ニホンナシ果実(Pyrus serotina Rehder var.culta Rehder)の成熟に伴うソルビトール関連酵素とインベルターゼ活性の季節変動

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Seasonal Fluctuation of Sorbitol-related Enzymes and Invertase Activities Accompanying Maturation of Japanese Pear (*Pyrus serotina* Rehder var. *culta* Rehder) Fruit

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Summary

Activities and roles of the sorbitol-related enzymes, NAD^+-dependent sorbitol dehydrogenase, sorbitol oxidase, $NADP^+$-dependent sorbitol dehydrogenase and sorbitol-6-P dehydrogenase, and acid invertase were studied in Japanese pear (*Pyrus serotina* Reder var. *culta* Rehder) fruit. NAD^+-dependent sorbitol dehydrogenase that converts sorbitol to fructose had the highest activity of the 4 enzymes throughout the developing season. The activity rose in June, decreased with fruit expansion, and increased again with fruit maturation. Fluctuation in the enzyme activity correlated with the accumulation of fructose in immature fruit. This enzyme was suggested to be important for sorbitol metabolism and sugar accumulation in Japanese pear fruit.

Sorbitol oxidase activity, which was about one-tenth that of NAD^+-dependent sorbitol dehydrogenase, had high activity in immature fruit, decreased with fruit expansion and increased again with fruit maturation. Sorbitol-6-P dehydrogenase and $NADP^+$-dependent sorbitol dehydrogenase activities were detected a little. The differences in sorbitol metabolism among Rosaceae plants were discussed, based on the seasonal fluctuations of these enzyme activities. Acid invertase activity was distinctly higher than that of sorbitol-related enzymes in fruit, and its role in sugar translocation and metabolism was discussed.

Introduction

Sorbitol is known to play a significant role in the translocation of photosynthate in the family Rosaceae (3, 9, 12). What kind of sugar the sorbitol loading into fruit is converted into, and how much of the sorbitol changes into other sugars have a direct relation to fruit taste, such as the intensity or quality of sweetness. Therefore, it is important to elucidate the pathway, site and rate of sorbitol metabolism. Some research has been done on the sorbitol enzymes contained in leaves (Fig. 1). Loescher et al reported the function of Sorbitol-6-P dehydrogenase (S6PDH) and NAD^+-dependent sorbitol dehydrogenase (NAD^+-SDH) in apple leaves (10). Hirai (5) and Yamaki (15) also showed the function of S6-PDH on loquat fruit and apple cotyledons, respectively. Moreover, Yamaki found sorbitol oxidase (SOX) (14) and $NADP^+$-dependent sorbitol dehydrogenase ($NADP^+$-SDH) (16) in apple leaves.

However, there are few reports concerning sorbitol enzymes in sink tissue like fruit(Fig. 1). Negm and Loescher (11) first detected NAD^+-SDH activity in apple callus tissue. Hirai(6) found S6PDH in loquat fruit and showed that activity clearly increased with the fruit maturation. Recently, we examined four sorbitol relating enzymes in apple fruit and leaves throughout the season, resultantly showed that NAD^+-SDH and SOX were so important in fruit, while S6PDH was most important in leaves (17). Now, the data are insufficient to know how these enzymes function to metabolyze the sorbitol loading into fruit. The metabolism of sorbitol may be different among

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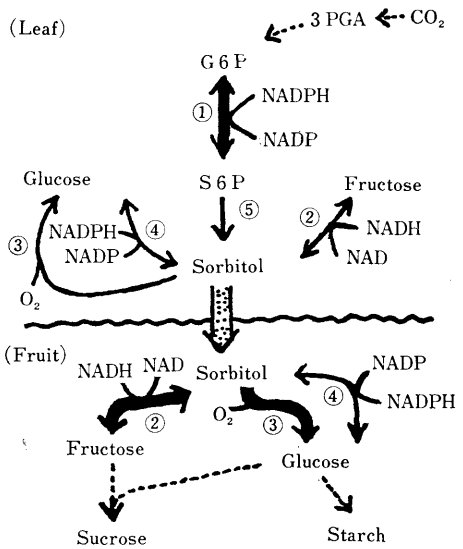

Fig. 1. Sorbitol relating enzymes and sorbitol metabolic pathway on leaf and fruit. ①: Sorbitol-6-P dehydrogenase(S6PDH), ②: NAD^+dependent sorbitol dehydrogenase (NAD^+-SDH), ③: Sorbitol oxidase (SOX), ④: $NADP^+$dependent sorbitol dehydrogenase ($NADP^+$-SDH), ⑤: Sorbitol-6-P specific acid phosphatase.

species of fruit (3). Thus, in the present paper, the seasonal changes in mainly NAD^+-SDH and SOX activities of Japanese pear fruit are examined, and discussed in relation to those in apple fruit or others.

