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Purification and Properties of NAD-Dependent Sorbitol Dehydrogenase from Apple Fruit

Hideaki Yamaguchi, Yoshinori Kanayama and Shohei Yamaki

Laboratory of Horticultural Science, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-01 Japan

This is the first report of the purification of NAD-dependent sorbitol dehydrogenase (NAD-SDH) from a plant source. The enzyme was extracted from apple (*Malus domestica* cv. Ourin) fruit and purified until it appeared as a single polypeptide chain on a gel after SDS-PAGE. From the apparent molecular mass of 62 kDa obtained by SDS-PAGE and that of 120 kDa by gel filtration, the enzyme appeared to be a homodimer. Maximum rates of oxidation of sorbitol and reduction of fructose were observed at pH 9.6 and pH 6.0, respectively. The K_m for oxidation of sorbitol was 40.3 mM and that for reduction of fructose was 215 mM. The maximum rate of oxidation of sorbitol was about 10 times higher than that of the reduction of fructose. The results of the kinetic analysis strongly suggest that in vivo the enzyme would favor the conversion of sorbitol to fructose over the reverse reaction. None of the divalent cations tested had any effect on the oxidation of sorbitol by NAD-SDH. The reaction catalyzed by NAD-SDH was not specific to sorbitol and other substrates could also be oxidized. Among the tested substrates, ethyl alcohol had a particularly high affinity for the enzyme.

Key words: Apple (*Malus domestica*) — Purification — Sorbitol — Sorbitol dehydrogenase.

Sorbitol is a widely distributed soluble carbohydrate that is found in a number of plants (Lewis and Smith 1967, Washüttl et al. 1973), being particularly abundant in plants of the Rosaceae (Plouvier 1963, Chong 1971, Yamaki et al. 1979, Wallart 1980). As a major product of photosynthesis, that is translocated from leaves (Webb and Burley 1962, Bieleski 1969, 1982, Chong and Taper 1971, Loescher 1987), sorbitol plays an important role in the metabolism of photosynthates in apple (Yamaki 1980, Yamaki and Ishikawa 1986).

Studies of the changes in and the factors that control the activities of enzymes responsible for the synthesis and degradation of photosynthates, which are translocated to sink tissues, could lead to a better understanding of processes that control the storage of photosynthates (Hawker 1971). NAD-dependent sorbitol dehydrogenase (NAD-SDH; EC 1.1.1.14) catalyzes the oxidation of sorbitol and the reduction of fructose (Negm and Loescher 1979). In the developing maize kernel, sucrose arriving from the phloem is metabolized to yield fructose as one of the products (Doehlert 1987). NAD-SDH has been partially purified from maize endosperm and its possible significance in the utilization of fructose has been discussed (Doehlert 1987). NAD-SDH has been detected in apple callus (Negm and Loesher 1979), and seasonal changes in its activity has been reported (Yamaki and Ishikawa 1986). Sorbitol accounts for about 80% of the total soluble carbohydrate in apple leaves, spurs, and peduncles but only about 3% to 8% in the fruit. This difference has been attributed to the high activity of NAD-SDH in the fruit (Yamaki 1986). NAD-SDH allows the utilization of the major translocated carbohydrate, namely sorbitol, for the synthesis of the major sugar that is accumulated, namely fructose, in apple fruit (Knee 1993). These earlier results and observations substantiate the importance of NAD-SDH in the regulation of sorbitol metabolism.

In this report we describe the purification of NAD-SDH from apple fruit and some characteristics of the enzyme.

Abbreviations: NAD-SDH, NAD-dependent sorbitol dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

Materials and Methods

Plant material—Apple (*Malus domestica*, cv. Ourin)

fruits at a pre-climacteric stage were obtained from the Fruit Tree Research Station, Morioka, Iwate, Japan, and stored in a cold storage room (O_2, CO_2, and humidity were at the ambient levels; temperature was 0°C.) until use.

