

Changes in the Amounts of the NAD-dependent Sorbitol Dehydrogenase and Its Involvement in the Development of Apple Fruit

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Abstract. Seasonal changes in the amounts of the NAD-dependent sorbitol dehydrogenase (NAD-SDH) (enzyme code, 1.1.1.14) protein in developing apple (*Malus pumila* Mill var. *domestica* Schneid) fruit were determined by immunoblotting analysis. The amounts of the enzyme protein were very low in young fruit and rose as fruit matured. The weak correlation between enzyme protein and NAD-SDH activity and also the changes in NAD-SDH specific activity suggested that there could be posttranslational modification to the pre-existing enzyme or isoenzyme(s) of NAD-SDH. The changes in the amounts of NAD-SDH protein did not show the same pattern as those in relative growth rate, which is used to express sink activity, especially in young fruit. The role of NAD-SDH on sink activity in apple fruit, therefore, could not be explained simply by the amount and activity of the enzyme. In young fruit, it seems that enzymes other than NAD-SDH would be more directly related with fruit growth.

Sorbitol is the primary photosynthetic product and is the major translocated sugar in many species of the Rosaceae including peach, pear, and apple (Loescher, 1987; Zimmermann and Zeigler, 1975). NAD-dependent sorbitol dehydrogenase (NAD-SDH) is found primarily in sink tissue and is responsible for the oxidation and eventual use of sorbitol (Loescher, 1987). Extraction and characterization of the enzyme have been attempted (Doehlert, 1987; Kuo et al., 1990; Negm and Loescher, 1979), and the enzyme recently was purified from plant tissue (Yamaguchi et al., 1994), allowing molecular approaches to determine its role and function. NAD-SDH is an important regulator of sorbitol metabolism (Yamaguchi et al., 1994) and has been reported in developing Japanese pear (Yamaki and Moriguchi, 1989) and apple (Berüter, 1985; Yamaki and Ishikawa, 1986) fruit. Immunoblotting analysis using antibodies raised the previously purified enzyme to characterize the amount of the enzyme protein in developing fruit could help to describe sorbitol metabolism in sink tissue.

Fruit growth typically is described as a single- or double-sigmoid curve, reflecting changes in organ fresh weight (FW) (Schechter et al., 1993). Most fruit development studies are concerned primarily with FW increase, so growth rates have been measured mostly by increase in fruit diameter or FW (DeJong and Goudriaan, 1989). The factors that contribute to sink activity are not well understood, but regulation of sink activity at the tissue or cellular level could occur during carbohydrate unloading from the phloem, during uptake and membrane transport of carbohydrate by sink cells, or at points of metabolic conversion (Daie, 1985; Ho, 1988). Relative growth rate (RGR) has been used to express sink

activity (Archbold, 1992) and to give a more complete picture of the growth pattern (Archbold, 1992; DeJong and Goudriaan, 1989; Schechter et al., 1993). By measuring fruit dry weight (DW) RGR could be determined.

