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Short Communication

Cloning of NAD-Dependent Sorbitol Dehydrogenase from Apple Fruit and Gene Expression

Kunio Yamada, Yasuhito Oura, Hitoshi Mori and Shohei Yamaki [1]

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, 464-8601 Japan

Partial amino acid sequences of NAD-dependent sorbitol dehydrogenase (NAD-SDH) were used to identify a full-length cDNA from apple fruit. This clone consisted of 1,433 bp containing an open reading frame of 1,137 bp that could code for a polypeptide with 379 amino acids. To our knowledge, this is the first report about cloning of NAD-SDH cDNA from a plant source. The deduced amino acids from cDNA revealed 43.7% identity to human NAD-SDH. The activity of this enzyme to convert sorbitol to fructose with the reduction of NAD was certified by the fusion protein of this clone expressed in *Escherichia coli*. Northern blot analysis showed that the mRNA was expressed in matured apple fruit.

Key words: Apple (*Malus domestica*) — cDNA cloning — NAD-dependent sorbitol dehydrogenase (EC 1.1.1.14) — Sorbitol.

Sorbitol accounts for about 80% of the total soluble carbohydrate in apple leaves, spurs, and peduncles but only 3% to 8% in the fruit throughout the growing season. This means that sorbitol imported into fruit is not stored as sorbitol but converted to other metabolites. Fructose is the main sugar accumulated in the fruit, comprising 45% to 60% of the total soluble carbohydrate. Lack of sorbitol in the matured fruit has been attributed to the high NAD-dependent sorbitol dehydrogenase (NAD-SDH) activity (Yamaki and Ishikawa 1986). Since the first detection from a plant source (Negm and Loescher 1979), NAD-SDH has been reported to be one of the key enzymes in sorbitol metabolism in the Rosaceae plant (Loescher et al. 1982, Yamaguchi et al. 1994, Yamaki and Moriguchi 1989).

Here, we report the cloning of NAD-SDH cDNA and the gene expression of this mRNA in apple fruit. To our knowledge, this is the first paper describing the cloning of NAD-SDH cDNA from plants, although NAD-SDH cDNAs have already been cloned and sequenced from animals and insect (Jeffery et al. 1984, Karlsson et al. 1991, Niimi et al. 1993).

NAD-SDH protein was purified from prematured apple (*Malus domestica*) fruits according to the method described by Yamaguchi et al. (1994). The final preparation was separated by SDS-PAGE. The Coomassie Brilliant Blue R-250 (CBB)-stained polypeptide bands were excised from the gel and were digested with trypsin in the gel (Hellman et al. 1995). The digested peptides were recovered from the gel and separated by the SMART-System (Pharmacia, Sweden). To determine the amino acid sequences, purified peptide fractions were dotted on ProSorb (Applied Biosystems, U.S.A.) and subjected to a gas phase sequencer (model 476A, Applied Biosystems, U.S.A.).

The total RNAs were extracted from prematured apple fruit by the phenol-SDS method (Nakajima et al. 1988) followed by cetyltrimethylammonium bromide method (Murray and Thompson 1980), and polyadenylated RNA (poly(A)$^+$ RNA) was purified by oligo(dT)-cellulose chromatography (Aviv and Leder 1972, Slater 1984). Sense and antisense primers were designed according to the peptide sequences obtained from the tryptic digest and used for PCRs in combination. Using the following primers, we prepared the PCR product, which was later used as a probe for library screening and Northern blot analysis; sense primer 5′-CA(T/C)TT(A/G)GTICCIGGIGA-3′ and antisense primer 5′-A(A/G)IGT(A/G)AACATIAC(T/C)TT-3′ (I: inosine). A cDNA library was constructed in the plasmid vector pBluescript SK (Stratagene, U.S.A.) by a vector-primer method with poly(A)$^+$ RNA as template, as described by Mori et al. (1991). The clones were sequenced by the dideoxy chain-termination method (Sanger et al. 1977) with an automated DNA sequencer (Li-Cor Inc., model 4000, U.S.A.).

