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Sorbitol- and Other Sugar-induced Expressions of the NAD+-dependent Sorbitol Dehydrogenase Gene in Japanese Pear Fruit

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Abstract. The regulation of NAD+-dependent sorbitol dehydrogenase (NAD-SDH, EC 1.1.1.14) by sugar was investigated by using sliced tissues of japanese pear (*Pyrus serotina* Nakai cv. Kousui) fruit in order to determine its role in the mechanism of sugar accumulation in fruit tissue. The results of the activities and steady-state levels of the protein and mRNA indicate that NAD-SDH in japanese pear fruit is among the sugar-inducible genes. By preincubating the sliced tissues for 16 hours in a medium without sugar, NAD-SDH activity declined and reached a stable level that was maintained for up to 40 hours. The washing procedure also reduced the sugar concentration in the apoplast and cytosol of the sliced tissues to low concentrations and enabled them to be manipulated by exogenous applications of carbohydrate solutions. Incubation of tissues in 50 or 100 mM sorbitol for 8 hours led to enhanced expression of the NAD-SDH gene as determined by increased mRNA and protein levels and enhanced enzyme activity. The presence of 100 mM glucose, sucrose, or mannitol also gave significant stimulation on the levels of activity, protein, and mRNA of NAD-SDH compared with those of control tissues bathed in media in which the osmotic potential had been adjusted to that of the sugar solutions by adding polyethylene glycol. However, fructose was ineffective in stimulating NAD-SDH activities and the level of the protein was not enhanced but the level of mRNA was increased. Therefore, it is suggested that NAD-SDH gene transcription is enhanced by each sugar investigated, and fructose appears to be unique as it also influences NAD-SDH at a post-transcriptional level.

One of the main processes governing plant growth and development is the regulation of assimilate accumulation and partitioning that is mainly determined by sink strength (Gifford and Evans, 1981). Sink strength is the ability of the sink organ to competitively attract assimilates (Ho, 1988). Whereas acid invertase and sucrose synthase are important in maintaining the sink strength in sucrose-translocating plants, NAD+-dependent sorbitol dehydrogenase (NAD-SDH, EC 1.1.1.14) has been ascribed a key role in the regulation of sink activity in sorbitol-translocating plants (Loescher et al., 1982; Yamaki and Ishikawa, 1986). NAD-SDH has been purified and characterized from japanese pear fruit, and comprises four subunits of the 40-kDa polypeptide (Oura et al., 2000). Its cDNA clones have been isolated from the fruits of apple (*Malus pumila* Mill. var. *domestica* Schneid.) (Yamada et al., 1998), peach (*Prunus persica* L.) (Yamada et al., 2001), and loquat (*Eriobotrya japonica* Lindl.) (Bantog et al., 2000). Four isogenes of NAD-SDH are in apple (Park et al., 2002).

Sugars modulate gene expression in plants (Koch, 1996). Particularly evident is stimulation in activities of enzymes participating in sugar metabolism in sink organs. The acid invertase gene is up-regulated in *Chenopodium rubrum* L. (Roitsch et al., 1995). Enzyme activation and gene induction of sucrose synthase have been reported for eggplant fruit (*Solanum melongena* L.) (Lee et al., 1997) and leaves (Claussen et al., 1986), potato (*Solanum tuberosum* L.) leaves and petioles (Salanoubat and Belliard, 1989), potato tuber (Fu et al., 1995), maize (*Zea mays* L.) (Koch et al., 1992), and *Chenopodium rubrum* (Godt et al., 1995). Sucrose-phosphate synthase induction is at the gene level in the sugar beet (*Beta vulgaris* L.) petiole (Hesse et al., 1995). However, in sucrose synthase and invertase, some isozymes are present that are regulated independently by different sugars (Fu et al., 1995; Roitsch et al., 1995). That is, some isozymes are up-regulated and some isozymes are down-regulated by each sugar.

In a suspension culture of the callus of celery (*Apium graveolens* L.), mRNA expression of mannitol dehydrogenase, which converts mannitol to fructose, is repressed by glucose, and is derepressed once glucose in the media has been consumed, the so-called famine condition (Prata et al., 1997). In the presence of hexose, mannitol dehydrogenase expression is transcriptionally repressed with the result that hexoses are preferentially consumed and mannitol pools are conserved. NAD-SDH activity in apple fruit was found to be enhanced by sorbitol and glucose but not by fructose (Archbold, 1999). Furthermore, reduced supply of sorbitol in girdled apple fruit led to decreased NAD-SDH activity (Berüter and Feusi, 1997). The expression of NAD-SDH may be regulated at the gene level by sorbitol and other sugars. Therefore, in this study, we investigated how sugars influence the expression of NAD-SDH at the gene level by using the sliced tissue of immature japanese pear fruit.

