Gene Expression of NAD⁺ – Dependent Sorbitol Dehydrogenase and NADP⁺ – Dependent Sorbitol – 6 – Phosphate Dehydrogenase During Development of Loquat (*Eriobotrya japonica* Lindl.) Fruit

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Summary

The aim of this study is to clarify the roles of NAD⁺ – dependent sorbitol dehydrogenase (NAD – SDH) and NADP⁺ – dependent sorbitol – 6 – phosphate dehydrogenase (S6PDH) in fruit development, and the regulatory mechanism(s) underlying their expression during development using loquat as the study material.

The cDNA of NAD – SDH, cloned from loquat fruit, consisted of 1,572 bp and contained an open reading frame of 1,023 bp capable of encoding a protein of 371 amino acids. The deduced amino acid sequence has 97.8% identity to that of an apple fruit.

The activity of NAD – SDH is a function of fruit development, i.e. the increase in protein of this enzyme synchronized consistently with that of activity showing no posttranslational modification throughout the developmental stages. Furthermore, NAD – SDH activity correlated with the mRNA levels, indicating that the key regulatory step of the activity is at the transcriptional level. The increase in NAD – SDH with fruit development plays a dominantly important role during fruit maturation and sugar accumulation.

The trend of S6PDH activity during fruit development paralleled that of NAD – SDH activity. It correlated with protein and mRNA levels revealing that the regulation of the activity is mainly at the transcriptional level like NAD – SDH. However, the role of S6PDH in fruit is not yet clear.

Key Words: cDNA of sorbitol dehydrogenase, gene expression, loquat fruit, sorbitol dehydrogenase, sorbitol – 6 – phosphate dehydrogenase.

Introduction

Sorbitol is the major photosynthetic product and translocating sugar alcohol in Rosaceae plants (Williams et al., 1967). The main enzymes involved in sorbitol metabolism are NADP⁺ – dependent sorbitol – 6 – phosphate dehydrogenase (S6PDH) and NAD⁺ – dependent sorbitol dehydrogenase (NAD – SDH) (Hirai, 1979; Negm and Loescher, 1979). Enzymatic analysis (Negm and Loescher, 1981) and tracer studies (Hansen and Ryugo, 1979) indicate that the ability to synthesize sorbitol is largely confined to leaves. Localization of S6PDH in the chloroplasts and cytosol from apple cotyledon (Yamaki, 1981) is further evidence for this conclusion. On the other hand, sorbitol utilization is mainly associated in sink tissues and mediated by the NAD – SDH (Loescher, 1987). Characterization studies on the activity of S6PDH and NAD – SDH in a number of Rosaceous fruits showed that their relative expression

is in accordance with their source and sink – to – source transition such as in the leaf developmental stage in apple (Loescher et al., 1982) and peach (Merlo and Passera, 1991; Sakanishi et al., 1998), and sink function, respectively.

Contrary to the above results, we found in our previous results (Bantog et al., 1999) that S6PDH activity in loquat fruit, a strong sink organ, was unexpectedly high compared with those in other Rosaceae fruits like apple (Yamaki and Ishikawa, 1986) and Japanese pear (Yamaki and Moriguchi, 1989); it increased concomitantly with fruit maturation. Likewise, NAD – SDH activity consistently increased with fruit development, unlike other Rosaceae fruits in which there was a transient reduction of activity in the midstage. Sorbitol – related enzymes changed seasonally and apparently regulated the sugar levels during the development of loquat fruit. Hence, these findings suggest that NAD – SDH and S6PDH have role(s) unique to the loquat fruit and specific to its developmental stages, and may further imply that the gene expression of said enzymes is developmentally regulated.

The ultimate goal of this undertaking is to understand

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the biochemical roles and the regulatory mechanisms of NAD-SDH and S6PDH expression in loquat. Hence, this study specifically investigated (1) the cDNA clone of NAD-SDH from loquat fruit; and (2) the temporal expression profiles of NAD-SDH and S6PDH in fruits at various stages of development, based on enzymatic activity and levels of protein and mRNA.

Materials and Methods

Plant material

Fruits were harvested on April 28, May 15, 19, 23, 26, 30, and June 2, 1998 from a privately-owned orchard in Minamichita, Aichi Prefecture, Japan. After they were sorted, peeled, halved and seeded, the pulp was frozen in liquid nitrogen and stored at -80°C until used.

Enzyme extraction and assay

Enzyme extraction and assay were carried out at 4°C and 25°C , respectively. The extraction and assay methods of NAD-SDH and S6PDH are described in a previous paper (Bantog et al., 1999).