NAD-SDH protein was purified from prematured apple (*Malus domestica* cv. Ourin) fruit according to the method described by Yamaguchi et al. (1994). After gel filtration on Superose 6, the fractions of NAD-SDH activities were subjected to SDS-PAGE. There were a few silver-nitrate-stained peptide bands (Fig. 1B) with elution profiles coinciding with NAD-SDH activities (Fig. 1A). Each active fraction after Superose 6 gel filtration was concentrated by

Abbreviations: CBB, Coomassie Brilliant Blue R-250; DAF, days after flowering; NAD-SDH, NAD-dependent sorbitol dehydrogenase; poly(A)$^+$ RNA, polyadenylated RNA.

The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL, GeneBank under accession number AB016256.

[1] Author for correspondence. FAX: +81-52-789-4025.

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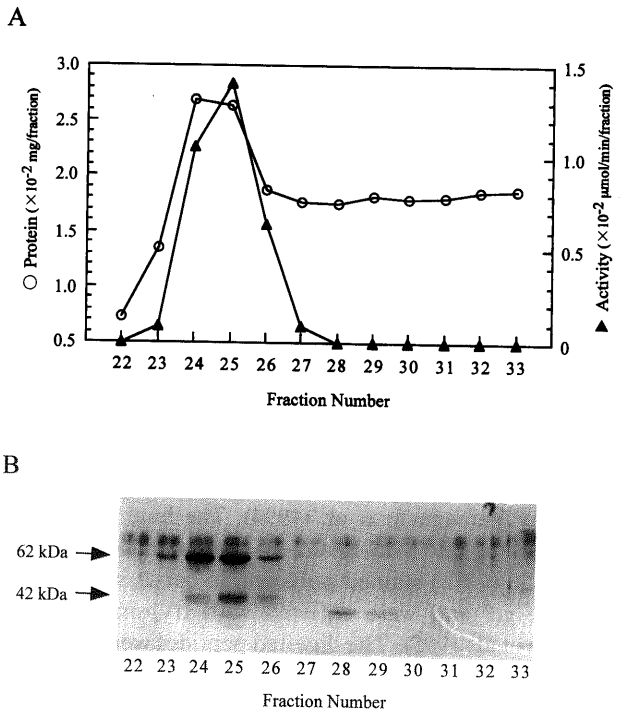

Fig. 1 Elution profile of NAD-SDH activity on Superose 6 gel filtration (A) and SDS-PAGE of each fraction (B). Fractions of 0.5 ml were collected. NAD-SDH activity was measured by the method of Yamaguchi et al. (1994) and protein contents were determined using bovine serum albumin as a standard (Bradford 1976). SDS-PAGE was performed as described by Laemmli (1970) in slabs of 10% acrylamide. Gel was stained with silver nitrate.

lyophilization until about 1/10 volume. The concentrated samples were separated by SDS-PAGE and the gel was stained with CBB. Two major bands (62 kDa and 42 kDa) corresponding to the activities were excised from the gel and were digested with trypsin as described above. The amino acid sequences of these fragments were determined and compared to the amino acid sequences of NAD-SDHs isolated from animals. After digestion of the 42 kDa polypeptide with trypsin, five fragments (Fragment A to E, Fig. 2 underlined) were obtained by the SMART-System. The amino acid sequences of these five fragments revealed high similarity to NAD-SDHs from humans, other mammals and silkworm, but the partial amino acid sequences of the 62 kDa polypeptide were not similar to these NAD-SDHs (data not shown).