Materials and Methods

Materials and sample preparation. Fruit of japanese pear ('Kousui') were harvested from Nagoya Univ. (Nagoya, Japan) orchard on 6 July 2001, at which stage the fruit were ≈45 g fresh weight and were just starting expansion growth. After harvesting, the fruit were kept at 4 °C for several days. The fruit were peeled and were cut lengthwise into four pieces, and the hard cores were removed. Both blunt and narrow ends of the pieces were removed. Sliced tissues of 5 mm thickness were prepared and were put into a washing medium composed of 10 mM MES-KOH buffer (pH 6.5) containing 10 mM Na-ascorbate.

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Incubation of sliced tissue. Sliced tissues (20 g) were incubated twice in medium A (10 mM MES-KOH buffer (pH 6.5), 2 mM dithiothreitol, 2 mM $CaCl_2$) for 3 min to wash them. For the sugar-releasing experiment, the sliced tissues were incubated for 40 h at 25 °C in medium A, which was exchanged after 1, 6, 16, 24, and 32 h. At each time, sugars contained in the incubation media were assayed. For experiments investigating the effects of sugar on NAD-SDH expression, the sliced tissues were preincubated for 16 h at 25 °C as described above. Then 100 mM sorbitol, glucose, fructose, sucrose, or mannitol, or 10% polyethylene glycol 8000 (PEG) was supplied in media A to keep the osmotic potential equal to 100 mM sugar in medium A, as described by Archbold (1999). The sliced tissues were incubated for 24 h with the media exchanged every 8 h. After incubation, the sliced tissues were washed once with distilled water, frozen in liquid nitrogen, and stored at –80 °C until used for assay.

Total RNA extraction and amplification of japanese pear NAD-SDH CDNA fragment. Total RNA was extracted from 10 g frozen sliced tissues by using the phenol-sodium dodecyl sulfate (SDS) method (Nakajima et al., 1988) combined with the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). An NAD-SDH cDNA fragment of japanese pear fruit was amplified by RT-PCR as follows. From the total RNA, the first strand cDNA was synthesized using oligo-adaptor primer as recommended by the TaKaRa RNA PCR Kit (AMV) ver. 2.1 (Takara Bio. Inc., Shiga, Japan). Two oligonucleotide primers (sense primers: A:5′-GTTGGCATATGTGGCAGTGATGTT-3′; antisense primers: C:5′-GCGGCAGTTTCAGAGGTG-3′) were designed from the apple NAD-SDH cDNA sequence (AC No. AB016256; Yamada et al., 1998). PCR was done with primers A and C and using the first strand cDNA as a template. An amplified fragment [≈840 base pairs (bp)] was cloned into the pT7BlueT vector (Novagen, Madison, Wisc.) by using the TA cloning method (Ichihara and Kurosawa, 1993) and was sequenced by using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, Calif.) with an automatic DNA sequencer (model 373; Perkin-Elmer). The deduced amino acid sequence of japanese pear NAD-SDH cDNA fragment showed an approximately 90%, 90%, 65%, 65%, 32%, 25%, and 23% identity to that of apple, loquat, arabidopsis, peach, silk moth, human, and yeast NAD-SDH, respectively (data not shown). This plasmid DNA was used as a template in a PCR that with sense primer B:5′-TGGTCGTGGGAGCAGGACCTATATG-3′ and antisense primers C to create an amplified fragment (≈440 bp) as a probe.

Enzyme extraction and assay. The enzyme was extracted by using a modified Lo Bianco et al. (1998) method. Sliced tissues (10 g) were homogenized in 10 mL of 0.2 M Tris-HCl buffer (pH 9.0) containing 2 mM 2-mercaptoethanol, 8% (v/v) glycerol, and 1% (w/v) polyvinylpolypyrolidone. The homogenate was centrifuged at 10,000 g_n for 20 min. The supernatant was collected, its pH was adjusted to 9.5, and it was passed over a Sephadex G-25 column (1.3 cm D × 2.6 cm; Amersham Pharmacia Biotech Co., Uppsala, Sweden). The eluate was used as the crude enzyme and soluble protein solution. NAD-SDH activity was assayed by measuring reduced NAD at 340 nm by using the method of Yamaki and Ishikawa (1986).

Protein determination. The amount of protein in the crude enzyme preparation was measured by using the method of Bensadoun and Weinstein (1976) with bovine serum albumin as a standard.