Soluble protein extraction and protein determination

The protein of fruit tissues was extracted according to Bantog et al. (1999) and the soluble protein was measured by the trichloroacetic acid (TCA) method of Lowry et al. (1951), using bovine serum albumin (BSA) as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed as described by Laemmli (1970) in 10% separating and 4.5% stacking gels. Immunoblotting was done following the method of Towbin et al. (1979). The primary antibodies used were polyclonal antibodies raised in rabbit against the apple NAD-SDH (Yamada et al., 1999) and the apple S6PDH prepared from mouse (Kanayama et al., 1995). Binding of antibodies was detected by the colorimetric reaction with alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G (IgG) (Promega Co., USA) for NAD-SDH and S6PDH analyses, respectively.

RNA extraction and northern blotting

Total RNA was extracted from fruits at various developmental stages following the phenol-SDS method (Nakajima et al., 1988) and the hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Ten μg of total RNA was subjected to electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde, then transferred to a Hybond-N+ membrane (Amersham, Little Chalfont, England). The membranes were hybridized to [^{32}P]-labeled cDNA probes of NAD-SDH and S6PDH prepared from loquat in a solution containing 50%

formamide, 20 X SSPE (3 mM NaCl, 173 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and 25 mM EDTA $\cdot 2\text{Na}$, pH 7.4), 0.5% SDS, 20 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA as the blocking agent, and 5% Irish cream liqueur (R. and A. Bailey & Co., Dublin, Ireland) at 37°C for 18 hr. The blots were washed with 2 X SSPE plus 0.1% SDS twice at room temperature for 15 min and twice again with the same solution at 55°C for 30 min. The results of hybridization were visualized with an image analyzer (BAS 2000; Fuji Film Co., Ltd., Tokyo, Japan). The same blot was used for NAD-SDH and S6PDH hybridization. The cDNA probes were prepared through polymerase chain reaction (PCR) using loquat cDNA as the template, and oligonucleotide primers (underlined in Fig. 1) designed according to apple NAD-SDH cDNA (Yamada et al., 1998) and apple S6PDH cDNA (Kanayama et al., 1992, 5'-CGAGATGCCGGTCATC-GGTCTC-3' and 5'-TGGAAGGTAGACTGGTACG-ATAC-3'), respectively.

Cloning of NAD-SDH cDNA from loquat fruit

Sense and antisense primers designed according to apple NAD-SDH (Yamada et al., 1999) (underlined in Fig. 1) were used for reverse transcription and polymerase chain reaction (RT-PCR) in the presence of poly(A)⁺ RNA isolated from loquat fruit. Gene specific primers were newly synthesized from the nucleotide sequence of the PCR product; a rapid amplification of cDNA ends (RACE) PCR (Frohman, 1993) was carried out to determine the 5'- and 3'-ends of the PCR product. The clones were sequenced by the ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster, CA, USA) using an automatic DNA sequencer (Model 373, Perkin-Elmer).

Results

Nucleotide and amino acid sequence of NAD-SDH cDNA

One fragment from poly(A)⁺ RNA isolated from loquat fruit was cloned using oligonucleotide primers designed according to apple NAD-SDH. Using RACE-PCR, the full length of cDNA sequence was determined (Fig. 1). The cDNA of NAD-SDH consisted of 1,572 bp and contained an open reading frame of 1,023 bp, capable of encoding a protein of 371 amino acids.

Correlation between the activity, protein and transcript of NAD-SDH during fruit development

The activity of NAD-SDH increased concomitantly with maturation, i.e. it was low at the beginning of fruit development, attained its peak on May 26, slightly decreased on May 30 then increased again on June 2 to a level higher than previous samples except that taken on May 26 (Fig. 2A). Immunological assay, done using soluble protein based on g FW, revealed that the apple NAD-SDH antibody cross-reacted variably with the