A PCR product was prepared using degenerated primers derived from the two tryptic fragments of the 42 kDa polypeptide (Fragments A and E), and used as a probe to screen an apple fruit cDNA library. This PCR product was confirmed to code a part of the 42 kDa polypeptide, because the deduced amino acid sequence of the PCR product corresponded with five tryptic fragments of the 42 kDa polypeptide (Fragment A to E). After screening about 40,000 colonies of cDNA library using PCR product as a probe, two independent clones were isolated. From these two positive clones, the complete nucleotide sequence and the deduced amino acid sequence of NAD-SDH were determined (Fig. 2). The cDNA of NAD-SDH consisted of 1,433 bp and contained an open reading frame of 1,137 bp capable of encoding a protein of 379 amino acids. The internal peptide sequences of NAD-SDH digested with trypsin are underlined in Figure 2 (Fragment A to E). From the structural analysis and mutagenesis studies of the rat NAD-SDH, Karlsson and Höög (1993) proposed that the catalytic zinc atom interacts with amino acid residues Glu175, His89 and Cys64. Since the characteristic amino acid sequence of zinc-containing alcohol dehydrogenase signature (G-H-E-X(2)-G-X(5)-(G/A)-X(2)-(I/V/S/A/C)) (Carr and Markham 1995) was located from the 88th to 102nd deduced amino acid residue, this indicated that apple NAD-SDH also belongs to the zinc-containing alcohol dehydrogenase family like other NAD-SDHs in animals. A computer search using the SWISS-PROT protein sequence database Release 35.0 revealed that NAD-SDH from apple fruit has high identity (about 40%) to the animal and the human NAD-SDH (Jeffery et al. 1984, Karlsson et al. 1991, Lee et al. 1994) (Fig. 3).

A coding region of NAD-SDH cDNA was subcloned into plasmid vector (pET-32a). The fusion protein of

Table 1 The enzyme activity of fusion protein of NAD-SDH cDNA expressed in *E. coli*

Reaction time (min)	pET-32a without insert	NAD-SDH cDNA in the pET-32a	
	NADH production (nmol)	NADH production (nmol)	Fructose production (nmol)
0	0.0	0.0	0.0
60	0.0	16.4	20.7
90	0.0	33.0	34.2
120	0.0	47.4	45.6

E. coli harboring the pET-32a vector inserting the NAD-SDH cDNA or the pET-32a vector without insert were grown with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The activity of NAD-SDH was detected by the method of Yamaguchi et al. (1994). Fructose produced in the reaction was analyzed by the enzyme-coupling method (Bergmeyer et al. 1974).

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   1  CTGCATCTTCACTATTTACTCGCAGCCTGAGAAAACAAAGAGAGAAAAATGGGAAAGGGAGGCATGTCTGATGGA    75
   1                                                    M  G  K  G  G  M  S  D  G     9

  76  GATCATGCTGATCGCTGTTGTGGGGAAGCAATAAATGGTGATGTTCAACAAGAGAACATGGCTGCTTGGCTTCTT   150
  10   D  H  A  D  R  C  C  G  E  A  I  N  G  D  V  Q  Q  E  N  M  A  A  W  L  L    34

 151  GGTGTTAAAAACCTCAAGATTCAACCTTACAAGCTCCCTAATCTTGGACCCCATGATGTTAGAGTCCGGCTGAGG   225
  35   G  V  K  N  L  K  I  Q  P  Y  K  L  P  N  L  G  P  H  D  V  R  V  R  L  R    59

 226  GCTGTTGGCATATGTGGCAGTGATGTTCACCACTTCAAGAACATGAGGTGTGTAGATTTTATAGTTAAAGAGCCA   300
  60   A  V  G  I  C  G  S  D  V  H  H  F  K  N  M  R  C  V  D  F  I  V  K  E  P    84

 301  ATGGTTATTGGGCATGAGTGTGCTGGGATCATAGAGGAAGTTGGGAGTGAGGTCAAGCATTTGGTGCCGGGGGAT   375
  85   M  V  I  G  H  E  C  A  G  I  I  E  E  V  G  S  E  V  K  H  L  V  P  G  D   109
                                                                  Fragment A
 376  CGTGTGGCACTAGAGCCTGGTATCAGTTGCAAGCGATGCAACCTCTGCAAACAAGGCCGGTACAATCTATGCCGC   450
 110   R  V  A  L  E  P  G  I  S  C  K  R  C  N  L  C  K  Q  G  R  Y  N  L  C  R   134