SDS-PAGE and immunoblotting. SDS-PAGE was done as described by Laemmli (1970) in 12.5% (w/v) acrylamide gels. Immunoblotting was done by using the method of Towbin et al. (1979). The polyclonal antibody used for immunoblotting was raised in rabbit against NAD-SDH expressed in *E. coli* transformed with apple NAD-SDH cDNA (Yamada et al., 1999). Binding of antibodies was detected by a colorimetric reaction with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies.

RNA blot analysis. Total RNA (20 μg) was electrophoresed on 1.0% agarose gels in the presence of 0.66 M formaldehyde and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech Co., Little Chalfont, England). The membrane was hybridized to ^{32}P-labeled japanese pear NAD-SDH cDNA fragment amplified by RT-PCR as described above. Hybridization was carried out in 6xSSPE (1xSSPE; 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM ethylenediaminetetraacetate, pH 7.4), 50%(v/v) formamide, 5% (v/v) Irish cream liquer (R. and A. Bailey & Co., Dublin, Ireland), 0.5% (w/v) SDS, and 20 $\mu g \cdot mL^{-1}$ denatured salmon sperm DNA at 45 °C for 20 h. The membrane was washed twice with 2xSSPE/0.1% (w/v) SDS at 65 °C for 20 min, twice. The results of hybridization were analyzed by using an imaging analyzer (BAS2000; Fuji Film Co., Tokyo).

Sugar assay. To measure the amounts of each sugar, aliquots of the incubation media were filtered (0.45 μm; Adavantec, Tokyo) before injection on to a HPLC system (655A-11LC; Hitachi, Tokyo) fitted with a Sugar SP0810 column (Showa Denko Co., Tokyo). The analysis was done at a flow rate of 0.8 $mL \cdot min^{-1}$ of distilled water at 80 °C with a refractive index detector. The amount of each sugar was calculated from standard curves of authentic sugars.

Results

Removal of sugars from sliced tissue during the incubation. To determine correctly the effect of each sugar added to the incubation media, the concentrations of sugars in the apoplast and cytosol of sliced tissues have to be kept at low levels. Therefore, the sliced tissues were incubated in media without sugars. During the first 1-h incubation, sugars were released from the tissues, and the total sugar concentration in the media was 17 mM. During the next 5-h incubation from 1 to 6 h and the subsequent 10-h incubation from 6 to 16 h, the amount of sugar released each hour fell. After 16 h incubation, only a little sugar was released and the sugar concentration in the media collected during each 8-h incubation from 16 to 24 h, from 24 to 32 h, and from 32 to 40 h was below 2 mM (Table 1). Therefore, for subsequent experiments, the sliced tissues were preincubated for 16 h in media without sugar, then were incubated for the different periods in media containing various sugars. The media containing the sugars was exchanged every 8 h.

Table 1. Concentration of each sugar released into medium A during incubation of sliced tissues. The medium was renewed after 1, 6, 16, 24, and 32 h incubation. Each value is the mean of two independent experiments. tr: means <1 mM.

Sugar (mM)	0–1 h	1–6 h	6–16 h	16–24 h	24–32 h	32–40 h
Sorbitol	5.9	9.4	8.7	1.4	tr	tr
Fructose	8.4	13.8	12.9	1.9	tr	tr
Glucose	2.2	3.2	3.5	tr	tr	tr
Sucrose	tr	tr	tr	tr	tr	tr

Stabilization of NAD-SDH activity in sliced tissue. To mitigate the fluctuation of NAD-SDH activity caused by artificial stress during the preparation of sliced tissues, the slices were incubated without sugar in the same medum as for the sugar removed experiment. After 6 h incubation, NAD-SDH activity was reduced to 60% of the control (0 h incubation) (Fig. 1). Thereafter, the activity remained almost constant during the incubation period from 6 to 40 h. Thus, we decided that a 16 h preincubation with renewal of media at 1 and 6 h was enough to get the stable activity and to avoid the influence of released sugars in the media.

Effect of sorbitol concentration on the expression of NAD-SDH. Sorbitol at 50, 100, or 200 mM was added to the medium after 16 h preincubation of sliced tissues (Fig.2a). Then, the tissues were incubated for 24 h with media renewal every 8 h. The NAD-SDH activity increased 1.5, 1.4, and 1.6 times the control activity (0 mM sorbitol) at 50, 100, and 200 mM sorbitol, respectively. Immunoblotting after SDS-PAGE showed two reactive bands at ≈40 kDa, which we considered to be caused by the NAD-SDH protein. NAD-SDH protein bands also increased at each sorbitol concentration (Fig. 2b). The steady-state level of NAD-SDH mRNA estimated by northern blot analysis increased to 1.5, 2.1, and 1.9 times the control at 50, 100, and 200 mM sorbitol, respectively (Fig. 2c) together with the increase in the activity and protein of NAD-SDH.