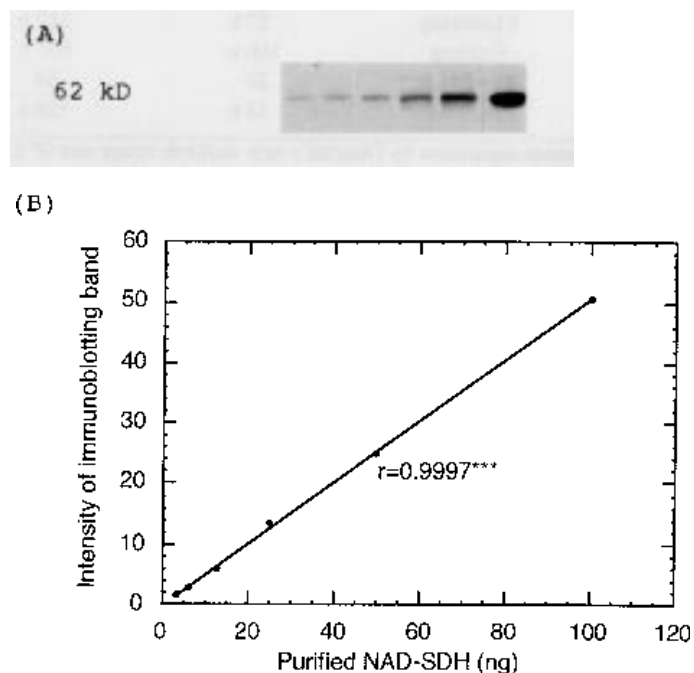


Fig. 1. Detection of NAD-SDH in the purified enzyme preparation by immunoblotting analysis. (A) The intensity of the immunoblotting band. From left to right 3.13, 6.25, 12.5, 25, 50, or 100 ng of the purified NAD-SDH was loaded on each lane. (B) The ordinate shows the intensity of the immunoblotting band calculated by the computer system. Correlation coefficient significant at $P = 0.001$.

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Our objective was to measure the amount of the NAD-SDH protein during development of apple fruit relative to fruit growth rates.

Materials and Methods

Plant materials. 'Orin', 'Alpsotome', 'Jonathan', and 'Jonagold' apple fruit were harvested at the orchard of the Fruit Tree Research Station; Ministry of Agriculture, Forestry, and Fisheries; Morioka;

Iwate; Japan. Full bloom of 'Orin' occurred on 12 May 1994 and that of 'Alpsotome', 'Jonathan', and 'Jonagold' occurred on 13 May 1994. Trees received routine horticultural care, including pruning, fruit thinning, fertilization, irrigation, and pest control.

Enzyme extraction and assay. Fruit samples ranging from 20 to 50 g fresh weight were used for extraction of the enzyme. Peeled and cored apple flesh was homogenized in 0.15 M KH_2PO_4 -NaOH buffer (pH 8.0) that contained 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Na-L-ascorbate, 10 mM 2-mercaptoethanol, and 5% PolyclarSB-100 (Gokyo Sangyo Co., Tokyo). The homogenate was squeezed through a layer of fine cloth and the filtrate was centrifuged at $13,000\times g$ for 20 min. The supernatant was collected as a crude extract and stored at -80°C until it was used as the source of NAD-SDH for immunoblotting analysis for apple fruit.

NAD-SDH activity was barely detectable in the crude extract, so partial purification was performed to obtain NAD-SDH for the assay of enzymatic activity for apple fruit. The crude extract was passed through a column of Sephadex G-25 (Pharmacia Co., Uppsala, Sweden) to remove phenolic compounds. The filtrate was brought to 40% saturation with $(\text{NH}_4)_2\text{SO}_4$ then centrifuged at $13,000\times g$ for 20 min. The supernatant was mixed with Butyl-Toyopearl 650 $^\circ\text{C}$ (Tosoh Co., Tokyo), which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) plus $(\text{NH}_4)_2\text{SO}_4$ to 40% saturation, 0.2 mM PMSF, and 2 mM 2-mercaptoethanol to adsorb proteins to the resin. The Butyl-Toyopearl 650 $^\circ\text{C}$ with the adsorbed proteins was packed in a column and proteins were eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM PMSF and 2 mM 2-mercaptoethanol. Active fractions were collected and used as the source of NAD-SDH for the assay of NAD-SDH activity in apple fruit. The activity of NAD-SDH was determined spectrophotometrically by following the reduction of NAD in the presence of sorbitol at 340 nm as described by Yamaguchi et al. (1994). All assays were performed at 25°C . All the values of the assays of NAD-SDH activity and the amount of the