Extraction and purification of the enzyme—The enzyme was extracted in a cold room in which the temperature was maintained at 4°C. A total of 800 g of peeled and cored apple flesh was homogenized in 800 ml of 0.15 M KH_2PO_4-NaOH buffer (pH 8.0) that contained 2 mM PMSF, 10 mM Na-L-ascorbate, 10 mM 2-mercaptoethanol, and 80 g of Polyclar AT (Gokyo Sangyo Co., Tokyo, Japan). The homogenate was squeezed through a layer of fine cloth and the filtrate was centrifuged at 13,000 × *g* for 15 min. The supernatant was passed through a column (12.5 cm i.d. × 5.5 cm) of Sephadex G-25 (Pharmacia Co., Uppsala, Sweden) to remove phenolic compounds. The filtrate was brought to 40% saturation with $(NH_4)_2SO_4$, centrifuged at 13,000 × *g* for 20 min, and the pellet was discarded. The supernatant was mixed with 60 ml of Butyl-Toyopearl 650 C (Tosoh Co., Tokyo, Japan), which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) plus $(NH_4)_2SO_4$ to 40% saturation, 0.2 mM PMSF, and 2 mM 2-mercaptoethanol, and the protein was adsorbed to the resin. The Butyl-Toyopearl 650 C with the adsorbed proteins was packed in a column (5 cm i.d. × 3.5 cm) and proteins were eluted with 10 mM Tris-HCl buffer (pH 8.0) that contained 0.2 mM PMSF and 2 mM 2-mercaptoethanol (buffer A). Ten-ml fractions were collected and assayed for NAD-SDH activity. Fractions with activity were pooled, dialyzed against buffer A, and loaded on a column (1.5 cm i.d. × 2 cm) of DEAE-cellulose DE-52 (Whatman Co., Maidstone, England) that had been equilibrated with buffer A. The column was washed with buffer A and eluted with buffer A that contained 0.5 M KCl. Five-ml fractions were collected and NAD-SDH was assayed. The fractions with activity were pooled, dialyzed against buffer A, and loaded on a column (1.0 cm i.d. × 3.0 cm) of Blue Sepharose CL-6B (Pharmacia) that had been equilibrated with buffer A plus 2 mM DTT and 10% (v/v) glycerol (buffer B). The column was washed with buffer B, eluted with buffer B that contained 0.5 M KCl, and the eluate was discarded. The column was again washed with buffer B and then eluted with buffer B plus 30 mM NAD. One-ml fractions were collected and assayed for NAD-SDH activity. The fractions with activity were pooled, concentrated in a collodion bag (Sartorius AG., Göttingen, Germany), and loaded on an FPLC column (1.0 cm i.d. × 30.0 cm) of Superose 6 (Pharmacia) that had been equilibrated with buffer B. The column was eluted with the same buffer. Fractions of 0.3 ml were collected and assayed for NAD-SDH activity. The active fractions coinciding with the peak of the protein (absorbance at 280 nm) were pooled as purified NAD-SDH.

Assays of enzymatic activity—The activity of NAD-SDH was determined spectrophotometrically either by following the reduction of NAD in the presence of sorbitol or by following the oxidation of NADH in the presence of fructose at 340 nm. The reaction mixture (0.6 ml) contained 68 mM Tris-HCl (pH 9.0), 1 mM NAD, 400 mM sorbitol, and an aliquot of the preparation of NAD-SDH (for the reduction of NAD), or 62 mM Tris-acetate (pH 6.0), 0.05 mM NADH, 400 mM fructose, and an aliquot of the preparation of NAD-SDH (for the oxidation of NADH). All assays were performed at 25°C.

Quantitation of protein—Protein content was determined by the method of Read and Northcote (1981). Bovine serum albumin was used as the standard.

SDS-PAGE—SDS-PAGE on a 10% slab gel with a 4.5% stacking gel was performed by the method described by Laemmli (1970). Proteins were detected by staining with Coomassie brilliant blue R-250.