Time course analysis on the expression of NAD-SDH with sorbitol. The NAD-SDH activity increased slightly after 8 h incubation of sliced tissues in 100 mM sorbitol and increased more after 16 and 24 h incubation (Fig. 3a), but incubation without sorbitol did not enhance the activity, which remained constant for 24 h after preincubation (Fig. 1). That is, the induction of activity started after 8 h incubation. The amount of NAD-SDH protein estimated by immunoblot analysis increased 1.8 times at 8 h incubation (Fig. 3b). The transcript increased before the increase in activity, and was 1.3, 1.5, and 1.4 times the control after incubation for 8, 16, and 24 h, respectively (Fig. 3c).

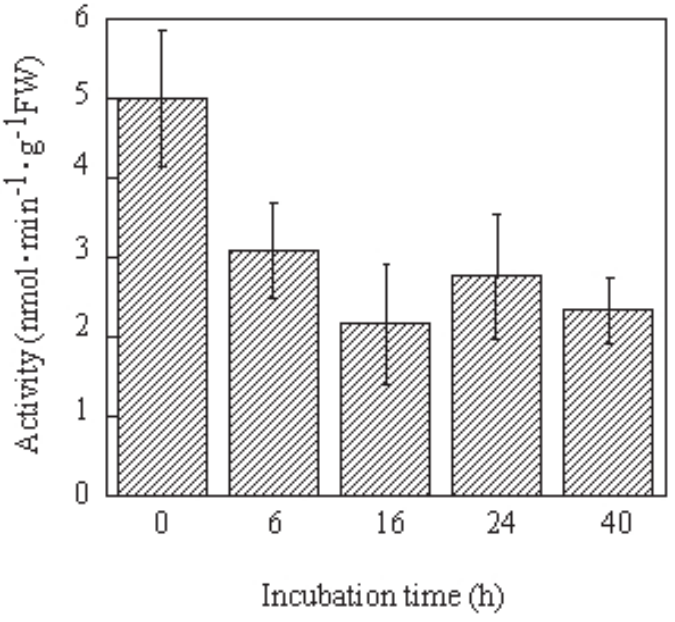

Fig. 1. Time course of NAD-SDH activity in sliced tissues incubated in a medium without sugars. Sliced tissues were incubated in medium A for 0, 6, 16, 24, and 40 h at 25 °C. Medium A was renewed at each of these times. Each value is the mean ± SE of three independent experiments. FW = fresh weight.

