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Purification and characterization of a NAD$^+$-dependent sorbitol dehydrogenase from Japanese pear fruit

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Abstract

NAD$^+$-dependent sorbitol dehydrogenase NAD-SDH, EC 1.1.1.14) from Japanese pear fruit was purified to apparent homogeneity (single band by SDS-PAGE with silver staining), and had a specific activity of 916.7 nKatal/mg protein. The molecular of the native enzyme was calculated to be 160 kDa by gel filtration, whereas SDS-PAGE gave a subunit size of 40 kDa, indicating that the native enzyme is a homotetramer. The protein immunologically reacted with an antibody raised in rabbit against the fusion protein expressed in *E. coli* harboring an apple NAD-SDH cDNA. The K_m values for sorbitol and fructose were 96.4 ± 8.60 and 4239 ± 33.5 mM, respectively, and optimum pH for sorbitol oxidation was 9.0 and 7.0 for fructose reduction. Pear NAD-SDH had a very narrow substrate specificity, that is, sorbitol, L-iditol, xylitol and L-threitol were oxidized but not any of the other alcohols tested. These data suggest the structural importance of an S configuration at C-2 and an R configuration at C-4 in the substrate(s). Its enzymatic activity was strongly inhibited both by heavy metal ions such as mercury, and by thiol compounds, such as L-cysteine. However, the addition of zinc ion reversed the enzyme inactivation caused by addition of L-cysteine.

Keywords: *Pyrus serotina*; Rosaceae; Japanese pear; Enzyme purification; NAD$^+$-dependent sorbitol dehydrogenase (Ec 1.1.1.14); Sorbitol

1. Introduction

In Rosaceae plants, sorbitol is the main photoassimilate translocated from leaves to sink tissues (Webb and Burley, 1962; Bieleski and Redgwell, 1985). The translocated sorbitol 'unloaded' in fruit tissue is eventually converted to fructose via a NAD$^+$-dependent sorbitol dehydrogenase NAD-SDH, EC 1.1.1.14) (Loescher et al., 1982; Yamaki and Isikawa, 1986; Yamaki and Moriguchi, 1989; Berüter et al., 1997). NAD-SDH, a key enzyme for the metabolic utilization of sorbitol, plays an improtant role in supplying carbon during fruit development.

NAD-SDH is present in animal, microorganisms and plant (Jeffery and Jörnvall, 1988). The enzyme was first partially purified from rat liver (Blakley, 1951), and has since been purified and characterized from many sources (Maret and Auld, 1988; Ng et al., 1992; Yamaguchi et al., 1994; Marini et al., 1997). The molecular weight of the native enzyme in bovine lens is 156 kDa with a subunit of 39 kDa, which suggests a homotetramer (Marini et al., 1997). The enzyme contains one zinc ion per subunit, which contributes to the catalytic activity of NAD-SDH (Karlsson et al., 1995; Marini et al., 1997). In plants, NAD-SDH was partially purified and characterized from apple callus (Negm and Loescher, 1979) corn kernel (Doehlert, 1987); subsequently, it was purified from apple fruits (Yamaguchi et al., 1994). In contrast to the bovine lens enzyme, the moleclar weight of the native apple enzyme is 120 kDa with 62 kDa subunits (Yamaguchi et al., 1994). However, the amino acid sequence of the 62 kDa subunit is still unknown and its cDNA has not yet been isolated.

Recently, a partially purified NAD-SDH from apple

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Table 1
Purification of NAD-SDH from Japanese pear fruit

Steps	Activity (nKatal[a])	Protein (mg)	Specific activity (nKatal/mg)	Purification (-fold)	Recovery (%)
Crude extract		240			
Butyl-Toyopearl	64.35	67.8	0.95	1	100
Blue-Sepharose	22.00	0.049	449.0	473	34.2
Superose 12	11.00	0.012	916.7	965	17.1

[a] 1 nKatal = 1 nmol reduced NAD^+/s.

fruit yielded a 40 kDa protein in addition to the 62 kDa protein previously detected (Yamada et al., 1998). This 40 kDa protein was cloned and found to have high homology with NDA-SDHs from many mammalian sources. Moreover, a fusion protein expressed in *E. coli* harboring apple NAD-SDH cDNA had NAD-SDH enzyme activity (Yamada et al., 1998). Based on these results, the 40 kDa protein was reported as the NAD-SDH in apple fruit.

