β-Galactosidase and Its Significance in Ripening of “Saijyo”
Japanese Persimmon Fruit

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The fruit extracts of ripening cv. Japanese Persimmon, “Saijyo”, contained a number of glycosidases and glycans. Among them, β-galactosidase appeared to be the most significant, and the activity increased in parallel with tissue ripening. Persimmon β-galactosidase was presented in at least three isoforms, β-galactosidase-I (pI = 4.88), β-galactosidase-II (pI = 6.76), and β-galactosidase-III (pI = 7.05). β-Galactosidase-III had exo-type galactanase activity, while the others did not.

The activity of endo-type glycans was a maximum in immature green or yellow fruits. The firmness of the pulp tissue decreased dramatically, and the amount of water-soluble polysaccharide (WSS) increased. The enzyme activities of exo-type glycosidases, especially β-galactosidase, appeared maximal in mature red fruits. The amount of extractable pectin remained unchanged, although the galactose content of the high-molecular-weight fraction in WSS decreased dramatically.

These results suggest that the ripening of persimmon was caused by the solubilization of pectic polysaccharide by endo-type glycans and digestion by exo-type glycosidases. β-Galactosidase, in particular, seemed to play a major role in ripening the fruit.

Key words: β-galactosidase; isozyme; persimmon; pectin; ripening

Fruit softening is induced by modification of the cell wall polysaccharides by cell wall-degrading enzymes. The solubilization and degradation of pectin, which comprises the middle-ramella of the plant cell wall and works as a binding agent between cell walls, has particularly been proposed to play an important role in the softening of fruit.1,3 The relationships between pectinase activity and tissue softening have already been reported for tomato and pear.4,5 On the other hand, Giovannoni et al. have reported that there was no relationship between the polygalacturonase (PGase) activity and softening of the pulp in mutated tomato.6 This result suggested that the solubilization of pectin and softening of the tissue were induced not only by digestion by PGase, but also by other factors.7

Apple fruit has very low PGase activity, although pectin is solubilized without degradation during ripening.8,9 Yoshioka et al.10 have reported that the galactosidase and arabinosidase activities increased in parallel with decreasing galactose and arabinose contents in the ripening of apple fruit, and suggested that hemicelluloses played a major role in softening of the fruit. Furthermore, β-galactosidase (galase), which is widely distributed in plants, has been studied for its enzymatic properties and functions in a large number of fruits.11,18

Persimmon is an ideal fruit for studying softening, because it softens dramatically and shows substantial changes in cell wall components and polyuronide solubilization during ripening. Kang et al.19 have found high galase activity in the Persimmon, ‘‘Fuyu’’, although its detailed properties and relation to fruit softening were not clarified.

In this paper, we report the significance of Persimmon glycosidases, especially galase, and clarify the relationship between enzyme activities and the digestion of cell wall polysaccharides in persimmon fruit during ripening.

Materials and Methods

Experimental materials. Sepharose CL-4B, DEAE-Sephadex, and Sephacryl S-300 HR were obtained from Pharmacia LKB Biotechnology (Sweden). TSKgel Sugar AXI, TSKguardgel Sugar AX, and CM-toyopearl 650M were obtained from Tosoh Co.
β-Galactosidase and Its Role in Ripening of Persimmon Fruit

Ltd. (Japan). Shodex Sugar SH-1821 and Shodex Sugar SG-1011P were obtained from Showa Denko Co. Ltd. (Japan). p-Nitrophenyl monosaccharides, 4-methylumbelliferyl(4-MeUmb)-β-D-galactoside, disaccharides, and trisaccharide were obtained from Sigma-Aldrich Co. Ltd. (USA). All other chemicals and reagents were obtained from Nakarai Chemicals Co. Ltd. (Japan).

Plant material. Immature green persimmon (Japanese persimmon cv. Saijo) were harvested and stored at 20°C until soft. After the firmness had been measured, the pericarp tissue and seeds were excised from the pulp and stored at -20°C until their extraction. The ripening process was distinguished by the change in pulp color from green to red.

