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Fluctuations in Activities of Three β-Galactosidase Isoforms from Ripening Avocado (*Persea americana*) Fruit and their Different Activities against its Cell Wall Polysaccharides as Substrates

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Summary

Since there is little information about the relationship between glycosidases and avocado fruit softening, we assayed some glycosidase activities during avocado fruit softening. β-Galactosidase, α-L-arabinofuranosidase and β-glucosidase activities increased during ripening; that of β-galactosidase activity closely paralleled the trend in fruit softening. Therefore, β-galactosidase was fractionated into three isoforms using hydrophobic chromatography and cation exchange chromatography; the isoforms were labeled AV-GAL I, AV-GAL II or AV-GAL III. AV-GAL III activity in the fruit, which was detected 4 days after harvest, increased with fruit softening, whereas AV-GAL I and II activities were detectable throughout the ripening period with little change. When the activities against four fractions of cell wall polysaccharides prepared from avocado fruit were assayed, AV-GAL III released a significant amount of monomeric D-galactose from guanidine thiocyanate (GTC) soluble cell wall polysaccharide, compared with AV-GAL I and II. AV-GAL I did not release detectable D-galactose from the four isolated cell wall polysaccharides, indicating that, among three β-galactosidase isoforms, AV-GAL III plays the most important role on the softening of avocado fruit.

Key Words: avocado (*Persea americana*), β-galactosidase, cell wall, fruit softening.

Introduction

Changes in the cell wall structure caused by some cell wall degrading enzymes lead to fruit softening during its ripening (Fischer and Bennett, 1991). Drastic changes were found in pectic polysaccharides of the cell wall component during fruit ripening, and polygalacturonase that hydrolyzes α-(1,4)-galacturonic bond is considered the key enzyme responsible for fruit softening. However, genetic improvements in polygalacturonase activity of tomato fruit have shown that polygalacturonase is not the sole determinant for fruit softening (Smith et al., 1988; Giovannoni et al., 1989). The loss of galactosyl and arabinosyl residues during fruit ripening has also been observed in many kinds of fruit, besides the depolymerization of pectic polymers hydrolyzed by polygalacturonase (apple: Knee, 1973; Yoshioka et al., 1994, tomato: Gross and Wallner, 1979; kiwifruit: Redgwell et al., 1992, Japanese pear: Yamaki et al., 1979). Galactose and arabinose are the major components of pectic side chains, and the release of those neutral sugars may increase the solubility of polyuronide or the susceptibility of cell wall polysaccharide against other cell wall hydrolases. Thus, the enzymes, β-galactosidase or α-L-arabinofuranosidase, which release those neutral sugar residues, may play an important role in fruit softening (Tateishi et al., 1996).

In higher plant, β-galactosidases, which are the only enzymes that cleave β-(1,4)-galactan bond to produce galactosyl residues from cell wall polysaccharides during ripening (Smith et al., 1998), have been extracted from several kinds of fruit and their biochemical properties well-characterized. β-Galactosidase isozymes are found in fruit of tomato (Pressy 1983), avocado (De Veau et al., 1993), apple (Yoshioka et al., 1995) and Japanese pear (Kitagawa et al., 1995), but not all β-galactosidases participate in the release of galactosyl residues from pectic polysaccharide during ripening. Thus, of three β-galactosidases (β-galactosidase I to III) purified from tomato fruit, only β-galactosidase II is responsible for fruit softening (Pressy 1983; Carey et al., 1995; Carrington and Pressy, 1996). In Japanese pear, β-galactosidase III plays the most important role for the release of galactosyl residues during fruit softening among the five fractionated β-galactosidases (β

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-galactosidase I to V) (Kitagawa et al., 1995).

Avocado is a typical climacteric fruit and its biochemical changes during ripening are well-characterized; however, reports about softening-related enzymes from this fruit except for cellulase, polygalacturonase and pectinmethylesterase are seldom found. Only De Veau et al. (1993) have reported detecting three β-galactosidase isozymes by 2 dimentional electrophoresis from avocado fruit and characterizing only one of them by using chelator soluble pectin, isolated from tomato fruit. However, other properties, such as the changes in activity during ripening or the activity against cell wall polysaccharides from avocado fruit were not obvious. In this study, we isolated three β-galactosidase isoforms from softening avocado fruit and assayed their activities during ripening. Furthermore, we tried to assign the roles of these isoforms to the fruit softening process by measuring their activities against polysaccharides fractionated from the cell wall of avocado fruit as substrates.