In Japanese pear fruit, NAD-SDH is the major enzyme for the oxidation of sorbitol to fructose. Its activity increases with sugar accumulation, contributing to fruit development (Yamaki and Moriguchi, 1989). This prompted us to use this fruit as a source of NAD-SDH.

2. Results and discussion

NAD-SDH from Japanese pear fruit was purified to appearent homogeneity via Butyl-Toyopearl hydrophobic, Blue-Sepharose affinity and Superose 12 gel filtration chromatography (Table 1). This resulted in NAD-SDH being purified about 1000-fold with a recovery of 17.1% and specific activity of 916.7 nKatal/mg protein. Its specific activity was similar to that of the bovine lens enzyme (866.8 nKatal/mg protein) (Marini et al., 1997). As shown in the elution profile of Superose 12 gel filtration, the highest NAD-SDH activity was detected in fraction 19 (Fig. 1). The SDS-PAGE analysis of equal volumes from fractions 16 to 22 from the Superose 12 gel filtration gave a single band of ca. 40 kDa, whose staining intensities corresponded well to the observed enzymatic activities (data not shown); no other silver-stained bands were visible.

Using the eluate from the Superose 12 gel filtration, the molecular weight of the native enzyme was estimated to be 160 kDa (Fig. 2). Since SDS-PAGE had given a single polypeptide band of ca. 40 kDa, the native NAD-SDH appeared to be a homotetramer, like others isolated from mammalian tissues. This anzyme is, however, different from one of the NAD-SDH forms purified from apple fruit, which has a native molecular mass of 120 and 60 kDa subunits (Yamaguchi et al., 1994). Note however, that apple fruit also contains a second NAD-SDH having the expected ca. 40 kDa subunit (Yamada et al., 1998).

Western blot analyses showed that the pear fruit NAD-SDH cross-reacted with antibodies raised in rabbit against the fusion protein expressed in *E. coli* which harbors the 40 kDa apple NAD-SDH subunit cDNA (Fig. 3).

The optimum pH for sorbitol oxidation (Fig. 4) was 9.0, although its activity not detected below pH 7.0. For fructose reduction, however, the optimum pH was 7.0 although activity was detected over a wide pH range (pH 5.0–8.0). These values are similar to those of NAD-SDHs from other plants (Negm and Loescher, 1979; Doehlert, 1987; Yamaguchi et al., 1994). although the optimum pH for fructose reduction determined here was higher.

The substrate concentration curves for various substances (sorbitol, fructose, NAD^+, NADH) displayed Michaelis–Menten kinetics and the K_m and V_{max} values were calculated from Lineweaver–Burk plots. The K_m values were 96.4 ± 8.60, 4239 ± 33.5, 0.17 ± 0.01 and 0.05 ± 0.004 mM for sorbitol, fructose, NAD^+ and NADH, respectively whereas the V_{max} values were 1.08 ± 0.075, 2.71 ± 0.340, $0.94 \pm 0{,}020$ and 0.86 ± 0.095 (μKatal/mg protein) for sorbitol, fructose, NAD^+ and NADH, respectively (Table 2). The K_m values for sorbitol and fructose are similar to those of

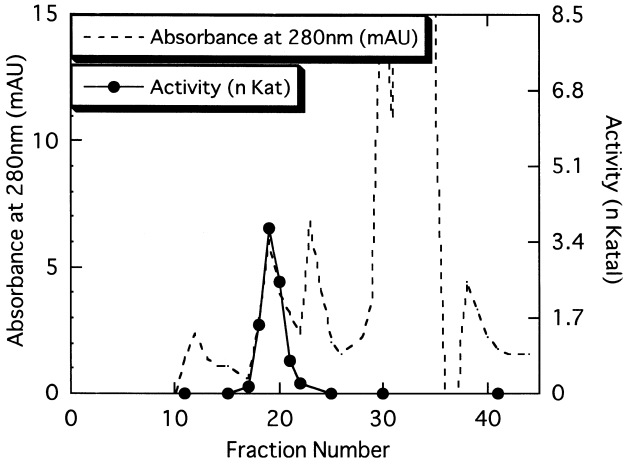

Fig. 1. Elution profile of NDA-SDH for Superose 12 gel chromatography. 0.5 ml fractions were collected.

