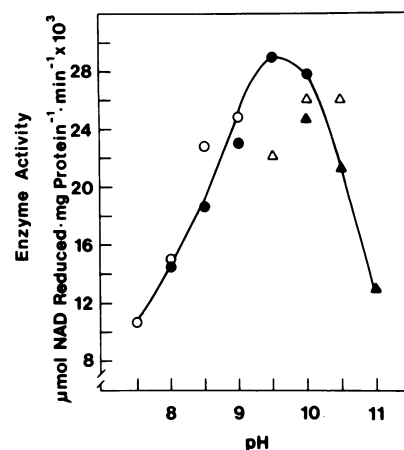[3] Abbreviation: Ammediol: 2-amino-2-methyl-1,3-propanediol.

FIG. 1. Response of sorbitol dehydrogenase to pH of assay mixture using sorbitol as substrate. Reaction mixtures were as described under "Materials and Methods." The following buffers were used at a concentration of 100 mM: Tris-HCl (O), Ammediol (●), glycine (△), and cyclohexylaminopropane sulfonic acid (▲).
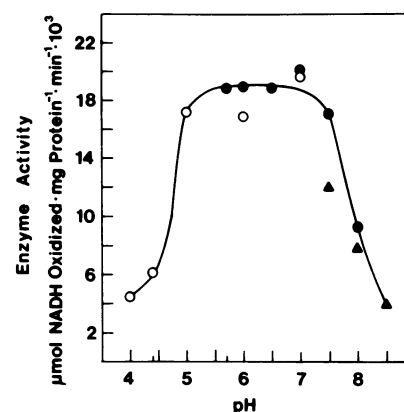

FIG. 2. Response of sorbitol dehydrogenase to pH of assay mixture using fructose as a substrate. Reaction mixtures were as described under "Materials and Methods." The following buffers were used at a concentration of 100 mM: citrate-phosphate (O), Na-phosphate (●), and Tris-HCl (▲).
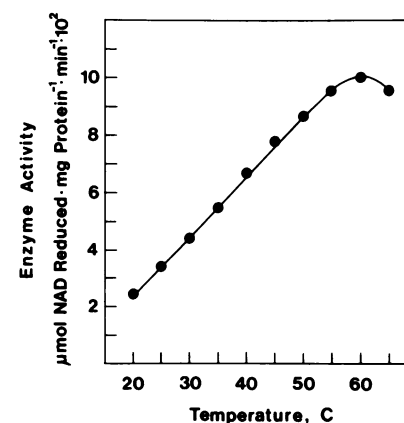

FIG. 3. Effects of temperature on sorbitol dehydrogenase activity using sorbitol as a substrate. Reaction mixtures were as described under "Materials and Methods" except that enzyme and NAD were incubated at each temperature for 5 min before addition of sorbitol.

polyols are shown in Table I. Other polyols that did not serve as substrates at 500 mM were glycerol, erythritol, D-arabitol, and myo-inositol. Galactitol was also inactive at 187 mM. The relative

rate of reduction of L-sorbose was 61% of that of D-fructose (Table I). No detectable activity was observed with D-tagatose. In addition, no reaction was observed with D-glucose, D-mannose, or D-xylose in the presence of either NAD or NADH.

Inhibitors. Tables II and III present the effects of various substances on enzyme activity. Ag^+, Hg^{2+}, and p-chloromercuribenzoate inhibited the oxidation of sorbitol as well as reduction of fructose. Iodoacetate completely inhibited oxidation of sorbitol in 0.1 M Tris-HCl at pH 9 and partially inhibited reduction of fructose. At pH 6.5, in phosphate buffer, reduction of fructose was inhibited 9 and 36% at 1 m and 10 mM iodoacetate, respectively.

Cysteine at 10 mM inhibited sorbitol oxidation completely in Tris-HCl at pH 9, but the inhibition could be overcome with 0.25 mM $ZnSO_4$. Fructose reduction was unaffected by cysteine at 1 mM and only slightly inhibited, i.e. 11% at 10 mM. EDTA (5 mM) partially inhibited fructose reduction (15% inhibition in phosphate buffer, pH 6.5), but was without effect on sorbitol oxidation.
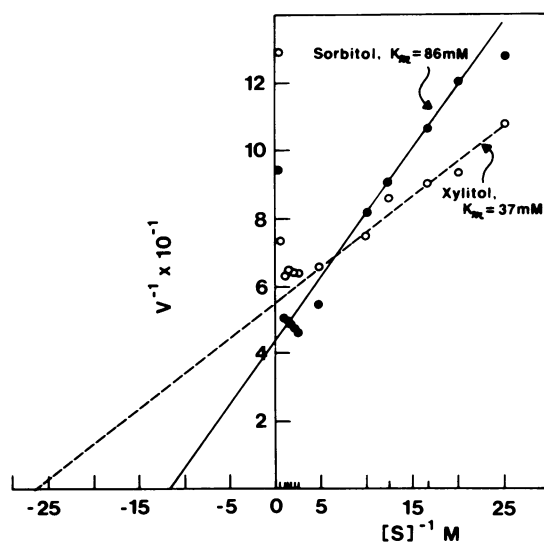
DISCUSSION