Materials and Methods

Plant material

Avocado (*Persea americana* Mill. cv. Fuerte) fruits, which were harvested in the Yamada Orchard, Shizuoka Prefecture in Japan, were allowed to ripen in a chamber at 20 ℃, and sampled daily to measure ethylene production and fruit firmness. Fruit firmness was measured by a Handy HIT (range 500-800, Fujihira kougyou, Japan) and a Fruit Pressure Tester FT-327 (Penetrometer, 11 mm plunger, Effegi, Italy). Peeled and sliced mesocarp was frozen in liquid nitrogen and stored at -85 ℃.

Enzyme extraction and fractionation of β-galactosidase

Enzyme extraction and fractionation were carried out in a chamber at 4 ℃ or in an ice-bath. Tissue samples were homogenized in 0.1 M K-phosphate buffer, pH 6.0, containing 1.0 M NaCl, 30 mM 2-mercaptoethanol and 0.1 % (w/v) Na-L-ascorbate, and then stirred for 1 hr to solubilize cell wall-bound enzymes. After centrifugation at 10,000 xg for 30 min, the resultant pellet was resuspended with some fresh extraction buffer followed by centrifugation at 10,000 xg for 30 min. After the supernatants were combined, solid ammonium sulfate was added to 25 % saturation and the mixture stirred for 1 hr. Upon centrifuging at 15,000xg for 30 min, the supernatant was loaded onto the BUTYL-TOYOPEARL 650C (Tosoh, Japan) column previously equilibrated with 10 mM K-phosphate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol and 25 % saturated ammonium sulfate. The column was washed with 20 % saturated ammonium sulfate in 10 mM K-phosphate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol. The absorbed enzymes, including β-galactosidase(s), were eluted with 10 % saturated ammonium sulfate in 10 mM K-phosphate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol followed by the same buffer without ammonium sulfate. Each active fraction was dialyzed separately against 10 mM Na-acetate buffer, pH 5.0, containing 10 mM 2-mercaptoethanol overnight and applied onto Mono S (Amersham Pharmacia Biotech, UK) column previously equilibrated with the same buffer used in dialysis. The absorbed proteins were eluted with a linear gradient of 0 to 0.8 M NaCl in the same buffer.

Assay of β-galactosidase and other glycosidases

Glycosidase activities were measured in the supernatant extracted by 1.0 M NaCl before the addition of ammonium sulfate (*see Enzyme extraction and fractionation of β-galactosidase*) according to the method with a small modification described by Tateishi et al. (1996). Briefly, 0.4 ml of reaction mixtures consisting of 100 mM Na-acetate, pH 4.5, 1.2 mM *p*-nitrophenylglycoside and the enzyme solution were incubated 34 ℃ for 40 min. The reaction was terminated by adding 0.6 ml of 0.2 M Na_2CO_3. The *p*-nitrophenylglycosides used as substrates were: *p*-nitrophenyl-β-D-galactopyranoside for β-galactosidase, *p*-nitrophenyl-α-D-galactopyranoside for α-galactosidase, *p*-nitrophenyl-α-L-arabinofuranoside for α-L-arabinofuranosidase, *p*-nitrophenyl-β-D-glucopyranoside for β-glucosidase and *p*-nitrophenyl-β-D-xylopyranoside for β-xylosidase. All substrates described above were purchased from Sigma (USA). The *p*-nitrophenol released was measured at 400 nm.