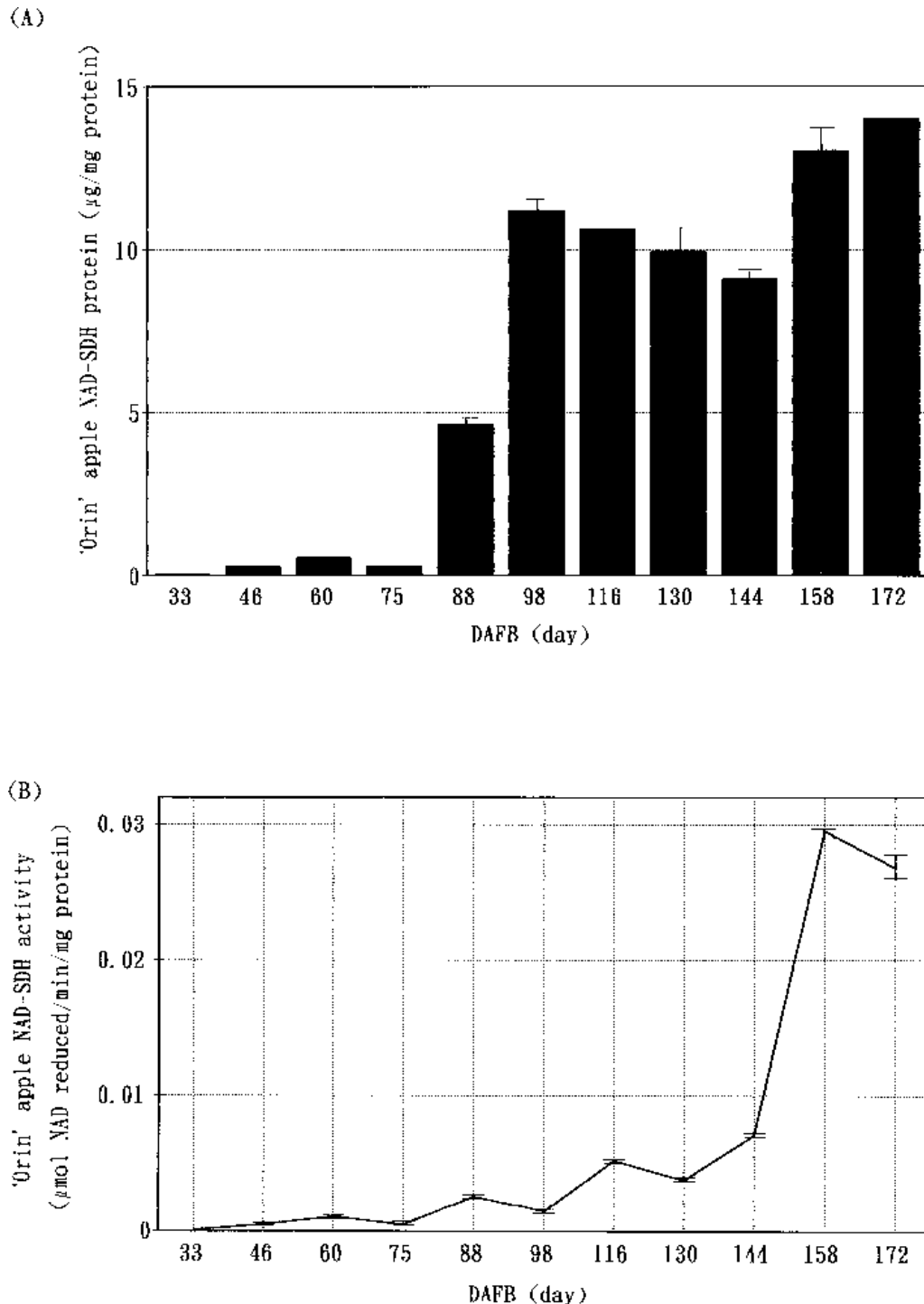


Fig. 2. The seasonal change in the amount of the NAD-SDH protein and NAD-SDH activity per milligram of extracted protein in 'Orin' apple fruit. (A) The amount of the NAD-SDH protein; each point is the mean \pm SE of three experiments. (B) NAD-SDH activity; each point is the mean \pm SE of three experiments.

NAD-SDH protein were based on three replicate samples from at least six fruit.

Quantitation of protein. Protein content was determined using bovine serum albumin as the standard (Read and Northcote, 1981).