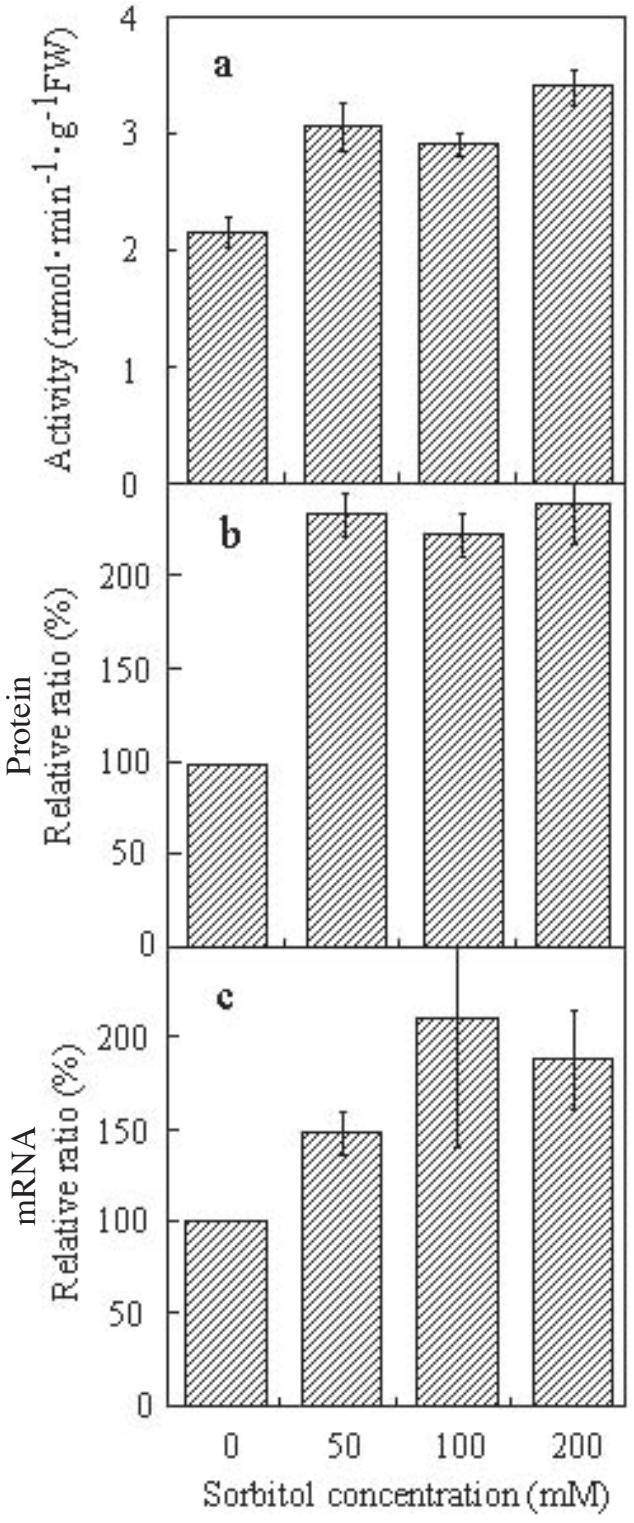

Fig. 2. Effect of sorbitol concentration to enhancement of activity, protein, and mRNA levels of NAD-SDH. Sliced tisuues were incubated in medium A with 0, 50, 100, and 200 mM sorbtiol for 24 h at 25 °C after 16 h preincubation. (**a**) Activity: extraction and assay for NAD-SDH activity was described in the materials and methods. (**b**) Protein: soluble protein (20 μg) in the enzyme extract was separated by SDS-PAGE. For immuno detection, rabbit antibody raised against apple NAD-SDH was used. The relative ratio to the control (0 mM sorbitol) was shown. (**c**) mRNA: total RNA (20 μg/lane) isolated from sliced tissues was hybridized with the radio-labeled partial NAD-SDH cDNA probe from japanese pear fruit. The relative ratio to the control (0 mM sorbitol) is shown. Each value of **a**, **b**, and **c** is the mean ± SE of three independent experiments. FW = fresh weight..