 451  AAGATGAAGTTTTTTGGCTCCCCTCCAAATAATGGTTGTCTGGCAAATCAGGTTGTCCATCCAGGAGATCTATGT   525
 135   K  M  K  F  F  G  S  P  P  N  N  G  C  L  A  N  Q  V  V  H  P  G  D  L  C   159

 526  TTTAAACTGCCAGACAATGTGAGTTTGGAGGAAGGCGCGATGTGTGAGCCCTTAAGTGTTGGTATTCATGCTTGT   600
 160   F  K  L  P  D  N  V  S  L  E  E  G  A  M  C  E  P  L  S  V  G  I  H  A  C   184

 601  CGCCGGGCAAATGTCTGTCAAGAAACAAATGTCTTGGTCGTGGGAGCAGGACCTATAGGACTTGTTACACTGCTA   675
 185   R  R  A  N  V  C  Q  E  T  N  V  L  V  V  G  A  G  P  I  G  L  V  T  L  L   209

 676  GCCGCTCGTGCTTTTGGGGCGCCCCGAATTGTCATTGCGGATGTGAATGACGAGCGTTTGTTGATTGCAAAGAGT   750
 210   A  A  R  A  F  G  A  P  R  I  V  I  A  D  V  N  D  E  R  L  L  I  A  K  S   234
                Fragment B
 751  CTTGGCGCAGATGCAGTCGTTAAGGTTTCAACAAATATTGAGGATGTAGCTGAAGAAGTGGCTAAGATACAAAAG   825
 235   L  G  A  D  A  V  V  K  V  S  T  N  I  E  D  V  A  E  E  V  A  K  I  Q  K   259

 826  GTTTTGGAAAATGGAGTGGATGTAACCTTCGACTGTGCAGGCTTTAACAAAACCATAACAACAGCTTTGAGTGCT   900
 260   V  L  E  N  G  V  D  V  T  F  D  C  A  G  F  N  K  T  I  T  T  A  L  S  A   284

 901  ACTCGTCCCGGAGGCAAAGTTTGCCTTGTGGGAATGGGTCAGAGAGAGATGACTCTCCCTCTCGCTACCAGAGAG   975
 285   T  R  P  G  G  K  V  C  L  V  G  M  G  Q  R  E  M  T  L  P  L  A  T  R  E   309

 976  ATTGATGTAATTGGAATTTTCCGATACCAGAACACATGGCCGCTGTGCCTTGAGTTTCTGAGAAGTGGTAAGATT  1050
 310   I  D  V  I  G  I  F  R  Y  Q  N  T  W  P  L  C  L  E  F  L  R  S  G  K  I   334

1051  GATGTGAAGCCCCTCATAACACATCGGTTTGGATTTTCTCAGAAGGAGGTGGAAGAAGCCTTTGAAACCAGTGCT  1125
 335   D  V  K  P  L  I  T  H  R  F  G  F  S  Q  K  E  V  E  E  A  F  E  T  S  A   359
        Fragment C                                    Fragment D
1126  CGCGGAGGCAATGCCATTAAGGTCATGTTCACCCTATTTCAAGCAAGAAGAATTAAACACTGACGATAAGCGAAA  1200
 360   R  G  G  N  A  I  K  V  M  F  T  L  F  Q  A  R  R  I  K  H  *               379
                           Fragment E
1201  ACCTATTGCCGATGATCTCTCCCCTACAGATTAACGGATTGAATGGAACAAATTTCAGGATCCAATCCAAGTTGT  1275
1276  CTGTGGGGCTGCCCATACTGTTCTTGTTGCAAATGATGGATTTAAGCTGTGGTCTTGGGGGAGAGGAAGGAGTGG  1350
1351  GGTTCTTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1425
1426  AAAAAAAA                                                                      1433
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Fig. 2 The nucleotide and deduced amino acid sequence of NAD-SDH cDNA from apple fruit. Deduced amino acids are shown in the one-letter code. The internal amino acid sequences determined for polypeptides digested with trypsin are underlined.