Making of polyclonal antibodies. NAD-SDH was purified by the method described previously (Yamaguchi et al., 1994). Purified enzyme was stored at -20°C until used to obtain antiserum. Antiserum was obtained by injecting mice with the homogenous NAD-SDH emulsified with Freund's complete adjuvant (the first injection), with Freund's incomplete adjuvant (the second injection), and without adjuvant (the third injection). Twenty micograms of the NAD-SDH protein was injected biweekly into each mouse. The antiserum obtained showed a single immunconjugate band with the purified NAD-SDH.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis. SDS-PAGE on a 10% slab gel with a 4.5% stacking gel was performed by the method described by Laemmli (1970). Immunoblotting was performed by the method described by Towbin et al. (1979). Polypeptides were electroblotted onto nitrocellulose membrane for 1.5 h at 9 V. The membrane was blocked for 45 min in Tris-buffered saline containing 0.05% Tween-20 (TBST) that contained 3% nonfat dry milk and incubated for 1 h at room temperature with the mouse anti-apple NAD-SDH antiserum. After being washed with TBST, the membrane again was incubated for 30 min at room temperature with goat anti-mouse immunoglobulin G. Visualization of the immunconjugate was performed with 33 μl of 5-bromo-4-chloro-3-indolyl phosphate, 66 μl of nitroblue tetrazolium, and 40 μl of 1 M MgCl_2 in 10 ml of Tris-HCl buffer. There was only one band specifically detectable with the antiserum. The images of the immunoblotting bands were produced by a charge coupled device (CCD) image sensor camera, and the intensity of the immunoblotting bands were calculated by a computer system (ACI Japan, Tokyo).

Determinations of FW, DW, and RGR. FW, DW, and RGR were analyzed quantitatively. For DW determinations, fruit was cut into sections and oven-dried at 75°C to a constant weight before being weighed. RGR was calculated using the following formula: $\text{RGR} = (\ln \text{DW}_2 - \ln \text{DW}_1) \div (T_2 - T_1)$; where T_2 and T_1 represent days after full bloom (DAFB) on each sampling date (Hunt, 1982; Schechter et al., 1993). All the values of FW and DW were based on at least eight fruit.

Results

Construction of a calibration curve for the amount of NAD-SDH protein. The amount of the NAD-SDH protein was determined by immunoblotting. A calibration curve for NAD-SDH was produced using purified apple NAD-SDH as the standard (Fig. 1 A and B). The intensity of the immunoblotting bands was correlated ($r = 0.9997$) with the amount of the NAD-SDH protein, which indicates that the amount of the NAD-SDH protein can be determined quantitatively by the intensity of the immunoblotting bands. These bands were equivalent to 0 to 100 ng NAD-SDH protein.

Changes in the amount of the NAD-SDH protein and NAD-SDH activity in developing 'Orin' apple fruit. Changes in the amount of the NAD-SDH protein in developing 'Orin' apple fruit were examined using antibodies raised against apple NAD-SDH. The amount of the NAD-SDH protein per milligram of total protein was low in young fruit, rose rapidly on 88 DAFB; then remained at a higher level from 98 DAFB through the end of the experiment (Fig. 2A). NAD-SDH activity per milligram of protein was low in young fruit and then increased gradually as fruit developed, with a rapid rise 144 DAFB (Fig. 2B). NAD-SDH specific activity was

Table 1. NAD-SDH specific activity in 'Orin' apple fruit during the 1994 growing season.

DAFB ²	NAD-SDH specific activity ($\mu\text{mol NAD reduced}/\text{min}/\mu\text{g NAD-SDH protein}$)
33	0.238
46	0.179
60	0.196
75	0.179
88	0.056
98	0.013
116	0.049
130	0.038
144	0.078
158	0.227
172	0.191

²Days after full bloom.

lower between 88 and 144 DAFB than either earlier or later (Table 1).

Changes in the amount of the NAD-SDH protein and RGR in developing 'Orin' apple fruit. 'Orin' apple increased to a maximum FW and DW of 240 and 39 g, respectively, by 172 DAFB (Fig. 3A). The amount of the NAD-SDH protein per gram of DW was initially low but started to rise 88 DAFB and remained high starting from 98 DAFB, except for a slight decrease 144 DAFB (Fig. 3B).

RGR decreased from 46 to 172 DAFB, but the decrease was not uniform (Fig. 3C). RGR was low 98 and 116 DAFB but increased 130 DAFB before declining again.