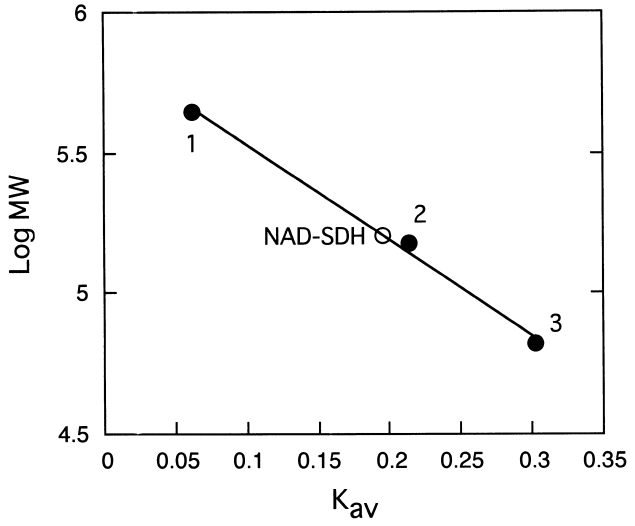

Fig. 2. Molecular mass estimation of NDA-SDH from Superose 12 gel chromatography. Standard protein markers: (1) apoferritin (443 kDa), (2) ADH (150 kDa), (3) BSA (66 kDa). K_{av} is defined as $V_e - V_0$ divided by $V_t - V_0$. V_e is the elution volume for individual protein, V_0 is the void volume, V_t is the bed volume of Superose 12.

apple callus tissue (Negm and Loescher, 1979), but two to ten times higher than those of apple fruit (Yamaguchi et al., 1994) corn kernel (Doehlert, 1987) and mammalian tissues (Maret and Auld, 1988; Marini et al., 1997; Ng et al., 1992). The K_m for fructose was much higher than for sorbitol, suggesting that this enzyme in vivo favors the reaction toward fructose formation. This supports the idea that NAD-SDH catalyzes the oxidation of sorbitol to fructose (Loescher et al., 1982; Yamaki and Ishikawa, 1986; Yamaki and Moriguchi, 1989; Berüter et al., 1997) indicating that this enzyme plays an important role in sorbitol metabolism in Japanese pear fruit.

The relative oxidation of various alcohols versus sorbitol was also examined, using 6-carbon (L-iditol, galactitol, D-mannitol), 5-carbon (xylitol, ribitol, L-arabitol), 4-carbon (erythritol, L-threitol), 3-crabon (glycerol) and 2-carbon alcohols (ethanol). L-iditol had the highest activity (88.3 ± 6.90% that of sorbitol) followed by xylitol (76.8 ± 4.35%) and L-threitol (40.5 ± 0.00%). Ribitol oxidation was low (13.7 ± 0.15%). Very little oxidation of L-arabitol, erythritol and ethanol was detected; D-mannitol, galactitol and glycerol were not oxidized (Table 3). From these results, it is suggested that the S configuration at C-2 and R configuration at C-4 of the alcohols is quite important for the reaction. This is supported by the results that sorbitol (C-2 (S), C-4 (R)) was oxidized but D-mannitol (C-2 (R), C-4 (R)) and galactitol (C-2 (S), C-4 (S)) were not. Furthermore, xylitol (C-2 (S),

Table 2
Kinetic characteristics of NAD-SDH from Japanese pear fruit[a]

Substrate	K_m(mM)	V_{max} (μKatal/mg protein)
Sorbitol	96.4 ± 8.60	1.08 ± 0.075
Fructose	4239 ± 33.5	2.71 ± 0.340
NAD^+	0.17 ± 0.010	0.94 ± 0.020
NADH	0.05 ± 0.004	0.86 ± 0.095

[a] All values represent the mean of duplicate independent experiments ± the variation in the replicates from the mean.