NAD-SDH with thioredoxin was expressed in the transformed *E. coli* (BL21). The crude extract from this *E. coli* showed the activity of sorbitol oxidation with the reduction of NAD. Further, the amount of NADH produced during the reaction corresponded with that of fructose which is the product of sorbitol oxidation by NAD-SDH (Table 1). The K_m value for sorbitol of this fusion protein (247 mM) was higher than that of purified NAD-SDH from apple fruit (40.3 mM) (Yamaguchi et al. 1994). The conformation of NAD-SDH may be different compared to native NAD-SDH from apple fruit since this fusion protein contained thioredoxin as an extra-protein. The extract from *E. coli* cells harboring plasmid vector (pET-32a) without insert showed no activity of sorbitol oxidation with the reduction of NAD (Table 1). These results indicated that the 42 kDa polypeptide coded by this cDNA had NAD-SDH activity by itself or its polymer.

The total RNAs extracted from apple fruit on 73 d

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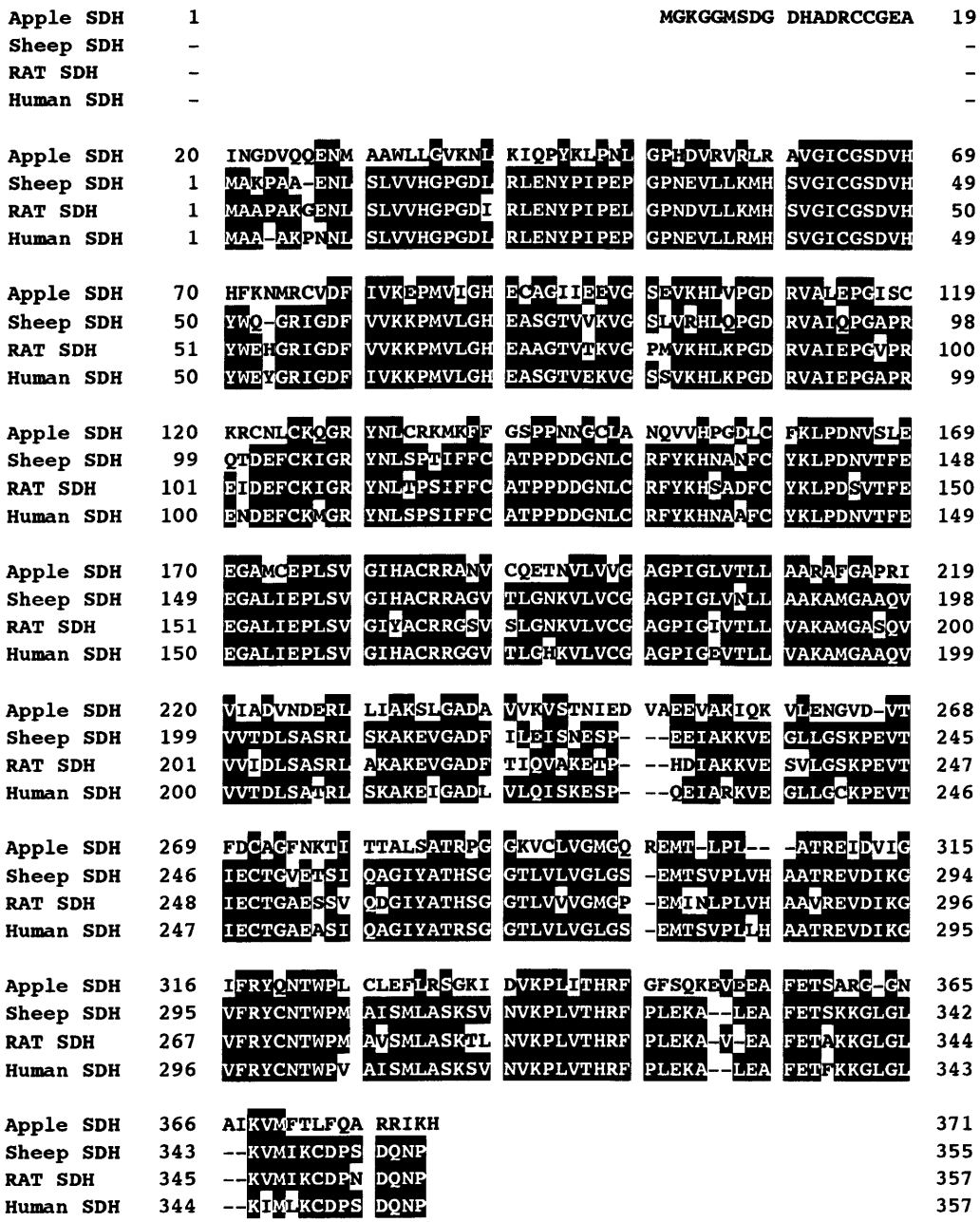