Changes in the amounts of the NAD-SDH protein and RGR in developing fruit of other apple cultivars. Total FW and DW accumulation varied among cultivars (Fig. 4A). 'Jonagold' showed the highest total FW and DW accumulation, while 'Alpsotome' showed the lowest total FW and DW accumulation, with intermediate FW and DW accumulation in 'Jonathan'. In all the cultivars studied, the amounts of the NAD-SDH protein were low in young fruit and became higher with fruit maturation. Amounts of the NAD-SDH protein per gram of DW also were higher in fruit with higher total FW and DW accumulation (Fig. 4B). RGRs for the three cultivars were fairly similar and showed the same general pattern of decrease in the time seen with 'Orin' (Fig. 4C).

Discussion

Sorbitol accounts for $\approx 80\%$ of the total soluble carbohydrate in apple leaves, spurs, and peduncles but only $\approx 3\%$ to 8% of soluble carbohydrate in the fruit throughout the growing season. This means that imported sorbitol into fruit is not stored as sorbitol but converted to other metabolites or consumed as an energy source. Fructose is the main sugar accumulated in the fruit, comprising 45% to 60% of the total fruit soluble carbohydrate. Lack of sorbitol in the mature fruit has been attributed to the high fruit 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 plants of the Rosaceae (Knee, 1993; Loescher, 1987; Yamaguchi et al., 1994; Yamaki and Ishikawa, 1986; Yamaki and Moriguchi, 1989). The change in the amount of the NAD-SDH protein in developing 'Orin' apple fruit was examined (Fig. 2A). A great deal of accumulation of the NAD-SDH protein appeared to precede (Fig. 2A) the rapid rise in NAD-SDH activity late in development