Materials and Methods

Materials

Samples of 20 to 60 fruit were harvested from 8 years old Japanese pear 'Kosui' throughout 1984 season. A portion of each fruit was subsampled and diced into small pieces. Composite fruit samples ranging from 20 to 100 g fresh weight were used for enzyme assay and sugar determination. All enzyme assays and sugar determination were duplicated.

Enzyme extraction

NAD^+-SDH, $NADP^+$-SDH and S6PDH were extracted by homogenizing pear fruit in 0.2 M K-phosphate buffer (pH 7.8) containing 10 mM K-ascorbate, 1 mM dithiothreitol (DTT) and 10% of Polyclar AT. After centrifugation at 12,000 ×g for 20 min, the supernatant was passed through Sephadex G-25 (coarse) column to remove phenolic compounds. The filtrated sample was precipitated by saturated ammonium sulfate. Then, this was dialyzed against 0.01 M Tris-HCl buffer (pH 7.2) containing 1 mM DTT. The protein in the dialyzate was adsorbed to DEAE-cellulose (Whatman DE-52) which had beed washed previously with 0.01 M Tris-HCl buffer (pH 8.0). The enzyme eluted by 0.01 M Tris-HCl buffer (pH 7.2) containing 0.2 M KCl, were concentrated in a collodion bag and used as the source of NAD^+-SDH, $NADP^+$-SDH and S6PDH.

SOX and acid invertase were extracted by homogenizing the fruit in 0.1 M K-phosphate buffer (pH 7.0) containing 10 mM K-ascorbate, 1 mM DTT and 0.3% Triton X-100. After centrifugation at 12,000 ×g for 20 min, the precipitate was rehomogenized with 0.1 M K-phosphate buffer (pH 7.0) containing 10 mM K-ascorbate, 1 mM DTT and 1 M NaCl. The homogenate was centrifuged again at 12,000 ×g for 20 min. The combined supernatant was fractionated with saturated ammonium sulfate. The precipitated protein was dialyzed against 0.01 M Tris-HCl buffer (pH 7.0) containing 1 mM DTT. The soluble enzyme that was concentrated in a collodion bag served as the source of soluble SOX and soluble acid invertase. Remained residue after centrifugation was dialyzed against same buffer, and served as the source of bound SOX and bound acid invertase.

Enzyme assay

NAD^+-SDH activity was determined by the increase in absorbance at 340 nm in a mixture of 30 mM Tris-HCl buffer (pH 9.6), 1 mM NAD^+ and 235 mM sorbitol. S6PDH (15) or $NADP^+$-SDH activity (16) was determined by the method described previously. SOX and acid invertase activities were assayed by determining the amount of glucose produced from sorbitol and sucrose, respectively, using the enzyme coupling method described previously (17). One unit of dehydrogenase was defined as the amount of enzyme that reduces 1 μmole NAD^+($NADP^+$) per hour, and that as SOX or acid invertase as the amount of enzyme which produces 1 μmole glucose per hour.

Determination of sugar content and composition

The amounts and kinds of sugar in the ethanolic extract were determined by derivatizing sugars with trimethylsilyl (TMS) and

injecting an aliquot into a GLC (Simadzu GC-4B) equipped with a column of 5% silicon gum, SE 30-Chromosorb-W.

Results

Sugar accumulation

Sugar content of Japanese pear fruit per g fresh weight increased gradually with fruit development. As a result, the sugar concentration of mature fruit on Sep 10 became about 5 times higher than that of immature one on Jun 7. Sorbitol translocated from leaves converted to other sugars with fruit development (Fig. 2). In the immature stage (Jun 7 to Jul 10), it was difficult to accumulate other sugars except sorbitol in fruit. More than 70 % of total sugar deposited as a sorbitol comparing with sorbitol remaining at only 20% of total sugars in matured stage. Fructose and glucose began to accumulate on July 10, then increased rapidly with fruit expansion to finally comprise 40% and 15% of total sugars, respectively. Sucrose rose rapidly accompanying with fruit maturation (Aug 20) to comprise finally about 30% of total sugar.