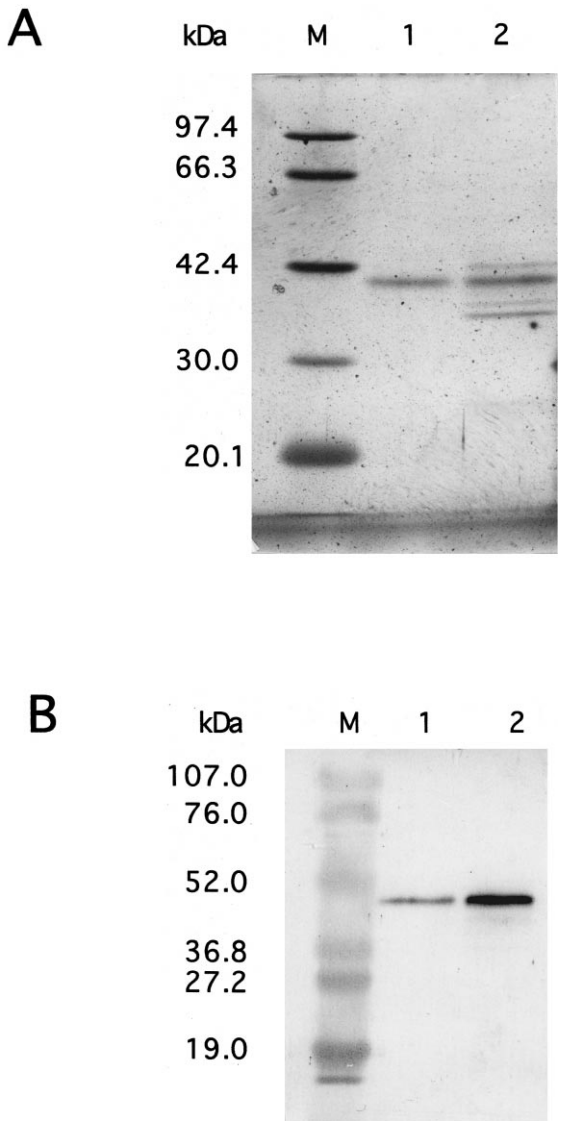

Fig. 3. SDS-PAGE (A) and Western-blotting (B) of purified NDA-SDH from pear fruit. Protein from each purification step was subjected to SDS-PAGE in a 12% polyacrylamide gel and stained with silver nitrate (A). After SDS-PAGE, the proteins were blotted onto a cellulose nitrate membrane. The membrane was immunostained with the antibody raised from rabbit against the fusion protein expressed in *E. coli* transforming apple NAD-SDH cDNA (B). Lane 1, after Superose 12 chromatography (0.18 μg protein); lane 2, after Blue-Sepharose chromatography (0.31 μg protein). Left molecular size markers; markers for (A) are the same as those for Fig. 1, and markers for (B) are phosphorylase B (107.0 kDa), BSA (76.0 kDa), ovalbumin (52.0 kDa), carbonic anhydrase (36.8 kDa), soybean trypsin (27.2 kDa) and lysozyme (19.0 kDa).