Preparation and sequential extraction of cell wall polysaccharides

Avocado fruit cell wall polysaccharides were prepared according to Huber and O'Donoghue (1993) using fruits 2 and 3 days after harvest. Fruits were homogenized in ice-cold 80 % ethanol, and endogenous enzymes in 80 % ethanol insoluble substances (AIS) were inactivated in Tris-buffered phenol, pH 7.0. The suspension was readjusted to 80 % ethanol and AIS were reprecipitated at -30 ℃. The AIS were transferred to chloroform:methanol (1:1, v/v) solution, filtered and the residue washed with acetone and then dried in an oven. The resultant cell wall materials were sequentially extracted with 50 mM cyclohexane-*trans*-1,2-diamine-*N,N,N',N'*-tetra acetic acid (CDTA), 50 mM Na_2CO_3, 6 M GTC and 4 M KOH as described by Redgwell et al. (1988). Extracted cell wall polysaccharides were dialyzed, concentrated, and then freeze-dried.

Activity against native cell wall polysaccharides and quantitative determination of D-galactose

Reaction mixtures consisting of 2 mg each extracted substrate (chemically solubilized cell wall polysaccharides), 1.0 ml of 50 mM Na-acetate, pH 5.0, including 50 mM NaCl and purified avocado β-galactosidase isolated from the mixed fruits 6 and 7 days after harvest, to which 25 μl of toluene was added as a bacteriostat.

Reaction mixtures were incubated for 72 hr at 37 ℃ ; the reaction was stopped by dipping the tube with reactants into boiling water for 10 min. Control samples were run identically but with heat-denatured β-galactosidase. Samples were centrifuged at 15,000 xg for 30 min, and the free D-galactose in the supernatant was measured using D-galactose dehydrogenase (EC 1.1.1.48) from *Pseudomanas fluorescens* (Kurz and Wallenfels, 1974).

Results

Changes in glycosidase activities during fruit softening

Fruit firmness, measured by Handy HIT and Penetrometer, showed that softening was detected 4 and 5 days after harvest, respectively (Fig. 1), when ethylene production was also detectable (data not shown). At 20 ℃, firmness rapidly decreased 4 to 7 days after harvest; fruits were considered over-ripe by 9 days after harvest. The rates of rapid fruit softening were similar with both instruments (Fig. 1).

All glycosidase activities were detected in firm fruit 0 to 3 days after harvest (Fig. 2). In particular, changes in β-galactosidase activity paralleled fruit softening (Fig. 2). The activity of β-galactosidase, α-L-arabinofuranosidase and β-glucosidase increased with fruit softening and reached a plateau about 6 days after harvest, but α-galactosidase and β-xylosidase activities changed little (Fig. 2).

Fractionation of β-galactosidase

Almost all β-galactosidase activity was extracted from fruit cell wall sampled 5 days after harvest by adding 1.0 M NaCl to the extraction buffer. A small amount of residual cell wall-bound β-galactosidase remained insoluble even when chelator solvent or a higher concentration of NaCl or both were used (data not shown). The addition of 25 % saturated ammonium sulfate sufficiently bound β-galactosidase to the ligands of hydrophobic chromatography BUTYL-TOYOPEARL. Thus, two β-galactosidase active fractions were obtained by a step down gradient of 10 % and 0 % saturated ammonium sulfate; no activity was detected in the pass through and the eluate of 20 % saturated ammonium sulfate (Fig. 3). Subsequently the active fractions from 10 % saturated ammonium sulfate were loaded onto Mono S column, eluted with 0 to 0.8 M NaCl in a linear gradient, yielded two peaks of β-galactosidase activities; no β-galactosidase activity was detected in pass through. The two β-galactosidase fractions were labeled AV-GAL I and AV-GAL II, respectively, according to the order of their elution. When the mixture of active fractions obtained by 0 % saturated ammonium sulfate elution from BUTYL-TOYOPEARL column was loaded onto Mono S column, only one peak of β-galactosidase activity was