1	TGAGTCCGAGAAAACAAAGAGAGAAAGATGGGAAAGGGAGGCATGTCTGATGGAGATCATGCTGATCGCTGTTGT	75
1	M G K G G M S D G D H A D R C C	16
76	GGGGAAGCAATAAATGGCGATGTTCAACAAGAGAACATGGCTGCTTGGCTTCTTGGTGTAAAAACCTCAAGATT	150
17	G E A I N G D V Q Q E N M A A W L L G V K N L K I	41
151	CAACCTTACAAGCTCCCTAATCTTGGACCCCATGATGTTAGAGTCCGGCTGAAGGCTGTTGGCATATGTGGCAGT	225
42	Q P Y K L P N L G P H D V R V R L K A V G I C G S	66
226	<u>GATGTT</u> CACCACTTCAAGAACATGAGGTGTGTAGATTTTATAGTTAAAGAGCCAATGGTTATTGGGCATGAGTGT	300
67	D V H H F K N M R C V D F I V K E P M V I G H E C	91
301	GCTGGGATCATAGAGGAAGTTGGGAGTGAGGTGCGAGGATTTGGTGCCAGGCGATCGTGTGGCACTAGAGCCTGGT	375
92	A G I I E E V G S E V E D L V P G D R V A L E P G	116
376	ATCAGTTGCAAGCGATGCAACCTCTGCAAAACAAGGCCGGTACAATCTATGCCGCAAGATGAAGTTTTTGGTTC	450
117	I S C K R C N L C K Q G R Y N L C R K M K F F G S	141
451	CCTCCAAATAATGGTTGTCTGGCAAATCAGGTGTCCATCCAGGAGATCTATGTTTTAACTGCCAGACAATGTG	525
142	P P N N G C L A N Q V V H P G D L C F K L P D N V	166
526	AGTTTGGAGGAAGGCGCGATGTGTGAGCCCTTAAGTGTGGTATTCATGCTTGTGCGCGGCAAATGTCTGTCAA	600
167	S L E E G A M C E P L S V G I H A C R R A N V C Q	191
601	GAAACAAATGTCTTGGTCGTGGGAGCAGGACCTATAGGACTGTTACACTGCTAGCCGCTCGTGCTTTTGGGGCG	675
192	E T N V L V V G A G P I G L V T L L A A R A F G A	216
676	CCCCGAATTGTCATTGCGGATGTGAATGACGAGCGTTTGTGCGATTGCAAAGAGTCTTGGCGCGGATGAAGTCGTT	750
217	P R I V I A D V N D E R L S I A K S L G A D E V V	241
751	AAGGTTTCAACAAATATTGAGGATTTAGCTGAGGAAGTGGCTACGATACAAAAGGTTTTGGAAAATGGAGTGGAT	825
242	K V S T N I E D L A E E V A T I Q K V L E N G V D	266
826	GTAAGCTTCGACTGTGCAGGCTTTGACAAAACATAACAACAGCTTTGAGTGCTACTCGTCCCGGAGGCAAAGTT	900
267	V S F D C A G F D K T I T T A L S A T R P G G K V	291
901	TGCCTTGTGGGAATGGGTCAGAGAGAGATGACTCTCCCTCTCGCGACCAGAGAGATTGATGTAATTGGAATTTTC	975
292	C L V G M G Q R E M T L P L A T R E I D V I G I F	316
976	CGATACCAGAACACATGGCCCCCTGTGCCTTGAGTTTCTGAGAAGTGGTAAGATTGATGTGAAGCCCCCTCATAACA	1050
317	R Y Q N T W P L C L E F L R S G K I D V K P L I T	341
1051	<u>CATCGG</u> TTTGGATTCTCTCAGAAAGGAGGTGGAAGAAGCCTTTGAAACCAGTGCTCGCGGAGGCAATGCCATTAAG	1125
342	H R F G F S Q K E V E E A F E T S A R G G N A I K	366
1126	GTCATGTTTAACTGTAAAATCGATCGGATACAAGGATATTTGTCATTGGACCAATCTTTGGTGTGTTTTTG	1200
367	V M F N L *	371
1201	TTTGCCCTTAGTAATTTGGGGTTTCTTTTTGGGAATGTGTTATTGGCACTCCAAAAATTCATTTTGTATTCCAAA	1275
1276	CTTCTACAATTATAAAGAAAAATACACCTGTGAGGAGTGTAGAATGAGATTTGTGAAGTGCTAATAACAGTTTC	1350
1351	CAAGTTAGTAATTTGGGTTTGTGGTTCTCTTGGTGTGTACTTATTATTAACCTTTTTTTTCTCTTTTTTTTAA	1425
1426	AAATTAGTTGTCATCATGAACAATTTAGAAATTTGCTTGTGTGCATGTAGTGAATTATGATGTCGGATAAGCTTT	1500
1501	TTTCTTCTACTCAATGCGATCTGGGTTCAAACCTTCGCTTTTCTTAATGTAATAATATTGTTTACCAAAAT	1572

Fig. 1. The nucleotide and deduced amino acid sequences of loquat NAD-SDH cDNA. The nucleotide sequence is shown in the upper line. Deduced amino acids (lower line) are shown in the one-letter code. * means the stop codon. Oligonucleotide primers for PCR are underlined.