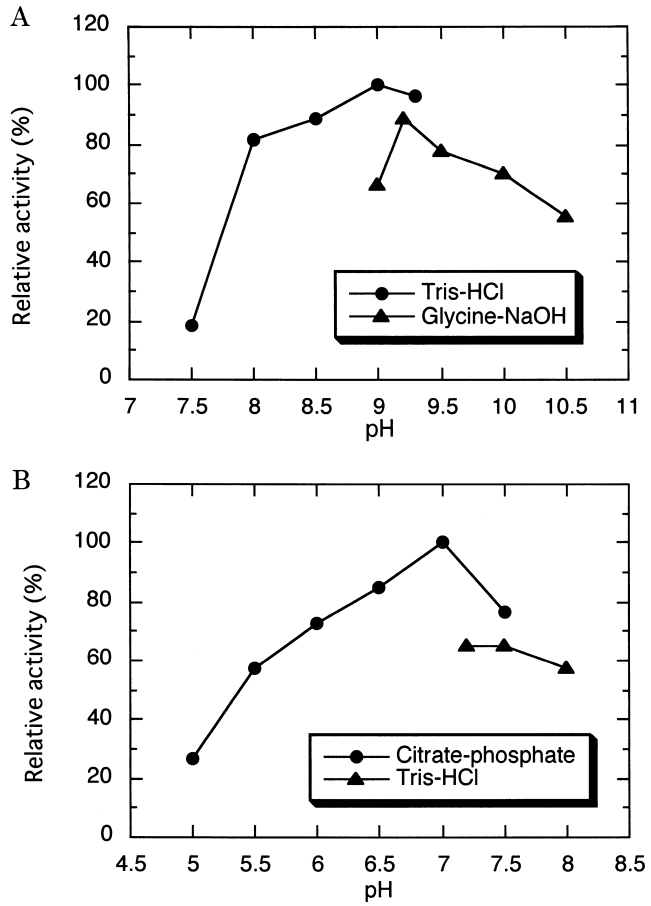

Fig. 4. Effect of pH on the sorbitol oxidation (A) and fructose reduction activities of NAD-SDH (B). Reaction mixture was as described in Section 3 except that 100 mM Tris–HCl and 100 mM glycine–NaOH buffers were used for pH 7.5 – 9.3 and pH 9.0 – 10.5, respectively for (A) whereas 100 mM citrate–phosphate and 100 mM Tris–HCl buffers were used for pH 5.0–7.5 and pH 7.2–8.0, respectively, for (B).

Table 3
Substrate specificity of NAD-SDH activity[a]

Substrate	Relative activity (%)
D-Sorbitol	100 ± 0.00
L-Iditol	88.3 ± 6.90
Xylitol	76.8 ± 4.35
L-Threitol	40.5 ± 0.00
Ribitol	13.7 ± 0.15
L-Arabitol	1.50 ± 0.10
Erythritol	0.75 ± 0.05
Ethanol	2.75 ± 0.45
D-Mannitol	n.d.[b]
Galactitol	n.d.
Glycerol	n.d.

[a] The oxidation of some substrates was determined as described in Section 3 except that substrate concentration was 600 mM. All values represent the mean of duplicate independent experiments ± the variation in the replicates from the mean.
[b] n.d., not detected.

C-4 (R)) was oxidized but L-arabitol (C-2 (S), C-4 (S)) was not. The configuration at C-3 is also important because the difference between xylitol and L-threitol (oxidized) and ribitol and erythritol (not oxidized) depends on the configuration at C-3. This specificity was previously reported for NAD-SDH from other sources (Maret and Auld, 1988; Marini et al., 1997; Lindstad et al., 1998).

All of the metal ions examined inhibited the oxidation of sorbitol (Table 4). Mercury ions completely inhibited SDH activity indicating that it is a thiol enzyme. Activity in the presence of zinc ions was reduced to 23.9 ± 1.90% of the control. Once ions (Mn^{2+}, Mg^{2+}, Ba^{2+}, and Ca^{2+}) inhibited NAD-SDH activity, but to lesser degree. Mammalian NAD-SDHs contain one zinc atom per subunit, which contributes to the catalytic activity of the enzyme (Marini et al., 1997; Karlsson et al., 1995) and are also involved in the primary interaction of thiol inhibitors with the enzyme active site (Lindstad and McKinley-McKee, 1996). The requirement for a metal ion in the active site of NAD-SDH of Japanese pear fruit was demonstrated using the thiol, L-cysteine, which forms a mercaptide with zinc ions in the active site and inhibits NAD-SDH activity through competition with sorbitol (Lindstad et al., 1994; Lindstad and Mckinley-Mckee, 1996). 10 mM L-cysteine inhibited NAD-SDH activity to 4.65 ± 0.25% of the control (Table 5). It was also found that only zinc ions reversed the inhibition by L-cysteine. These results indirectly suggest that NAD-SDH in Japanese pear fruit may also contain zinc ions at the active site.