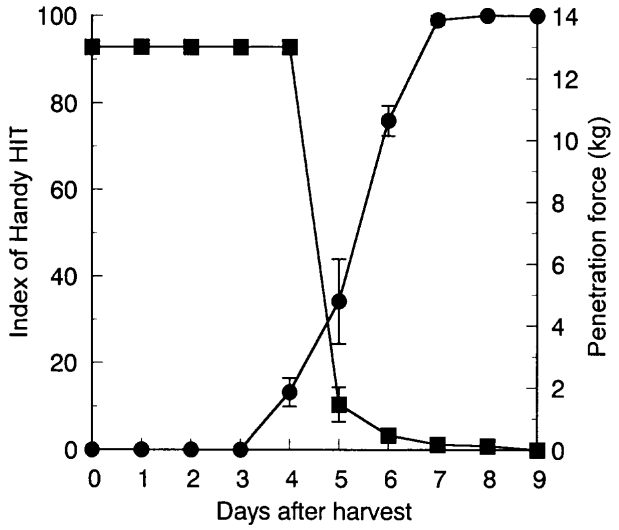

Fig. 1. The decrease of fruit firmness during ripening. The index of Handy HIT (●) are the mean of six positions of each fruit and the values of Penetrometer (■) are the mean of four positions of each fruit. Vertical bars indicate the SE. Detectable ethylene production from the fruit was initially observed at 4 days after harvest and peaked at 5 days after harvest.

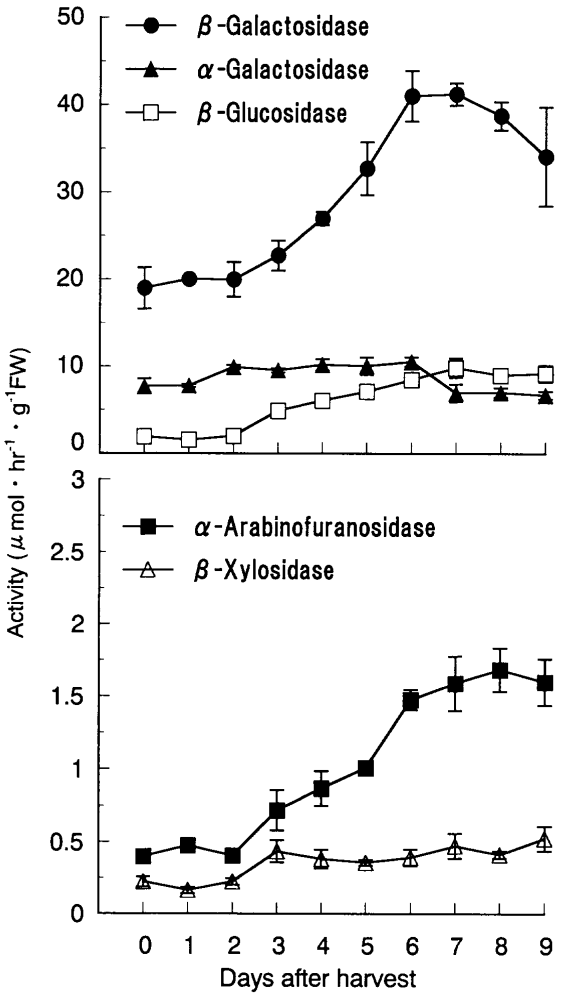

Fig. 2. Changes in glycosidases activities during fruit ripening. The values are the mean of three independent measurements. Bars indicate the SE. The activity was represented as μmol *p*-nitrophenol released from *p*-nitrophenyl-glycosides (see Materials and Methods).