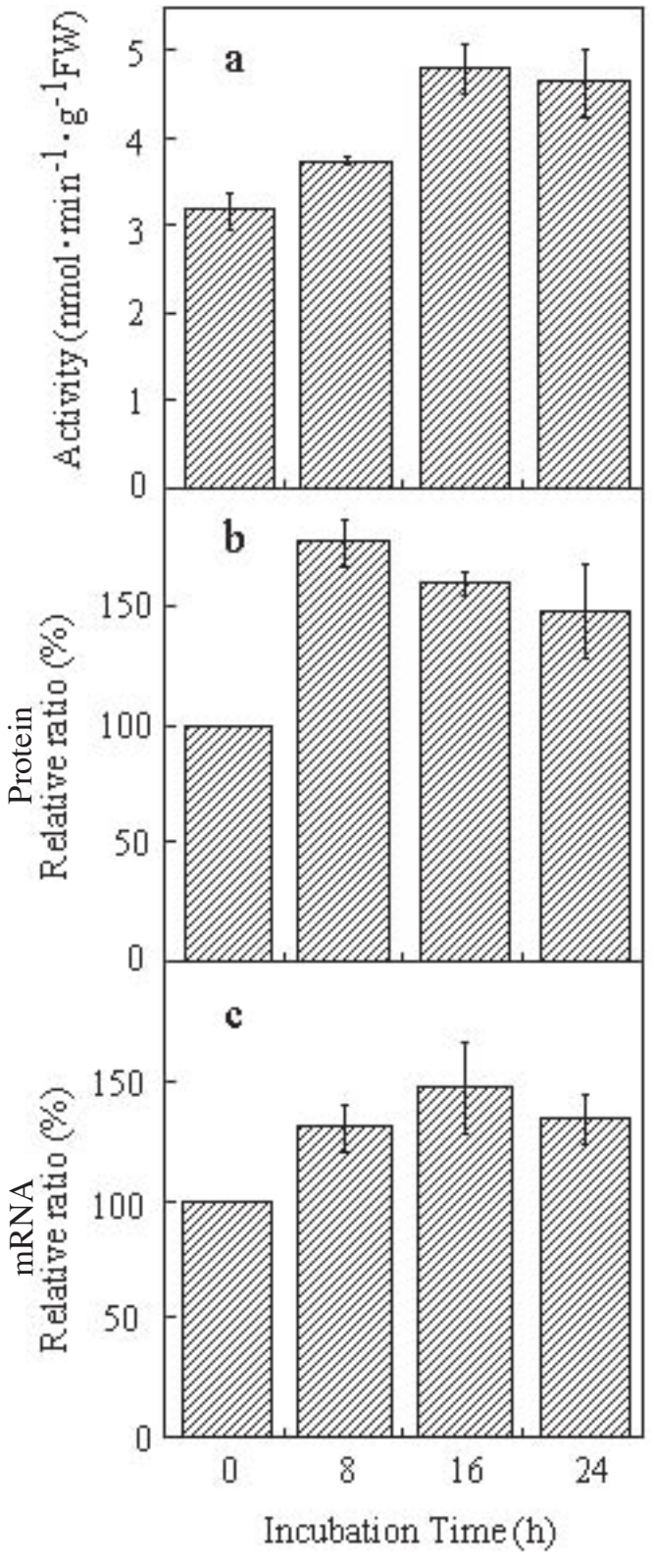

Fig. 3. Time course analysis to enhancement of activity, protein and mRNA levels of NAD-SDH. Sliced tissues were incubated in medium A with 100 mM sorbtiol for 0, 8, 16, and 24 h at 25 °C after 16 h preincubation. (**a**) NAD-SDH activity. (**b**) The relative ratio of NAD-SDH protein to the control (0 h incubation) by immunoblot analysis. (**c**) The relative ratio of NAD-SDH mRNA to the control by RNA blot analysis. The details are described in Fig. 2. FW = fresh weight.

Effect of various sugars on the expression of NAD-SDH. From the results of sorbitol concentration and the incubation time, we decided to incubate for 24 h in 100 mM sugar after 16 h preincubation in sugar-free media for subsequent experiments. The changes in the activity, protein, and mRNA levels of NAD-SDH were examined by adding 100 mM of sorbitol, glucose, fructose, sucrose, or mannitol and were compared with the control containing PEG at the same osmotic potential as the sugars supplied. The activity was stimulated to 1.3 and 1.5 times the control (with PEG) by adding mannitol and sorbitol, respectively, and was stimulated by ≈1.2 times by the addition of glucose or sucrose, but the activity was not enhanced by the addition of fructose (Fig. 4). The activity in tissues incubated with PEG corresponded to the activity in media without sugar or PEG. Mannitol, sorbitol, and glucose enhanced protein levels 1.8, 1.7, and 1.5 times, respectively, when compared with the control, but sucrose and fructose enhanced the protein levels only slightly as estimated by immunoblot analysis (Fig. 5). Northern blot analysis revealed that the levels of mRNA were enhanced by the addition of each sugar, including fructose. The levels of mRNA increased 2, 1.9, 1.9, 1.7, and 1.4 times the control in the presence of glucose, fructose, sorbitol, sucrose, and mannitol, respectively (Fig. 6).

Discussion

To study the regulation of gene expression of NAD-SDH by sugar, we must solve the following important problems. 1) Eliminate the influence of sugars released from sliced tissue on the sugar concentration of the incubation media: The concentration of sugars released from sliced tissues into the media must be maintained below 5 mM (<10% of applied sugar concentration in media) to remove the influence of sugars supplied exogenously.

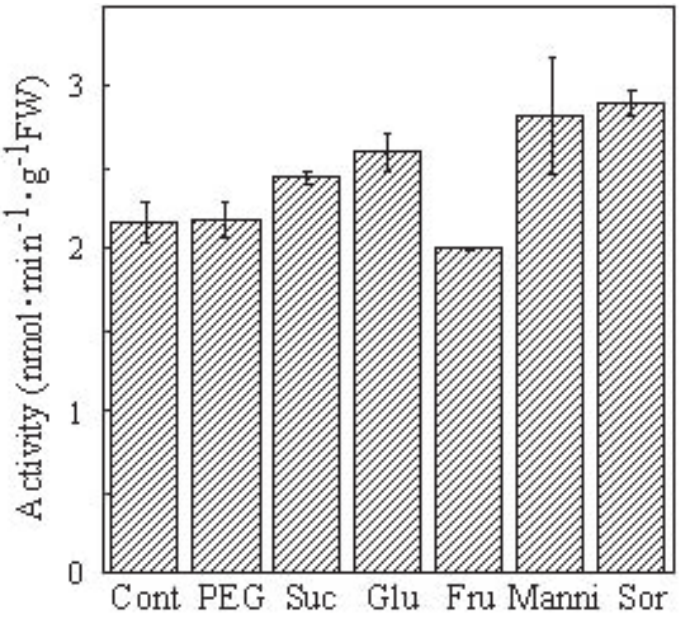

Fig. 4. Enhancement of NAD-SDH activity by adding various sugars. Sliced tissues were incubated in medium A without sugar (control) or with 10% polyethyleneglycol (PEG), 100 mM sucrose (suc), 100 mM glucose (glu), 100 mM fructose (fru), 100 mM mannitol (manni), and 100 mM sorbtiol (sor) for 24 h at 25 °C after 16 h preincubation. Each value is the mean ± SE of three independent experiments. FW = fresh weight.