Results and Discussion

Purification of NAD-SDH—The results of a typical purification are summarized in Table 1. Only one peak of NAD-SDH activity was detected at each chromatographic step. The enzyme was purified 158-fold and the recovery was 12% from the chromatography on Butyl-Toyopearl to that on Superose 6. The activity was barely detectable in the crude extract, perhaps because of the presence of some

Table 1 Summary of the purification of NAD-SDH from 800 g of apple fruit

Step	Activity[a] (μmol min^{-1})	Protein (mg)	Specific activity (μmol min^{-1} (mg protein)$^{-1}$)	Purification (-fold)
Crude extract		248		
Butyl-Toyopearl	0.517	75.5	0.007	1.0
DEAE-cellulose	0.357	37.0	0.010	1.4
Blue Sepharose	0.159	0.220	0.723	103.3
Superose 6	0.063	0.057	1.105	157.9

[a] The sorbitol-oxidation activity was estimated as described in Materials and Methods.

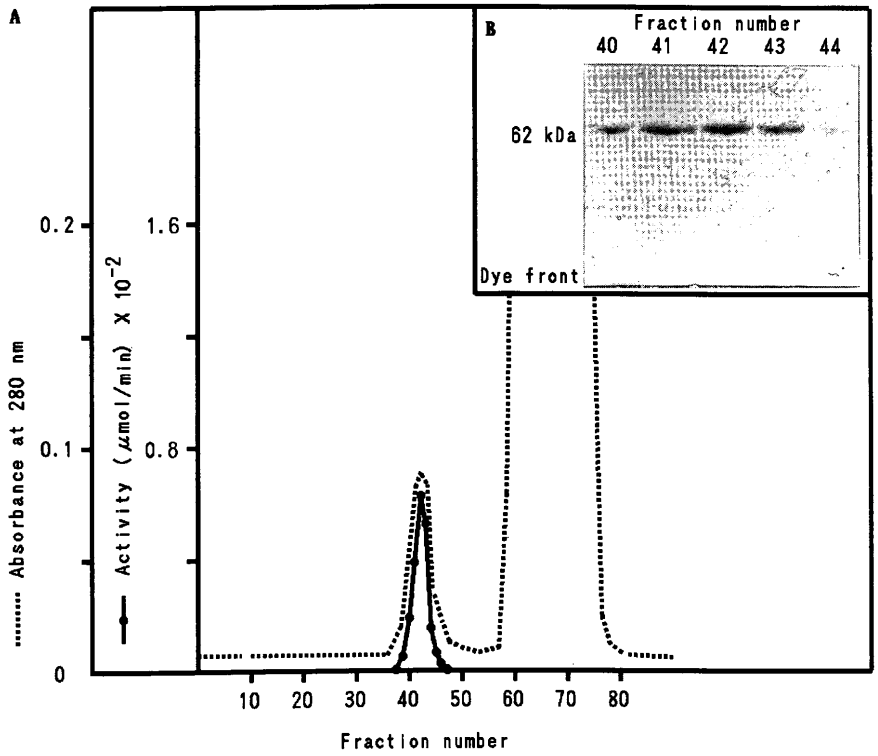

Fig. 1 Column chromatography on Superose 6 of NAD-SDH. (A) Profiles of protein concentration and NAD-SDH activity. (B) SDS-polyacrylamide gel (10%) stained for protein with Coomassie brilliant blue R-250.

inhibitors of the enzyme in the initial homogenate. The specific activity of the purified enzyme was 1.11 μmol of sorbitol oxidized min^{-1} (mg protein)$^{-1}$.

The profile of elution after FPLC on the column of Superose 6 is shown in Figure 1A. The highest activity of NAD-SDH was observed in fraction 42. The peak from fraction 57 to fraction 78 was β-NAD. SDS-PAGE of the preparation of enzyme in fractions 40 to 44 gave a single band. The intensity of stained bands on the gel corresponded to the strength of the activity of respective samples (Fig. 1B).