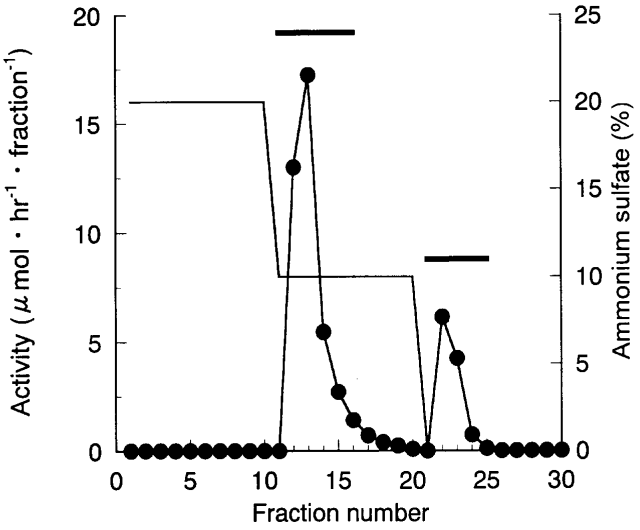

Fig. 3. Elution profile of β-galactosidase from BUTYL-TOYOPEARL column chromatography. β-Galactosidases were eluted by step down gradient of 10 % and 0 % saturated ammonium sulfate. Each fraction has 3.5 ml of eluate. Fraction number 12-15 and 22-24 (horizontal bars) were dialyzed and loaded on Mono S column separately. The activity is expressed as μmol *p*-nitrophenol released from *p*-nitrophenyl-β-D-galactopyranoside (see Materials and Methods).

obtained in the eluate; we labeled AV-GAL III. Thus, all three β-galactosidases were eluted in different fraction from Mono S column by a linear gradient of NaCl.

Changes in the activities of three β-galactosidase isoforms during fruit softening

When the above β-galactosidases were assayed, AV-GAL I activity, which was detected in pre-ripe hard fruits from 0 to 3 days after harvest, showed little change throughout the fruit ripening period (Fig. 4A). AV-GAL II activity, which was also detected in pre-ripe hard fruit at a lower rate than that of AV-GAL I, likewise, changed little during the same period. On the contrary, AV-GAL III activity, which was undetectable at 0 to 3 days after harvest, became apparent on the 4th day when measurable softening and ethylene production were recorded (Fig. 4B). AV-GAL III activity paralleled the trend of fruit softening, increasing from 3 to 7 days after harvest and then decreasing until the 9th day after harvest (Figs. 1, 4B).

The activity of three β-galactosidases against avocado fruit cell wall polysaccharides

When the AIS of avocado fruit cell wall was sequentially extracted using CDTA, Na_2CO_3, GTC and KOH, each substrate incubated with AV-GAL I yielded no detectable D-galactose (Table 1), whereas AV-GAL II released D-galactose from GTC-soluble polysaccharides and a small amount of D-galactose from Na_2CO_3- and KOH-soluble polysaccharide fractions. However, AV-GAL III released large amount of D-