Evidence for the detection and characterization of a polyol dehydrogenase from higher plant tissue is presented above. Only limited information has previously been available on enzymes oxidizing sorbitol and other polyols in higher plants. Kocourek *et al.* (13) obtained an active enzyme in acetone powder preparations of tobacco leaves that was capable of converting L-arabitol to L-ribulose. This enzyme was specific for L-arabitol and no reaction occurred with sorbitol, mannitol, xylitol, ribitol, or D-arabitol. In addition, two other reports on the isolation of an enzyme that oxidized sorbitol were published but few details were given (8, 16).

Most of the properties of sorbitol dehydrogenase isolated from apple callus tissue were quite similar to those reported for sorbitol dehydrogenases isolated from mammalian tissue, with the possible exception of the high K_m value for the apple callus enzyme. The K_m values for sorbitol dehydrogenases isolated from a variety of mammalian species have ranged between 0.28 and 9.8 mM (4, 12, 21, 23) for sorbitol, and 0.11 and 0.18 mM for xylitol (21, 23). In our studies, the K_m values for sorbitol and xylitol were 86 and 37 mM, respectively (Fig. 4). A K_m value (1.5 M) was obtained for fructose reduction in our studies, whereas Rehg and Torack (21)

FIG. 4. Lineweaver-Burk plot of sorbitol dehydrogenase activity as a function of sorbitol and xylitol concentrations. V = μmol NAD reduced per mg protein per min. Reaction mixtures were as described under "Materials and Methods" except sorbitol and xylitol concentrations were varied as indicated.
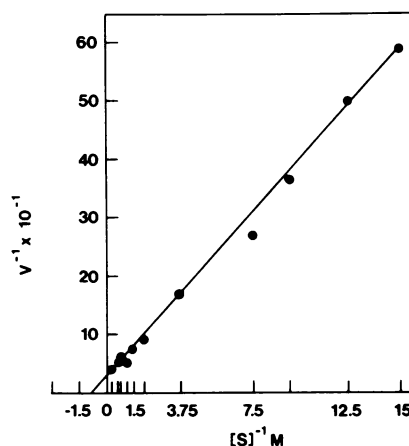
FIG. 5. Lineweaver-Burk plot of sorbitol dehydrogenase activity as a function of fructose concentration. V = μmol NADH oxidized per mg protein per min. Reaction mixtures were as described under "Materials and Methods" except fructose concentration was varied as indicated.

Table I.   Substrate Specificity of Sorbitol Dehydrogenase[1]

Assays were made at 25 C in a 3 ml reaction mixture containing:
a) for polyols, 93 mM Tris-HCl pH9, 1 mM NAD, 0.1 ml enzyme (dialyzed 30-70% fraction), and 500 mM polyol. L-Threitol was tested at 100 mM and compared to 100 mM sorbitol.
b) for ketoses, 93 mM Na phosphate pH 6.5, 0.1 mM NADH, 0.1 ml enzyme (dialyzed 30-70% fraction), and 500 mM ketose.

Substrate	Relative rate (%)	Substrate	Relative Rate (%)
Polyols		**Ketoses**	
D-Sorbitol	100	D-Fructose	100
D-Mannitol	8	L-Sorbose	61
Xylitol	84	D-Tagatose	0
L-Arabitol	48		
Ribitol	12		
L-Threitol	82		

[1]Polyols that did not serve as substrate were glycerol, erythritol, D-arabitol, galactitol, and myo-inositol.

Table II. Effect of Various Substances on Sorbitol Oxidation

Reaction mixture (3 ml) contained 90 mM Tris-HCl, pH 9, 1 mM NAD, 0.1 ml enzyme (dialyzed 30-70% fraction), and one of the test compounds listed below. The reaction mixture was incubated for 10 min at 25 C before addition of sorbitol (final concentration, 500 mM).