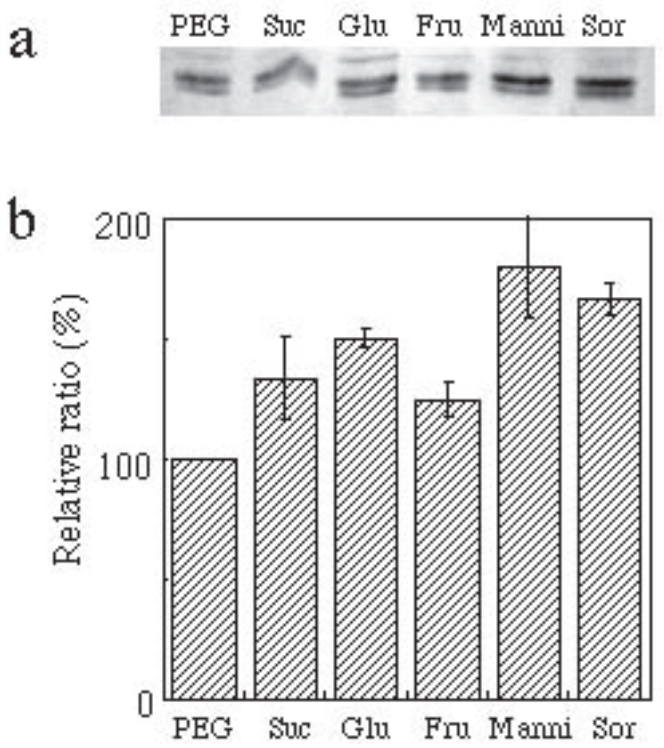

Fig. 5. Enhancement of NAD-SDH protein level by adding various sugars. (**a**) For immunoblot, soluble protein (20 μg) of the same extract as for Fig. 4 was used as described in Fig. 2b. (**b**) The relative ratio to the control (PEG). Each value in **b** is the mean ± SE of three independent experiments.

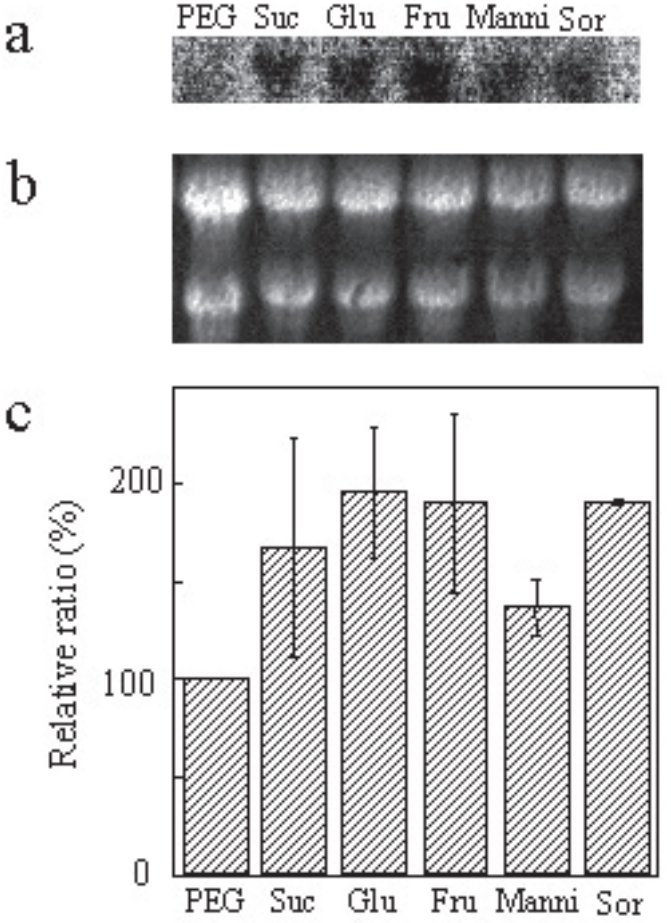

Fig. 6. Enhancement of NAD-SDH mRNA level by adding various sugars. (**a**) For NAD-SDH mRNA level, total RNA (20 μg/lane) isolated from sliced tissues incubated in the same conditons as for Fig. 4 was hybridized with the radio-labeled partial NAD-SDH cDNA probe from japanese pear fruit. (**b**) Ethidium bromide-stain RNA gel. (**c**) The relative ratio to the control (PEG). Each value is the mean ± SE of three independent experiments.