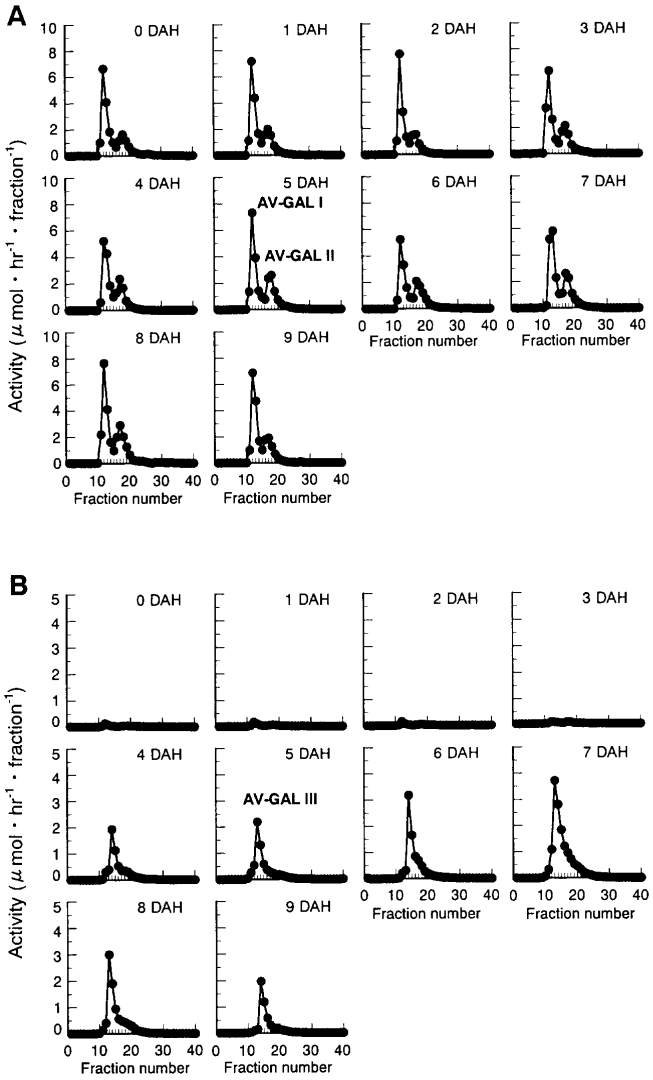

Fig. 4. Fractionation of β-galactosidase by Mono S column chromatography and changes in activities of three β-galactosidase isoforms during fruit ripening. (A) Elution profile of β-galactosidase by Mono S column. Active fractions were initially eluted with 10 % saturated ammonium sulfate through a BUTYL-TOYOPEARL column and then loaded onto a Mono S column. The first peak of β-galactosidase was labeled AV-GAL I and second one AV-GAL II. (B) Same as (A) except that the sample was initially eluted with 0 % ammonium sulfate through a BUTYL-TOYOPEARL column before being loaded onto the Mono S column. The β-galactosidase peak eluted was labeled AV-GAL III. The activity is expressed as μmol *p*-nitrophenol released from *p*-nitrophenyl-β-D-galactopyranoside (see Materials and Methods).

galactose from GTC-soluble polysaccharide and significant amount of D-galactose from Na_2CO_3- and KOH-soluble polysaccharides of cell wall fractions (Table 1). No D-galactose was detected in the reaction mixtures incubated with heat-denatured enzyme.

Discussion

In many kinds of fruit, galactose and arabinose, which are major components of pectic polysaccharide side chain, are released during ripening (Knee, 1973; Gross

Table 1. Activities of three β-galactosidase isoforms against polysaccharides fractions solubilized from cell wall of avocado fruit.

Substrate	AV-GAL I	AV-GAL II	AV-GAL III
CDTA-soluble	N. D.[z]	N. D.	N. D.
Na_2CO_3-soluble	N. D.	3.31 ± 1.42[y]	11.0 ± 2.25
GTC-soluble	N. D.	7.58 ± 1.56	79.8 ± 20.9
KOH-soluble	N. D.	1.20 ± 0.50	9.78 ± 2.39

[z]N. D.: Not detected.

[y]D-Galactose released from each substrate was represented as μg per reaction mixture. Values are the mean of three individual experiments ± SE. The activity of each β-galactosidase isoform contained in reaction mixtures was adjusted to the enzyme activity which is able to hydrolyze 2 μmol *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside per hr at 34 ℃.

and Wallner, 1979; Yamaki et al., 1979; Yoshioka et al., 1994; Redgwell et al., 1992). In this study, the three glycosidases, β-galactosidase, α-L-arabinofuranosidase and β-glucosidase, which were detectable in hard fruits, became highly active during fruit ripening (Fig. 2) indicating that the first two enzymes may be responsible for the release of galactosyl and arabinosyl residues. Thus, they play an important role in the softening of avocado fruit. Although the activity of the third enzyme, β-glucosidase, increased during fruit ripening, its role in fruit softening could not be clearly established.

β-Galactosidase isoforms are present in tomato (Pressy, 1983), avocado (De Veau et al., 1993), apple (Yoshioka et al., 1995) and Japanese pear (Kitagawa et al., 1995) fruits; one of the isoforms releases galactosyl residues from pectic polysaccharide during ripening. In this study, three β-galactosidase isoforms, AV-GAL I, II and III, were isolated (Figs. 3, 4). AV-GAL I and II were detectable 0 to 3 days after harvest, whereas AV-GAL III was not and the activities of the former two changed little subsequently, whereas that of the latter III was highly active (Fig. 4). Moreover, the change in AV-GAL III activity was coincident with ethylene production and fruit softening (Figs. 1, 4B). It is indicated that among three β-galactosidases isolated from avocado fruit, AV-GAL III may play a significant role for avocado fruit softening.