Dehydrogenase activities relating to sorbitol metabolism

Only NAD^+-dependent sorbitol dehydrogenase activity in three sorbitol-relating dehydrogenases was detected remarkably in Japanese pear fruit(Fig. 3). Other two dehydrogenase activities of S6PDH and $NADP^+$-dependent SDH were hardly to be detected throughout the seasons. The activity of NAD^+-SDH per g fresh weight rose once rapidly in immatured stage until it attained the maximum activity on 10th July, then reduced with fruit enlargement and subsequently rose again with fruit maturation after 20th August. When the activity was expressed on a whole fruit basis, it increased apparently over both the immature and enlargement stages without the decline occuring in the activity based on fresh weight. Moreover it rose extensively at the maturation stage.

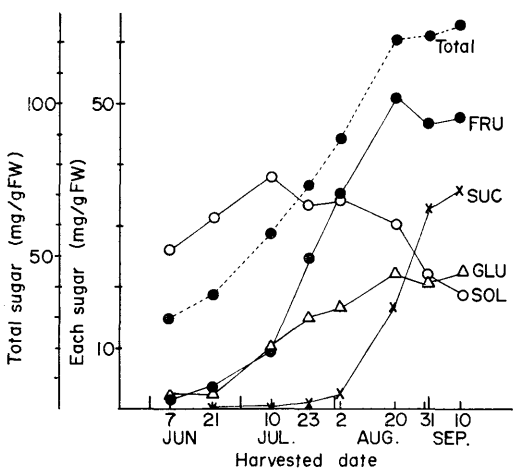

Fig. 2. Seasonal changes in soluble carbohydrates in Japanese pear fruit. ●: Fructose, ○: Sorbitol, ×: Sucrose, △: Glucose. Carbohydrate content are expressed as mg per g fresh weight. Each point is the mean of parallel experiments and its error is small.

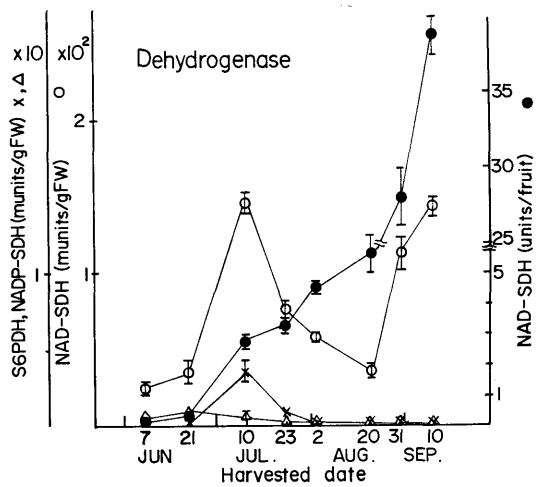

Fig. 3. Seasonal changes in NAD^+-SDH, S6PDH and $NADP^+$-SDH activities in Japanese pear fruit. Each point is the mean ±SE of parallel experiments. NAD^+-SDH(○), S6PDH(×) and $NADP^+$-SDH (△) activities were expressed as units per g fresh weight. NAD^+-SDH (●) activity was also expressed as units per whole fruit.

Sorbitol oxidase

Sorbitol oxidase was composed of two types of soluble and bound. In the immature stage, about 50% of total SOX activity comprised the bound type, while more than 70% did the bound type in the matured stage. The total SOX activity on a fresh weight basis was one-tenth of NAD^+-SDH activity. The activity was comparatively high in the immature fruit, decreased slightly with fruit expansion, and then rose clearly again with fruit maturation. This rising of activity was owing to that of the bound type (Fig. 4). When the activity was expressed on a whole fruit basis, the total

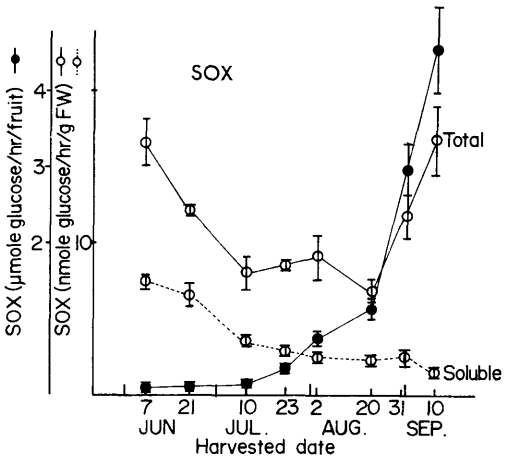

Fig. 4. Seasonal changes in SOX activity in Japanese pear fruit. Each plot is the mean ±SE of parallel experiments. —○—: Total activity of bound and soluble forms per fresh weight (munits/g fresh weight). ···○···: Activity of soluble form per fresh weight (munits/g fresh weight). —●—: Total activity of bound and soluble forms per fruit (units/whole fruit).