NAD-SDH protein of loquat fruit extracted at various stages of development (Fig. 2B). No trace of this protein was detected prior to May 19, but its expression which began on May 23, increased to a maximum level on May 26. The level of translated proteins decreased on May 30

and June 2; the final level was comparable or slightly higher than that of May 23. The steady-state levels of NAD-SDH mRNA fluctuated between various stages of fruit development (Fig. 2C). Transcript was not detected on April 28 to May 19, but occurred in a significant

Fig. 2. Fluctuation in gene expression of NAD-SDH in developing loquat fruits. (A) Enzyme activity during fruit development. Data are means $\pm$ SE of 2 replications; SE bars are present only when larger than the symbols. (B) Immunoblot analysis of NAD-SDH protein. Soluble protein equivalent to 40 mg FW was cross-reacted with the polyclonal antibody against apple NAD-SDH after SDS-PAGE. (C) Northern blot analysis of NAD-SDH mRNA. Total RNA (10 μ g.lane⁻¹) was hybridized with the radiolabeled NAD-SDH cDNA probe from loquat. (D) Ethidium bromide-stained RNA gel.

amount from May 23; its level remained the same until May 26. The transcript reached its maximum level on May 30 and then decreased slightly by June 2.

Correlation between the activity, protein and transcript of S6PDH during fruit development

S6PDH activity increased with maturation but decreased as the fruit ripened, i.e. the activity was low on April 28 until May 19 but increased progressively from May 19 until May 26 when it reached its highest level (22-fold higher). Subsequently, its activity decreased slightly at harvest on June 2 (Fig. 3A). The S6PDH antibody showed varied degrees of cross-reactivity throughout fruit development according to the western blotting data (Fig. 3B). There was relatively weak or no immunoreaction in fruits sampled on April 28 until May 19, but the protein bands became visible on May 23. After the highest protein amount occurred on

Fig. 3. Fluctuation in gene expression of S6PDH in developing loquat fruits. (A) Enzyme activity. Experimental conditions and samples used were the same as those in Fig. 2. (B) Immunoblot analysis of S6PDH protein. Soluble protein equivalent to 10 mg FW was cross-reacted with the polyclonal antibody raised against apple S6PDH after SDS-PAGE. (C) Northern blot analysis of S6PDH mRNA. Total RNA (10 μ g.lane⁻¹) was hybridized with radiolabeled S6PDH cDNA probe from loquat.

May 26, the band intensity subsequently decreased through May 30 to June 2 although these 2 are higher than that of May 23. Note that there are two protein bands depending on the developmental stage; the intensities of the bands varied noticeably from May 23 to June 2. The steady-state levels of S6PDH mRNA in fruits at different sampling dates (Fig. 3C) reveal that no evident hybridization of S6PDH cDNA probe occurred with the RNA samples from April 28 to May 19. A low signal was detected on May 23; the mRNA started to accumulate on May 26 yielding the highest signal on May 30. The transcript level subsequently decreased on June 2, but it remained higher than that on May 23.

Discussion

The cDNA clone of NAD-SDH was isolated earlier from apple fruit but the homology of its deduced amino acid with those of animals and human was comparatively low (Yamada et al., 1998). Therefore, data of cDNA clones from other sources, especially, Rosaceae plants are necessary to compare gene structure. To date, only cDNA from the above mentioned apple fruit was the first to be reported. Hence, the cDNA isolated from loquat fruit in this study is the second cloning of NAD-SDH. The deduced amino acid sequence of loquat NAD-SDH has 97.8% identity to that of apple (data not

shown).

NAD-SDH activity increased with fruit maturation (Fig. 2A) and the marked rise from May 23 to May 26 is responsible for the low sorbitol concentration in fruit (Bantog et al., 1999). The level of NAD-SDH protein correlates well with its enzymatic activity (Fig. 2A). Both NAD-SDH activity and its protein which were low until May 19 reached their highest levels on May 26 (Fig. 2B). This same correlation was observed in 'Fuji' apple fruit (Yamada et al., 1999). In both studies, no posttranslational modification apparently occurred. Northern blot analysis indicates that NAD-SDH mRNA was actively transcribed in mature but not in young fruits (Fig. 2C), similar to apples (Yamada et al., 1998). Except for the decrease from May 26 to June 2, changes in NAD-SDH activity and protein levels were closely correlated with the accumulation of transcripts (Fig. 2C). Likewise, Yamada et al. (1999) reported that NAD-SDH mRNA correlated well with the protein concentrations and the enzyme activity in 'Fuji' apple fruit. We found a similar correlation between mRNA with its protein and enzyme activity, suggesting that transcriptional ability may be the key regulatory step in the developmental occurrence of NAD-SDH activity.