Compound Added	Final Conc. (mM)	Relative Activity (%)
Control	---	100
AgNO$_3$	0.1	0
HgCl$_2$	1.0	0
p-chloromercuribenzoate	1.6	0
Iodoacetate	1.0	0
L-Cysteine	10.0	0
L-Cysteine + 0.25 mM ZnSO$_4$	10.0	100
L-Cysteine	1.0	91
EDTA	5.0	100

Table III. Effect of Various Substances on Fructose Reduction

Reaction mixture (3 ml) contained 90 mM Na-phosphate, pH 6.5, 0.1 mM NADH, 0.1 ml enzyme (dialyzed 30-70% fraction), and one of the test compounds listed below. The reaction mixture was incubated for 10 min at 25 C before addition of fructose (final concentration, 500 mM).

Compound Added	Final Conc. (mM)	Relative Activity (%)
Control	---	100
AgNO$_3$	0.1	0
HgCl$_2$	1.0	0
p-chloromercuribenzoate	1.6	0
Iodoacetate	10.0	64
Iodoacetate	1.0	91
L-Cysteine	10.0	89
L-Cysteine	1.0	100
EDTA	5.0	85

found the K_m value for fructose to be 77 mM with purified rat brain sorbitol dehydrogenase. The high K_m value for fructose is consistent with a reaction in favor of sorbitol oxidation rather than fructose reduction.

According to McCorkindale and Edson (17), mammalian sorbitol dehydrogenases catalyze ketose formation from acyclic polyols in which C_2 and C_4 of the polyol are in the L-configuration with respect to C_1. Our results with the apple callus enzyme conform to the rule (Table I) with the exception of L-arabitol, in which the secondary alcohol group at C_3 possesses a D-configuration with respect to C_1. Smith (23), however, did find that sheep liver sorbitol dehydrogenase oxidized L-arabitol, especially at high pH and high substrate concentration. The slow oxidation of D-mannitol (8% of sorbitol) was also observed by others (21, 23, 27) even though its structure is contrary to the rule for polyol oxidation (17).

Apple sorbitol dehydrogenase did oxidize L-threitol, a 4-carbon polyol, but erythritol was inactive as a substrate. Hollman (10) isolated an NAD-xylitol dehydrogenase from washed guinea pig mitochondria which oxidized L-threitol in addition to other polyols. Smith (23) suggested that the oxidation of L-threitol is consistent with the rule for polyol oxidation (17), provided it is assumed that the hydroxyl group of the primary alcohol at C_4 rotates into the same orientation as that on the C_4 on a longer polyol chain.

Xylitol did have a high affinity for the sorbitol dehydrogenase in our studies, just as has been reported for the enzymes isolated

from some animal systems. The metabolic significance of this in apple tissue deserves further study since there is only one report on the presence of xylitol in higher plants (24), and these results have been questioned (22).

Oxidation of sorbitol by apple callus sorbitol dehydrogenase was inhibited completely by heavy metal ions, sulfhydryl-binding agents, p-chloromercuribenzoate and iodoacetate (Table II). Similar results have been obtained for sorbitol dehydrogenases isolated from different animal tissues (4, 21, 23, 27). With the apple callus enzyme sorbitol oxidation was completely inhibited by cysteine (10 mM) at pH 9, and 0.25 mM ZnSO$_4$ reversed the inhibition (Table II), whereas fructose reduction was affected only slightly at pH 6.5. Thus, the apple callus sorbitol dehydrogenase, like most animal enzymes, apparently contains sulfhydryl groups and requires a metal ion for full activity.

The 30 to 70% fraction of our extract did contain a highly active NAD-dependent alcohol dehydrogenase. We found, however, that alcohol dehydrogenase can be distinguished from sorbitol dehydrogenase by: (a) different electrophoretic mobilities (data not shown); (b) complete inhibition of alcohol oxidation by hydroxylamine at 10 mM, whereas sorbitol oxidition was unaffected; and (c) complete inhibition of sorbitol oxidation by iodoacetate which had no effect on alcohol oxidation.

In other experiments (data not shown) we were able to detect sorbitol dehydrogenase activity in Granny Smith apple callus grown exclusively on glucose, fructose, or sucrose. This may suggest that the enzyme is not an inducible enzyme. Activity was

also detected in tissues from other apple cultivars as well as pear. Activity of pear enzyme preparations was approximately three times that of apple. Sour and sweet cherry tissues, however, which did not grow well on sorbitol containing media, had very low enzyme activity.

Finally, the characteristics of this enzyme are consistent with data obtained with labeling studies in various fruit tissues (1, 9). In those studies sorbitol transformations primarily involved conversions to hexose sugars and sucrose with fructose being the primary product (9). Although sorbitol-6-P has been implicated in synthesis of sorbitol in *Prunus* leaves (3, 20) we were unable to obtain any oxidation of this compound with our preparation from apple callus tissues. This suggests that different pathways may be involved in its synthesis in *Prunus* leaves and transformations in other tissues.

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