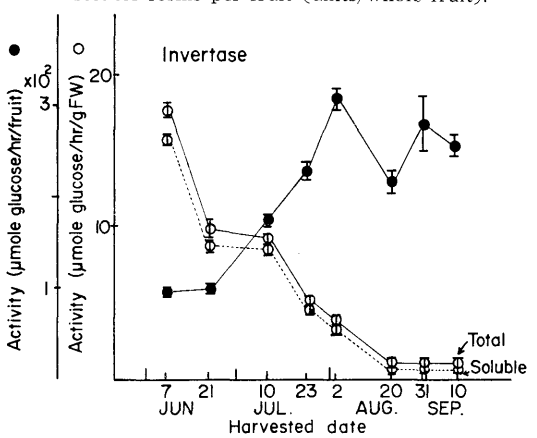

Fig. 5. Seasonal changes in acid invertase activity in Japanese pear fruit. Each point is the mean ±SE of parallel experiments. —○—: Total activity of bound and soluble forms per fresh weight(units/g fresh weight), ···○···: Activity of bound form per fresh weight (units/g fresh weight), —●—: Total activity of bound and soluble formes per fruit (units/whole fruit).

activity clearly increased with fruit development after being kept constant in the immature stage.

Acid invertase

Acid invertase was also composed of two types of bound and soluble. Throughout the season, more than 80% of total activity comprised the bound type. This activity was quite higher than other sorbitol relating enzymes. Total activity based on fresh weight was highest on 7th June, and then decreased drastically until 20th August. Thereafter, it was kept roughly constant up to harvest (Fig. 5). Total activity on a whole fruit basis gradually increased with fruit enlargement, and then was held in the same level during the mature stage.

Discussion

The role of sorbitol enzymes in friut

Sorbitol metabolism in Japanese pear fruit was certified to be excuted by mainly NAD^+–SDH and minorly SOX, which played an important role in apple fruit flesh (17). The intensity and the seasonal profile of NAD^+–SDH activity throughout the season was roughly same as those of apple fruit flesh(1,17). On the other hand, the activity of SOX was a half to one–forth of that activity in apple fruit, but the activity rose prominently with fruit maturation. The other enzyme activities of $NADP^+$–SDH and S6PDH were hard to detect throughout the season.

NAD^+–SDH activity increased clearly on immature stage, which just corresponded to the beginning of rapid enlargement, and correlated to the accumulation of fructose on the stage. So, this NAD^+–SDH seems to be functioning to the supply of fructose. Re-enhancement of NAD^+–SDH activity and the enhancement of SOX activity with fruit maturation paralleled to the rapid rising of sucrose. Both enzymes are supposed to play an active role in the supply of precursor for sucrose biosynthesis. The reduction of both enzyme activities per fresh weight accompanying fruit enlargement depend upon the dilution induced by fruit expansion. The activity of both enzymes per whole fruit continued to increase greatly during enlargement stage, and also can supply enough sugars.

The accumulation of sucrose with fruit maturation is so prominent, and may be depending upon the rising of sucrose synthetase or sucrose phosphate synthetase, as described in pear or apple fruit by Latche et al. (8). Simultaneously, a lot of fructose and glucose supplied by enhanced activities of NAD^+–SDH and SOX will make easy to synthesize sucrose. The other possibility of sucrose accumulation may be attributed to the concurrent reduction in

acid invertase activity.