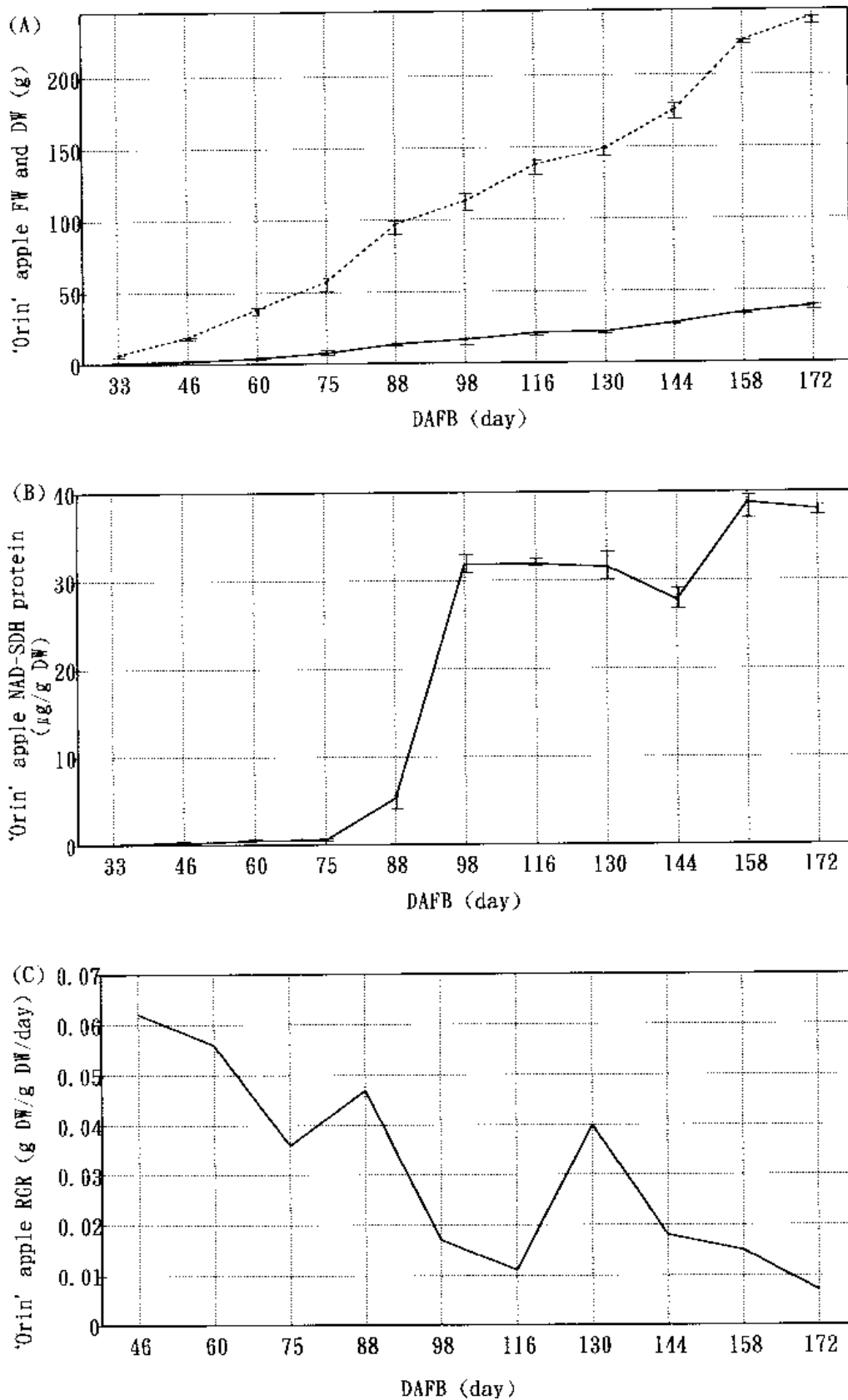


Fig. 3. Growth characteristics and the seasonal change in the amount of the NAD-SDH protein per gram of dry weight (DW) and relative growth rate (RGR) in 'Orin' apple fruit. (A) fresh weight (•••; FW) and DW (—). Vertical bars are the SE of the means of at least six fruit. (B) The amount of the NAD-SDH protein. Vertical bars are the SE of the means of three experiments. (C) RGR.

(158 and 172 DAFB) (Fig. 2B). These data seem to imply that NAD-SDH activity could be regulated by activation or inactivation of the pre-existing enzyme. NAD-SDH specific activity showed apparent changes during development of 'Orin' apple fruit (Table 1), suggesting posttranslational modification to the pre-existing enzyme or existence of isoenzyme(s) of NAD-SDH. However, no indication of isoenzyme(s) of NAD-SDH was detected in the purification process (Yamaguchi et al., 1994).

NAD-SDH protein per gram of DW (Fig. 3B) increased rapidly after 88 DAFB, a similar pattern to that seen in Fig. 2A when expressed on a per-unit protein basis; however, RGR (Fig. 3C) in 'Orin', apple fruit declined over time. Similar patterns were seen in other apple cultivars ('Alpsotome', 'Jonathan', and 'Jonagold') differing in fruit size (Fig. 4 B and C). These changes in NAD-SDH concentrations over time were not correlated with RGR of apple fruit, especially in young fruit. Beside sorbitol, sucrose has translocated into apple fruit (Zimmermann and Zeigler, 1975); however, it has been unclear how the concentration of sorbitol and sucrose in the phloem exudate changes through the developmental season of apple. If sorbitol is a main translocated sugar in young fruit, sorbitol oxidase converting sorbitol to glucose seems to be functioning since the activity in young fruit is higher than in older fruit (Yamaki and Ishikawa, 1986). If sucrose is a main translocated sugar in young fruit, acid invertase must be functioning because high activity is maintained compared to mature fruit (Yamaki and Ishikawa, 1986). Sucrose synthase also is important for sucrose unloading. These enzymes may be more directly correlated with changes in growth rates of apple fruit than NAD-SDH. Why NAD-SDH activity increased drastically as fruit matured and how posttranslational regulation of NAD-SDH occurs will have to be solved by further study.