Fig. 3 Comparison of deduced amino acid sequences of apple NAD-SDH cDNA with other NAD-SDHs. Alignment of NAD-SDH from apples, sheep (P07846), rats (P27867) and humans (Q00796). Dashes indicate gaps introduced to maximize alignment. Identical amino acid residues of two or more sequences are represented as white-on-black letters.

after flowering (DAF), 102 DAF and 165 DAF were subjected to electrophoresis. The Northern blot analysis showed a marked increase in the level of NAD-SDH mRNA in the matured fruit (165 DAF) and no detectable signal of NAD-SDH in the young fruit (73 DAF and 102 DAF) (Fig. 4A). The NAD-SDH activity has been reported to be very low in the young fruit and to increase rapidly in the matured fruit (Yamaguchi et al. 1996). Our result of the Northern blot analysis also showed a marked increase of NAD-SDH transcript in the matured apple fruit.

In this study, we isolated and purified NAD-SDH protein from apple fruit according to the work of Yamaguchi et al. (1994), except for staining of the gel with silver-nitrate after SDS-PAGE, and found a 42 kDa polypeptide in addition to the 62 kDa polypeptide they detected by CBB staining. It is clear that the 42 kDa polypeptide has

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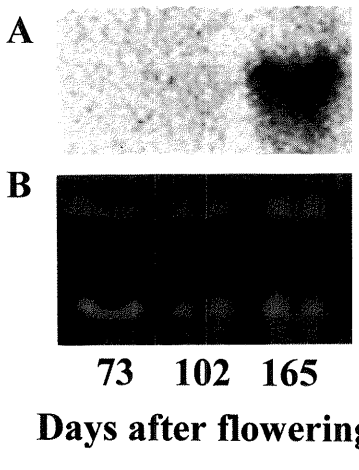

Fig. 4 RNA blot analysis of expression of a gene for NAD-SDH on apple fruit at various growth stages. The total RNAs were extracted from apple fruit at various growth stages (73 DAF, 102 DAF and 165 DAF). Twenty μg each of the total RNAs were subjected to electrophoresis on 1.0% agarose gel in the presence of 0.66 M formaldehyde and transferred to the Hybond-N+ (Amersham, U.K.). The membrane was hybridized to [^{32}P]-labeled NAD-SDH cDNA probe. Hybridization was carried out in 6× SSPE (SSPE; 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 50% formamide, 5% Irish Cream (R. and A. Bailey & Co., Dublin, Ireland), 0.5% SDS and 20 μg ml^{-1} denatured salmon sperm DNA at 42°C for 18 h. The membrane was washed with 2× SSPE/0.1% SDS at 65°C for 1 h. The results of hybridization were analyzed with an imaging analyzer (BAS2000; Fuji Film Co. Ltd., Tokyo, Japan) (shown in A). RNA gel stained with ethidium bromide is shown in B.

NAD-SDH activity by itself or its polymer, but unclear how the 62 kDa polypeptide functions. In the present study, we cloned the full-length cDNA for NAD-SDH from apple fruit and identified its nucleotide sequence. In the future, it is important to clarify the relationship between sugar accumulation and the expression of this gene in fruit and to elucidate the regulatory mechanism of this gene expression.