AV-GAL III released the most amount of D-galactose from Na_2CO_3-, GTC- and KOH-soluble polysaccharides, whereas AV-GAL I did not release any (Table 1). Such differences in β-galactosidase activity between artificial and native substrates were also observed in kiwifruit (Ross et al., 1993) and Japanese pear (Kitagawa et al., 1995). In this study we showed that AV-GAL III was highly active against GTC-soluble polysaccharide releasing much monomeric D-galactose (Table 1). The polysaccharides solubilized by GTC contained more highly branched rhamnogalacturonan backbones than CDTA- and Na_2CO_3-soluble polymers and also contained hemicellulosic polysaccharides (Redgwell et al., 1988). De Veau et al. (1993) found that treating tomato chelator soluble pectin with one of β-galactosidase isozymes from avocado resulted in the release of galactose and an increase in pectin solubility. Concurrently, a decrease in the molecular size of the tomato chelator soluble pectin was noted. However, we could not establish whether or not AV-GAL III corresponds to the isozyme. A similar downshift in pectin molecular weight caused by β-galactosidase was reported in melon fruit (Ranwala et al., 1992). During ripening, the downshift of pectin molecular size also observed in avocado fruit (Huber and O'Donoghue, 1993). Thus, the increased pectin solubility and decreased size of pectin molecules during the softening of avocado fruit may be attributed to the release of galactosyl residues by AV-GAL III from the GTC-soluble polysaccharides containing highly branched pectic side chains.

That AV-GAL I did not release detectable D-galactose from each polysaccharide fractionated from avocado cell wall contracts the finding of De Veau et al. (1993) (Table 1). AV-GAL I is distinguishable from a β-galactosidase found by De Veau et al. (1993) by its substrate specificity; all three avocado β-galactosidases isozymes have pectolytic activity against the tomato chelator soluble pectin. AV-GAL I did not release galactose from galactan polysaccharide but might hydrolyze galactose binding to aglycon. It is unlikely that AV-GAL I participates in avocado fruit softening because considerable activity was detected in pre-ripe hard fruits and this activity did not increase with fruit softening. The reaction of AV-GAL II against cell wall polysaccharides was similar to that of AV-GAL III (Table 1). AV-GAL II released a small amount of monomeric D-galactose from the GTC-soluble polysaccharide compared with AV-GAL III. Hence, the role of this isoform for fruit softening is not clear.

The increase in AV-GAL III activity with ethylene production and fruit softening, and its activity against polysaccharides prepared from avocado fruit cell wall indicated that among three β-galactosidase isoforms, AV-GAL III plays the most important role in fruit softening of avocado.

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アボカド果実におけるβ-ガラクトシダーゼアイソフォームの追熟に伴う活性変動と細胞壁多糖類に対する異なった反応特性

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摘　要

アボカド果実の軟化における各種グリコシダーゼの役割についてはほとんど知られていない．そこで，アボカド果実の軟化に伴う数種のグリコシダーゼ活性を測定した．β-ガラクトシダーゼ，α-L-アラビノフラノシダーゼおよびβ-グルコシダーゼ活性は果実の成熟に伴って上昇し，特に，β-ガラクトシダーゼ活性の変動は果実の軟化と一致した．疎水性クロマトグラフィーおよび陽イオン交換クロマトグラフィーによってβ-ガラクトシダーゼは3つのアイソフォームに分画され，これらをAV-GAL I，AV-GAL IIおよびAV-GAL IIIとした．AV-GAL IおよびAV-GAL II活性は，果実の追熟中のどのステージからも検出され，また，ほとんど変化しなかったのに対して，AV-GAL III活性は，採取後4日目の果実ではじめて検出され，果実の軟化に伴って増大した．アボカド果実の細胞壁から調整した4つの多糖類画分に対しての各アイソフォームの反応特性を調べたところ，AV-GAL IおよびAV-GAL IIと比較してAV-GAL IIIはグアニジンチオシアン酸塩(GTC)可溶性の多糖類から，D-ガラクトース単糖を効果的に遊離した．AV-GAL Iは4つの多糖類画分のどれからも，D-ガラクトースを遊離することはできなかった．従って，アボカド果実における3つのβ-ガラクトシダーゼアイソフォームのうち，AV-GAL IIIがアボカド果実の軟化に最も重要な役割を果たしていると考えられる．