Molecular mass of NAD-SDH and its subunit—The molecular mass of NAD-SDH was estimated to be 120 kDa by gel filtration (Fig. 2). By cotrast, that of the NAD-SDH from maize endosperm was reported to be 78 kDa (Doehlert 1987). SDS-PAGE gave a single band of a peptide of 62 kDa (Fig. 3). Therefore, NAD-SDH appeared to be a homodimer.

Properties of NAD-SDH—NAD-SDH had different pH optima (Fig. 4) for the oxidation of sorbitol (pH 9.6) and the reduction of fructose (pH 6.0). The pH values are similar to those of the activities from apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987).

The kinetics of the reactions catalyzed by NAD-SDH resembled Michaelis-Menten kinetics for both the oxidation of sorbitol and the reduction of fructose (Fig. 5). NAD-SDH had a K_m of 40.3 mM for sorbitol. The reported values of the K_m for sorbitol for the enzymes from

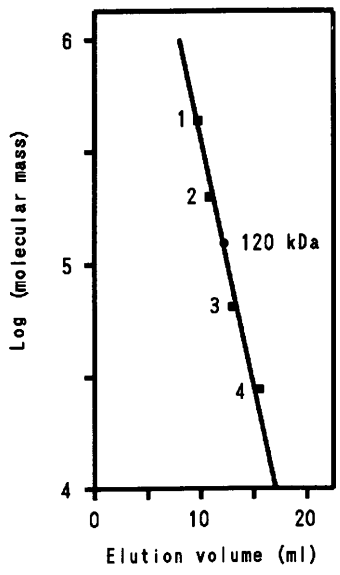

Fig. 2 Estimation of the molecular mass of NAD-SDH by gel filtration on Superose 6 using standard protein markers: 1, apoferritin (443 kDa); 2, β-amylase (200 kDa); 3, bovine serum albumin (66 kDa); 4, carbonic anhydrase (29 kDa).

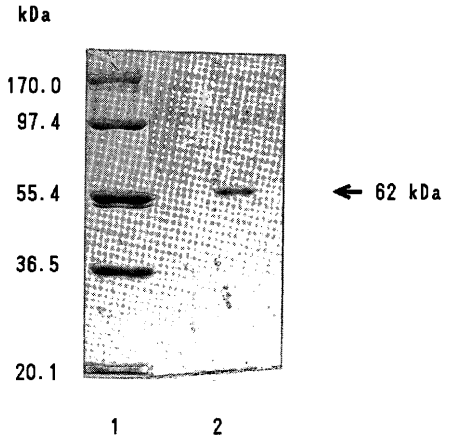

Fig. 3 Determination of the molecular mass of NAD-SDH on an SDS-polyacrylamide gel (10%) stained for protein with Coomassie brilliant blue R-250. (1) Size markers. (2) NAD-SDH.

apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987) were 86 mM and 8.45 mM, respectively. The maximum rate of oxidation of sorbitol was 1.31 μmol min^{-1} (mg protein)$^{-1}$, which is lower than the rate of 5.87 μmol min^{-1} (mg protein)$^{-1}$ reported for maize endosperm (Doehlert 1987). The K_m value for fructose was 215 mM, while the corresponding K_m values for the activity from apple callus tissue (Negm and Loescher 1979) and maize endosperm (Doehlert 1987) were 1.5 M and 136 mM, respectively. The maximum rate of reduction of fructose (0.12 μmol min^{-1} (mg protein)$^{-1}$) was lower than the reported rate of 21.2 μmol min^{-1} (mg protein)$^{-1}$ for the enzyme from maize endosperm (Doehlert 1987). The kinetic properties (Fig. 5), in particular the maximum rate of reduction of fructose, appeared to be different from those reported by Doehlert (1987). Doehlert (1987) reported that NAD-SDH from maize endosperm seemed to favor the conversion of fructose to sorbitol and suggested that the enzyme might function to metabolize some of the fructose produced from the degradation of the translocated sucrose. Hansen and Ryugo (1979) reported that, in prune fruit to which sorbitol is translocated, sorbitol was readily converted to other sugars. Our results (Fig. 5) suggest that in apple fruit also, in which sorbitol is the primary trans-