3. Experimental

3.1. Plant material

Mature Japanese pear (*Pyrus serotina* Rehder var. *culta* Redher cv. Kousui) fruit were obtained from the

Table 4
Effect of metal ions on NDA-SDH activity[a]

	Relative activity (%)
Control[b]	100 ± 0.00
1 mM $BaCl_2$	85.9 ± 1.95
1 mM $CaCl_2$	82.8 ± 1.10
1 mM $MnSO_4$	56.5 ± 1.60
1 mM $MgCl_2$	41.3 ± 2.60
1 mM $ZnSO_4$	23.9 ± 1.90
1 mM $HgCl_2$	n.d.[c]

[a] All values represent the mean of duplicate independent experiments ± the variation in the replicates from the mean.
[b] The control contained no added metal ions.
[c] n.d., not detected.

orchard at Nagoya University. Fleshy tissues were excised and stored at −80°C until use.

3.2. Enzyme purification

All procedures were carried out a 4°C, unless otherwise stated. Frozen fruit (400 g) was homogenized in 500 ml of 0.2 M potassium phosphate buffer (pH 8.0) that contained 2 mM PMSF, 10 mM sodium-L-ascorbate, 10 mM 2-mercaptoethanol and 25 g of polyclar SB-100. The homogenate was squeezed through a layer of fine cloth and the filtrate was centrifuged at 10,000 $\times g$ for 25 min. The supernatant was brought to 40% saturation with ammonium sulfate, then gently stirred for 30 min and centrifuged at 10,000 $\times g$ for 30 min. The supernatant was mixed with 60 ml of Butyl-Toyopearl 650C, which was equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 2 mM 2-mercaptoethanol and saturated to 40% with ammonium sulfate. The Butyl-Toyopearl 650C with the adsorbed proteins was packed in a column (5 cm i.d. × 3 cm) and proteins were eluted with 10 mM Tris–HCl buffer (pH 8.0) containing 2 mM 2-mercaptoethanol. The eluate was collected in 2 ml fractions. The fractions with activity were pooled and dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 2 mM DTT.

The enzyme solution was applied on a Blue-sepharose CL-6B column (1 cm i.d. × 7 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) (buffer A) containing 2 mM DTT and 10% (v/v) glycerol. The column was initially washed with 2 bed volumes of buffer A, and then with 2 bed volumes of buffer A containing 0.35 M KCl. The column was again washed with 2 bed volumes of buffer A, and the proteins were eluted with buffer A containing 10 mM NADH (1 ml fractions collected). The fractions with activity were pooled and concentrated using an Amicon YM50 ultrafiltration membrane. The concentrated solution was loaded on a FPLC Superose 12 column (1 cm i.d. × 30 cm) and elution used buffer A containing 0.05 M NADH and 0.15 M KCl. Fractions with activity were pooled as the purified NAD-SDH preparation.

Table 5
Effect of L-cysteine and some metal ions of NAD-SDH activity[a]

	Relative activity (%)
Control[b]	100 ± 0.00
1 mM L-Cysteine	73.9 ± 9.40
10 mM L-Cysteine	4.65 ± 0.25
10 mM L-Cysteine + 1mM $ZnSO_4$	118.5 ± 10.1
10 mM L-Cysteine + 1mM $CaCl_2$	5.2 ± 1.70
10 mM L-Cysteine + 1mM $MgCl_2$	2.3 ± 0.00
10 mM L-Cysteine + 1mM $BaCl_2$	2.3 ± 0.00
10 mM L-Cysteine + 1mM $MnSO_4$	n.d.[c]

[a] All values represent the mean of duplicate independent experiments ± the variation in the replicates from the mean.
[b] The control contained no L-cysteine or added metal ions.
[c] n.d., not detected.

3.3. Enzyme assay

NAD-SDH activity was determined spectrophotometrically by monitoring the production of NADH at 340 nm as described previously (Yamaguchi et al., 1994). One nKatal of enzyme activity is defined as the amount of enzyme needed to generate 1.0 nmol of NADH or NAD^+/min.

3.4. Determination of protein content

The protein content was determined according to Bradford (1976) using the Baradford Reagent (Sigma, USA). Bovine serum albumin was used as a standard.

3.5. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). Proteins were stained with a Silver Stain Plus kit (Bio-Rad Lab., USA).

3.6. Western-blot analysis

After SDS-PAGE, proteins in the gel were transferred to a nitrocellulose membrane. The membrane was immunostained with the antibody raised in rabbit against the fusion protein expressed in *E. coli* transforming apple NAD-SDH cDNA.

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