The relationship between the enzyme activity and accumulated sugar amount

We tried to estimate the amount of sorbitol which can be converted to other sugars per day by the activities of the extracted NAD^+-SDH and SOX. It is suggested that the present total activities of both enzymes are able to supply only about 70% of the soluble sugar accumulated in fruit at the immature stage, and about 20% at the enlargement or mature stage. This also was the same situation in apple fruit (17). However, since the conversion to other metabolites and the consumption by respiration must be considered, the above ratio will be more reduced. The lack of agreement stoichiometrically between the sugar accumulation and the total enzyme activities *in vitro* may be caused by the following reasons. 1) Both activities of NAD^+-SDH and SOX were inhibited during the extraction process; 2) an activator and other factors stimulating their activities are present *in vivo*; 3) other sorbitol enzymes exist in fruit or; 4) much of the sugar loaded into fruit are sugars other than sorbitol, such as sucrose. The first possibility can be expected from the facts that NAD^+-SDH activity in apple fruit could not be detected without DEAE-cellulose treatment, and $NADP^+$-SDH activity in apple leaf increased with the purification steps of DEAE-cellulose and 2, 5 ADP-Sepharose 4B column chromatography (16). In further experiment, it may be necessary to determine the enzyme amount by the enzyme-linked immunosorbent assay (ELISA) as reported by Hirai (7). For the second possibility, the activity of SOX localized on various membranes may be enhanced by attaching the membrane *in vivo* as observed in various membrane bound enzymes. The third possibility is not to be expected because other enzymes converting sorbitol to fructose or glucose have not been reported yet, even for animals or microorganisms. The forth possibility may be expected from the results that acid invertase activity in fruit was so high, and was enough to supply the amounts of glucose and fructose described in Fig. 4.

However, considering the photosynthetic assimilate loaded to the fruit, the soluble sugar detected in the stem and peduncle (17) was almost always composed of sorbitol, and sucrose was a rather minor component. Moreover, xylem and phloem sap of pear or apple have been reported to be composed mainly of sorbitol (2). So it is difficult to expect that sucrose is the main sugar loaded rather than sorbitol in Japanese pear. Thus, the contradiction remains to be resolved now.

Some difference of sorbitol metabolism

When the sugar accumulation pattern was compared between apple and Japanese pear fruit, the sorbitol loaded in the former did not almost remain as sorbitol throughout the seasons, but converted other sugars. On the other hand, the large part of sugar in the latter was held as sorbitol in the immature stage. Then, the conversion of sorbitol to other sugars was stimulated gradually fruit enlargement and maturation to accumulate a lot of fructose, glucose and sucrose. However, even on the mature stage, sorbitol remained in fruit was quite higher than that in apple fruit. This higher concentration of sorbitol may be led by the lower activity of SOX in Japanese pear fruit, since NAD^+-SDH activity had not a critical change between both fruit. Moreover, in peach fruit, we could not detect NAD^+-SDH and S6PDH activity, but rather detected the higher activity of SOX in preliminary experiment. And in loquat fruit S6PDH activity was high and rose with fruit maturation (6), while the activity was not almost detected in Japanese pear or apple (17). In addition, ^{14}C-sorbitol taken up into whole fruit was reported to be converted actively to fructose in apple (13) and glucose in French prune (4). Thus, there seems to be some difference in sorbitol metabolism among apple, pear, peach and loquat.

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摘 要

ニホンナシ果実の糖の蓄積に重要な働きをするNAD^+依存性ソルビトール脱水素酵素，ソルビトール酸化酵素，ソルビトール-6-P脱水素酵素，$NADP^+$依存性ソルビトール脱水素酵素そしてインベルターゼ活性の季節変動と糖の蓄積との関係を検討した．ソルビトールをフルクトースに変換するNAD^+依存性ソルビトール脱水素酵素がソルビトール関連酵素のなかで，果実の生長，成熟過程をとおして最も高い活性を示した．その活性は6月に上昇し，果実の肥大に伴って減少し，果実の成熟とともに再び増加した．この活性変動は未熟果でのフルクトースの蓄積に密接な関連を示し，この酵素はニホンナシ果実の糖の蓄積にたいして重要な役割を果していることが示唆された．ソルビトールをグルコースに変換するソルビトール酸化酵素はNDA^+依存性ソルビトール脱水素酵素の約10分の1の活性を持ち，幼果において高い活性を示し，果実の肥大とともに減少し，その成熟に伴って再び増加した．しかしながらソルビトール-6-P脱水素酵素，$NADP^+$ソルビトール脱水素酵素活性はほとんど検知出来なかった．これらのソルビトール関連酵素活性の季節変動に基づいて，他のバラ科果実のソルビトール代謝と比較しながら，ニホンナシ果実のソルビトールの代謝機構及び糖の蓄積機構を論議した．また酸性インベルターゼ活性はソルビトール関連酵素活性よりもはるかに高く，糖の転流，蓄積に対する役割を論議した．