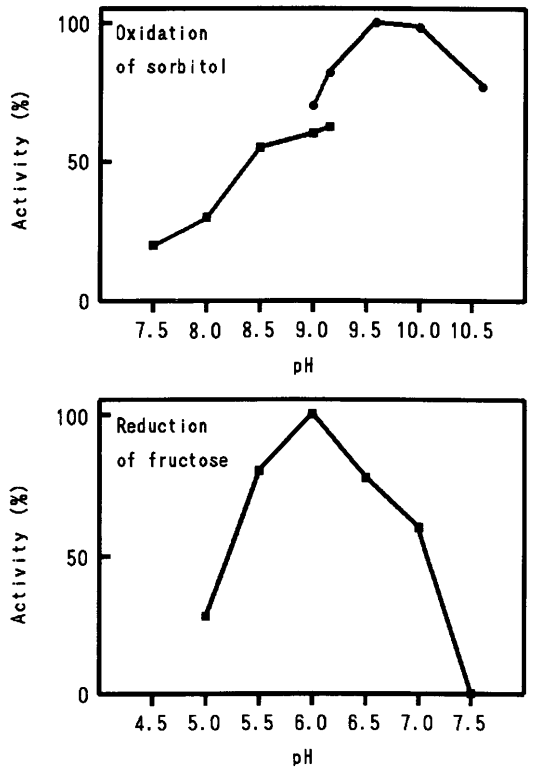

Fig. 4 Effect of pH on the activity of NAD-SDH. The maximum rate of oxidation of sorbitol was 1.16 μmol min^{-1} (mg protein)$^{-1}$ and that of reduction of fructose was 0.14 μmol min^{-1} (mg protein)$^{-1}$. Reaction mixtures were as described in Materials and Methods with the exception that the buffers used were 50 mM glycine-NaOH from pH 9.0 to 10.6, 50 mM Tris-HCl from pH 7.5 to 9.2, and 50 mM Tris-acetate from pH 5.0 to 7.5.

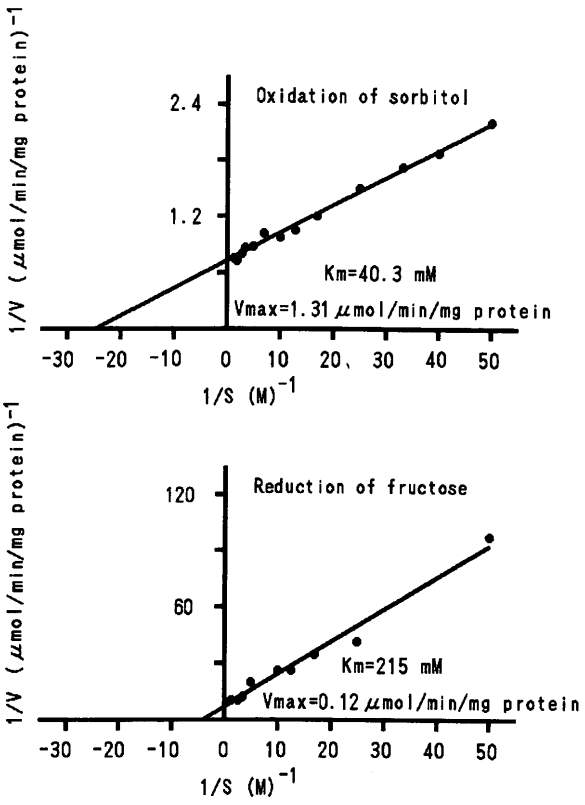

Fig. 5 Double-reciprocal plots of reaction velocity *versus* the concentration of sorbitol or fructose for NAD-SDH. The composition of each reaction mixture was as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