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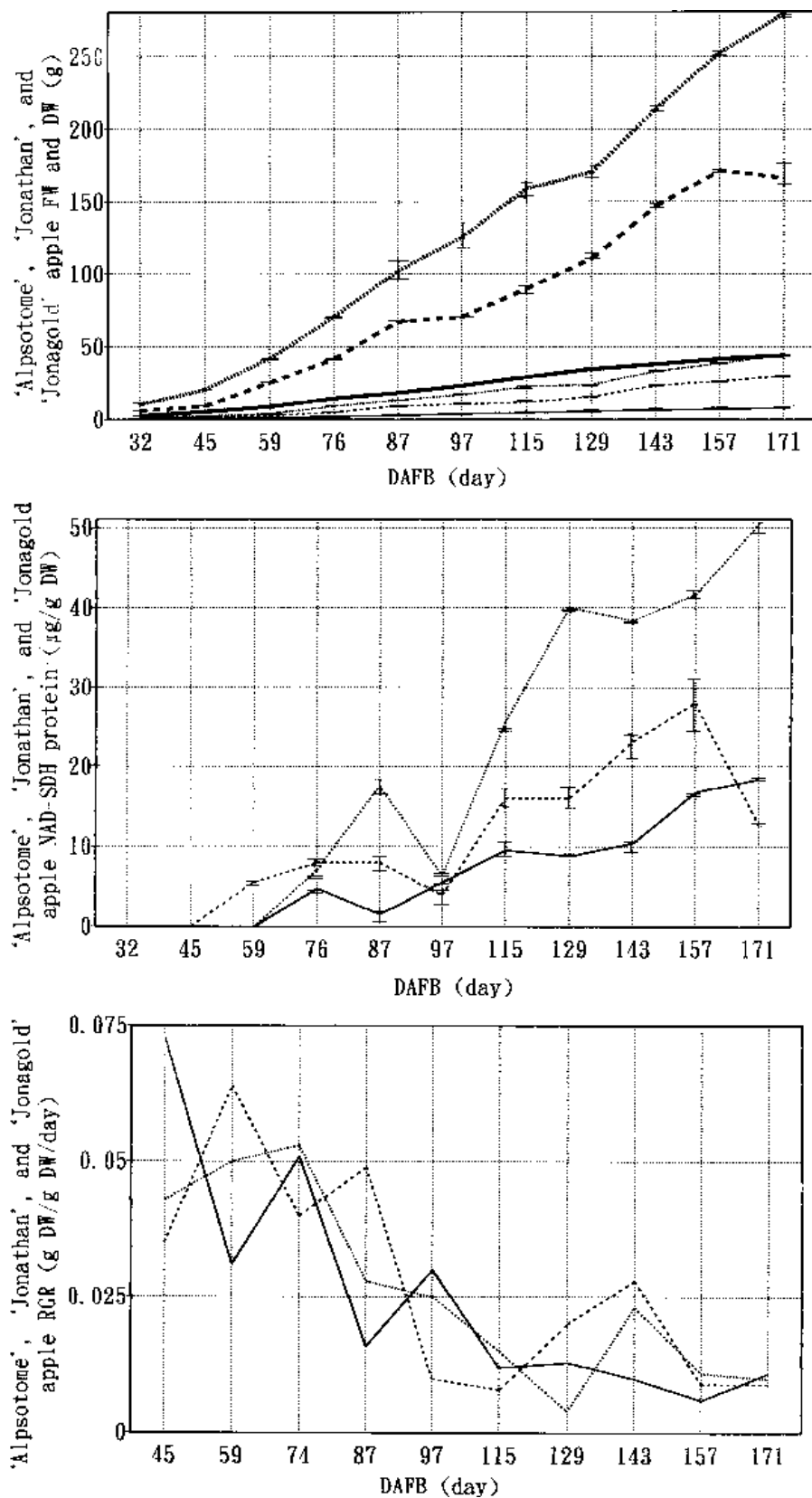


Fig. 4. Growth characteristics and the seasonal changes in the amounts of the NAD-SDH protein per gram of dry weight (DW) and relative growth rate (RGR) in 'Alpsotome', 'Jonathan', and 'Jonagold' apple fruit. (A) Fresh weight (FW) and DW. (B) The amounts of NAD-SDH protein. Vertical bars are the SE of the means of three experiments. (C) RGR 'Alpsotome' (—), 'Jonathan' (·····), and 'Jonagold' (•••••).

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