In disk tissues of apple (Yamaki and Ino, 1992) and pear (*Pyrus communis* L. var. *sativa* DC) (Yamaki et al., 1993) fruits, much sugar is released from the apoplast during the first incubation for several minutes, and is released from cytosol during incubation for 30 min. Thereafter the pools of sugars are gradually released from the vacuole during incubation for a long term. Therefore, the sugar concentration in the apoplast is almost the same as in the incubation media after several minutes. This study showed that the release of sugar after 16 h preincubation is very slow and the media concentrations remain below 2 mM sugars even after 8 h incubation. The concentration of released sugars is therefore insignificant when compared to those supplied exogenously in the treatment solutions. 2) The sugar concentration in the cytosol: In previous studies that used disk tissues of apple (Yamaki and Ino, 1992) and pear (Yamaki et al., 1993) fruits, the velocity constants of each sugar across the plasma membrane and tonoplast were measured by using compartment analysis methods. The velocity constant of each sugar across a plasma membrane in immature fruits was ≈100 times higher than across the tonoplast. This means that the plasma membrane is more permeable to each sugar than the tonoplast. In this study, after 16 h preincubation sugar released from sliced tissues had already reached a steady state. Thus, the sugar concentration in cytosol is expected to be almost equal to that in the apoplast, and that of the concentration in the apoplast. From the results of 1) and 2), we suggest that the sugar concentration in cytosol should be kept below 2 mM for each 8 h incubation after 16 h preincubation in sugar-free media and should be easily equilibrated with the concentration of sugars supplied in the media.

When the supply of photoassimilate for fruit is restricted by girdling stem between leaves and fruit and by defoliation near the fruit, NAD-SDH activity in fruit is reduced compared to the untreated control. The NAD-SDH activity increases when disk tissues prepared from fruit on girdled trees are incubated in media containing sorbitol (Archbold, 1999). In this study, 50 mM sorbitol enhanced the NAD-SDH activity, which was accompanied by an increase of NAD-SDH protein and mRNA levels. The enhancement of activity started after 8 h incubation and continued to increase up to 24 h, but the protein and mRNA levels were maximum after 8 h incubation. Therefore, the stimulation of NAD-SDH activity by adding sorbitol may be caused by the expression of mRNA.

NAD-SDH activity was stimulated by glucose but not by fructose, as has been described previously for apple fruit tissue (Archbold, 1999). A similar pattern was shown for the protein level. However, the expression of mRNA differed from both the activity and protein level because mRNA levels increased by fructose as well as by glucose. This suggests that lack of stimulation of NAD-SDH activity by fructose is caused by regulating on the translational levels. Sucrose stimulated the NAD-SDH activity and was accompanied by increased protein and mRNA levels. However, as sucrose can be easily converted to glucose and fructose by invertase, whether this stimulation was caused by sucrose itself or by its component of hexoses is unclear. Mannitol also stimulated the activity of NAD-SDH with accompanying

increases in protein and mRNA levels. As mannitol is usually used as an osmolyte, we investigated if the stimulation was due to changes in the osmotic potential of the media. Therefore, sliced tissues were incubated in media containing PEG at the same osmotic potential; stimulation of the activity, protein, and mRNA of NAD-SDH was not observed and their levels remained similar to the activity level in tissues incubated without PEG or sugars. Thus, we consider the stimulative expression of NAD-SDH at the transcriptional level is due to the sugar itself, and not to an osmotic influence.

The expression of mannitol dehydrogenase (MDH) mRNA in the callus of celery, which mainly uses mannitol as a translocating sugar, is repressed by adding glucose, and is derepressed under starvation conditions that develop once sugars in the media have been consumed during prolonged incubation (Prata et al., 1997). That is, when hexose is readily available, it is predominantly consumed and MDH expression is restricted. Consequently, mannitol accumulates in the tissues to protect them from osmotic stress (Stoop and Pharr, 1996). Further, hexokinase may function as the signal to repress the expression of the MDH gene (Prata et al., 1997). The expression of the NAD-SDH gene in the callus of the peach fruit is induced by hexose depletion (Kanamaru et al., 2002). In this study that used sliced tissues of japanese pear fruit, however, the expression of NAD-SDH mRNA was not repressed by hexose, but was stimulated. NAD-SDH activity was not stimulated by incubation without sugar, although the sugar concentration in the apoplast and cytosol was kept at very low levels that mimicked the starved condition. However, when the influx of loading sugars, including sorbitol, into fruit is restricted by girdling the stem, NAD-SDH activity falls (Berüter and Feusi, 1997) and is stimulated by adding sorbitol to disk tissues prepared from similarly treated fruit (Archbold, 1999). The results of this study of the NAD-SDH activity related to the expression at transcriptional and translational levels support these other studies.

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