Table 2 Effects of metal ions on the activity of NAD-SDH

Salt	Activity[a] (%)
No additions	100
1 mM $BaCl_2$	87
1 mM $CaCl_2$	72
10 mM $CaCl_2$	71
1 mM $MgCl_2$	54
10 mM $MgCl_2$	54
1 mM $ZnCl_2$	22
1 mM $HgCl_2$	9

[a] The sorbitol-oxidation activity (the results are averages from 4 experiments) was estimated as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

Table 3 Substrate specificity of NAD-SDH

Substrate	Activity[a] (%)
400 mM Sorbitol	100
400 mM Xylitol	40
400 mM L-Threitol	36
400 mM D-Mannitol	8
400 mM Ribitol	7
400 mM L-Arabitol	5
400 mM Ethyl alcohol	51

[a] The oxidation of some substrates (the results are averages from 4 experiments) was estimated as described in Materials and Methods with the exception that the buffer used for the sorbitol-oxidation reaction was 68 mM glycine-NaOH (pH 9.6).

located product of photosynthesis (Webb and Burley 1962, Bieleski 1969, 1982, Chong and Taper 1971, Loescher 1987), NAD-SDH would favor the conversion of sorbitol to fructose. The NAD-SDH in apple fruit might be different in terms of its metabolic function from that in maize endosperm.

Effects of divalent cations—None of the tested divalent cations had an activating effect on the oxidation of sorbitol by NAD-SDH (Table 2), as also reported by Doehlert (1987) for the NAD-SDH from maize endosperm. NAD-SDH from apple fruit is similar to the enzyme from maize endosperm in this respect. Zinc ions have been reported to reverse inhibition by cysteine (Negm and Loesher 1979). However, NAD-SDH was rather strongly inhibited by zinc ions. Calcium and magnesium ions also inhibited the oxidation of sorbitol by NAD-SDH.

Substrate specificity—The substrate specificity of NAD-SDH was examined and the reaction catalyzed by the enzyme proved not to be specific to sorbitol (Table 3). NAD-SDH from apple callus tissue (Negm and Loescher 1979) and from maize endosperm (Doehlert 1987) were also reported to oxidize some substrates other than sorbitol. Xylitol and L-threitol had a relatively high affinity for NAD-SDH (Table 3), as also reported by Negm and Loescher (1979) and Doehlert (1987). D-Mannitol and ribitol were oxidized at a comparatively low rate, just as reported by Negm and Loescher (1979). L-Arabitol was oxidized by NAD-SDH at a low rate (5% of the rate of oxidation of sorbitol), and the enzyme is similar in this respect to the enzyme from maize endosperm (Doehlert 1987) rather than to that from apple callus (Negm and Loescher 1979). Glycerol was not oxidized by NAD-SDH. The properties of NAD-SDH isolated from some animal systems have been reviewed and its close structural relationship to alcohol dehydrogenase has been discussed (Jeffery and Jörnvall 1988). Ethyl alcohol had a high affinity for NAD-SDH from apple fruit (51% of that of sorbitol). Further investigations are required to elucidate the structural, functional, and metabolic relationships between NAD-SDH and alcohol dehydrogenase in plant systems.

The present study yielded three prominent results. First, NAD-SDH was purified from plant tissue for the first time (Fig. 1A, Fig. 1B). Second, from the estimations of molecular mass (Fig. 2, Fig. 3) and its kinetic properties (Fig. 5), NAD-SDH from apple fruit appeared to be different from NAD-SDH from maize endosperm (Doehlert 1987), though some similarities were found in the effects of pH (Fig. 4) and divalent cations (Table 2), as well as in the substrate specificity (Table 3). Third, the kinetic properties (Fig. 5) suggested that the enzyme would favor the conversion of sorbitol to fructose, providing further evidence for the importance of the enzyme in the metabolism of the translocated sorbitol in apple fruit.

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