NAD-SDH gene is several times more plentiful in mature than in young fruits which indicates that NAD-SDH plays a temporal and important role in its sink function by providing carbon from sorbitol oxidation and the sustained import of sorbitol into the fruit as the carbon source required for cell enlargement and sugar accumulation at the later stage of fruit growth. Since loquat is a sorbitol-translocating plant, our findings suggest that NAD-SDH may be an indicator of sink strength; as such, it may control the sorbitol export from the source leaves, the sorbitol import by sink cells and the biochemical fate of other carbohydrates as well.

The increase in S6PDH activity (Fig. 3A) with fruit maturation has prompted us to corroborate it with the protein amount (Fig. 3B) and mRNA level (Fig. 3C). The trends in the activity and protein level paralleled each other, so that the profiles correlated well as did S6PDH during leaf ontogeny in loquat (Hirai, 1983). S6PDH activity, which is under tight developmental control and consistently regulated by the amount of protein, suggests the absence of posttranslational modification during loquat fruit development. Furthermore, the mRNA level (Fig. 3C) corresponded with the activity and protein level throughout the developmental stages. The close correlation between the enzyme activity, protein, and transcript levels indicates that the S6PDH activity may be highly regulated at the transcript level during fruit development as was S6PDH in developing peach leaves (Sakanishi et al., 1998). The increase of S6PDH activity which parallels with fruit maturation implies that it contributes to sugar accumulation. However, the mechanism underlying this uptake by cells is unknown. The expression of S6PDH gene at a high

level in fruit (Fig. 3C) may substantiate and support earlier enzymatic studies that sorbitol may be synthesized in the fruit itself (Hirai, 1980) and S6PDH may not be restricted to green tissues (Berüter, 1985). It is possible that glucose-6-phosphate present in the fruit, is converted to sorbitol-6-phosphate by S6PDH and finally converted to sorbitol by phosphatase. Furthermore, two distinct bands were detected in fruits although in peach leaves (Sakanishi et al., 1998) and apple seedlings (Kanayama et al., 1995), only one distinct band at 36 kDa was detected by immunoblotting. The bottom band in Fig. 3B which is unique only in loquat fruits may be responsible for the induction of S6PDH activity in maturing fruits (Fig. 3A). However, there are no reports of S6PDH isozymes to date. Thus, whether the two S6PDH bands in loquat fruit indicate the presence of isozymes awaits further verification.

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ビワ果実の生長・成熟に伴う NAD⁺ 依存性ソルビトール脱水素酵素と NADP⁺ 依存性ソルビトール-6-リン酸脱水素酵素の遺伝子発現

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摘 要

ビワ果実の生長・成熟に伴う NAD⁺ 依存性ソルビトール脱水素酵素 (NAD-SDH) と NADP⁺ 依存性ソルビトール-6-リン酸脱水素酵素 (S6PDH) の遺伝子レベルでの発現調節を検討した。

最初に NAD-SDH の cDNA 遺伝子をビワ果実から単離した。cDNA の全長塩基配列は 1572bp, そのタンパク質は 371 個のアミノ酸残基からなり, リンゴ果実のものと 97.8% の同一性があった。

NAD-SDH 活性は果実の生長・成熟の進行とともにその発現が調節されており, そして, そのタンパク質の変動は活性の変動と一致し, 翻訳後の修飾は認められなかった。さらに,

そのタンパク質の変動は mRNA の発現量の変動とも一致し, この酵素活性の主要な制御段階は mRNA 合成レベルであることを示唆した。成熟に伴う NAD-SDH の遺伝子レベルでの発現は, 果実の成熟および糖の集積に特に重要であることを示した。

果実の生長・成熟に伴う S6PDH 活性の変動は NAD-SDH 活性の変動と同様な傾向を示した。その活性の変動はタンパク質及び mRNA レベルでの変動と一致し, NAD-SDH と同様この酵素活性は主に mRNA 合成レベルで調節されていることを示唆した。しかしながら, 果実での S6PDH の役割についてはまだ明らかではない。