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References

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* 69: 1408–1412.

Bergmeyer, H.U., Bernt, E., Schmidt, F. and Stock, H. (1974) *Methods of Enzymatic Analysis*. Vol. 3. Edited by Bergmeyer, H.U. p. 1304–1307. Verlag Chemie Weinheim, Academic press Inc., New York.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Carr, I.M. and Markham, A.F. (1995) Molecular genetic analysis of the human sorbitol dehydrogenase gene. *Mammalian Genome* 6: 645–652.

Hellman, U., Wernstedt, C., Góñez, J. and Heldin, C.H. (1995) Improvement of "in-gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* 224: 451–455.

Jeffery, J., Cederlund, E. and Jornvall, H. (1984) Sorbitol dehydrogenase. The primary structure of the sheep-liver enzyme. *Eur. J. Biochem.* 140: 7–16.

Karlsson, C. and Höög, J.O. (1993) Zinc coordination in mammalian sorbitol dehydrogenase. Replacement of putative zinc ligands by site-directed mutagenesis. *Eur. J. Biochem.* 216: 103–107.

Karlsson, C., Jornvall, H. and Höög, J.O. (1991) Sorbitol dehydrogenase: cDNA coding for the rat enzyme. Variations within the alcohol dehydrogenase family independent of quaternary structure and metal content. *Eur. J. Biochem.* 198: 761–765.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lee, F.K., Cheung, M.C. and Chung, S. (1994) The human sorbitol dehydrogenase gene: cDNA cloning, sequence determination, and mapping by fluorescence in situ hybridization. *Genomics* 21: 354–358.

Loescher, W.H., Marlow, G.C. and Kennedy, R.A. (1982) Sorbitol metabolism and sink-source interconversions in developing apple leaves. *Plant Physiol.* 70: 335–339.

Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991) Pumpkin malate synthase. Cloning and sequencing of the cDNA and northern blot analysis. *Eur. J. Biochem.* 197: 331–336.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* 8: 4321–4325.

Nakajima, N., Nakagawa, N. and Imaseki, H. (1988) Molecular size of wound-induced 1-aminocyclopropane-1-carboxylate synthase. from Cucurbita maxima Duch. and change of translatable mRNA of the enzyme after wounding. *Plant Cell Physiol.* 29: 989–998.

Negm, F.B. and Loescher, W.H. (1979) Detection and characterization of sorbitol dehydrogenase from apple callus tissue. *Plant Physiol.* 64: 69–73.

Niimi, T., Yamashita, O. and Yaginuma, T. (1993) A cold-inducible Bombyx gene encoding a protein similar to mammalian sorbitol dehydrogenase. Yolk nuclei-dependent gene expression in diapause eggs. *Eur. J. Biochem.* 213: 1125–1131.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463–5467.

Slater, R.J. (1984) Purification of poly(A)-containing RNA by affinity chromatography. *In* Methods in Molecular Biology. Vol. 2. Edited by Walker, J.M. pp. 117–120. Humana Press Inc., Clifton, New Jersey.

Yamaguchi, H., Kanayama, Y., Soejima, J. and Yamaki, S. (1996) Changes in the amounts of the NAD-dependent sorbitol dehydrogenase and its involvement in the development of apple fruit. *J. Amer. Soc. Hort. Sci.* 121: 848–852.

Yamaguchi, H., Kanayama, Y. and Yamaki, S. (1994) Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit. *Plant Cell Physiol.* 35: 887–892.

Yamaki, S. and Ishikawa, K. (1986) Roles of four sorbitol related enzymes and invertase in the seasonal alteration of sugar metabolism in apple tissue. *J. Amer. Soc. Hort. Sci.* 111: 134–137.

Yamaki, S. and Moriguchi, T. (1989) Seasonal fluctuation of sorbitol-related enzymes and invertase activities accompanying maturation of Japanese pear (*Pyrus serotina* Rehder var. *culta* Rehder) fruit. *J. Japan. Soc. Hort. Sci.* 57: 602–607.

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