Tissue firmness. The firmness of each fruit prior to tissue sampling was measured by a Rheometer type R-DM-2 (San-kagaku Co. Ltd., Japan).

Preparation of the enzyme. Tissue (164 g) was homogenized in 492 ml of a 10 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl. The homogenate was centrifuged at 8,000 x g for 40 min at 4°C, the resulting supernatant being pooled and used as the enzyme source.

Enzyme assay. The assay mixture consisted of 0.5 ml of 50 mM sodium acetate containing 0.2 mg/ml of bovine serum albumin (BSA) at pH 4.0, and 0.2 ml of 10 mM of the corresponding p-nitrophenyl derivative. The reaction mixture was incubated at 30°C for 5 min before adding 0.1 ml of the enzyme solution. The enzyme activity was assayed during 10 min of incubation at the same temperature. A 0.5 ml amount of 0.5 M Na2CO3 was added to the reaction mixture in order to stop reaction, and the p-nitrophenol formed was determined from its absorbance at 405 nm. The glycosidase activity is expressed as μmol p-nitrophenol formed per min (unit) per mg fresh weight of fruit.

The PGase and galactanase activities were respectively assayed at pH 5.0 and 30°C with 0.2% citrus pectate and galactan (Sigma-Aldrich Co. Ltd., USA). The increase in reducing sugars was measured by the Somogyi method. In order to determine the hydrolytic type of PGase (exopolysaccharidase (exo-PG) or endopolysaccharidase (endo-PG)), the digested products of the pectates were analyzed by HPLC in a Shodex Sugar SH-1821 column.

Protein assay. Protein content was measured by the method of Lowry et al., BSA being used as a standard. In monitoring the effluent from column chromatography, the protein concentration was measured by the absorbance at 280 nm.

Purification of galase.

Step 1. All operations were done at 4°C. Ammonium sulfate was added to the crude extract (1423.1 mg, 601.3 units) to 25% saturation, and the mixture was allowed to stand overnight. The resulting precipitate was collected by centrifugation at 9,000 × g for 25 min. Ammonium sulfate was added to the resulting supernatant to 70% saturation, and the mixture was allowed to stand for 24 h. The resulting precipitate was collected by centrifugation and dissolved in 84 ml of a 10 mM sodium phosphate buffer (pH 7.2), and dialyzed against a 20 mM Tris-HCl buffer (pH 7.8) at 4°C for 1 day. A small amount of sediment, which had been formed during the dialysis, was removed by centrifugation (hereafter called the “salt extract”).

Step 2. The salt extract (430.8 mg, 387.6 units) was loaded into a DEAE-Sephacel column (1.9 cm × 27.8 cm) which had been pre-equilibrated with a 20 mM Tris-HCl buffer (pH 7.8). After the column had been washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl from 0.0 M (530 ml) to 0.5 M (530 ml) in the same buffer at pH 7.8. The flow rate was 20 ml/h, and 5.0-ml fractions were collected. The active fractions unbound and bound to the column were respectively pooled and designated as E1 and E2.

Step 3. The E2 fraction was dialyzed against a 10 mM sodium phosphate buffer (pH 7.2) containing 0.25 M KCl, and concentrated to 2.4 ml by an Amicon ultrafiltration system with a PM-10 filter. The concentrate was loaded into a Sephacryl S-300 column (1.5 cm × 95 cm) that had been equilibrated with the same buffer. The flow rate was 6 ml/h, and 1.0-ml fractions were collected. The active fractions were pooled, dialyzed against a 20 mM sodium acetate buffer (pH 5.0), and used as purified β-galactosidase-1 (galase-I).

Step 4. The E1 fraction was dialyzed against a 20 mM sodium acetate buffer (pH 5.0) and loaded into a CM-Toyopearl 650M column (1.5 cm × 18 cm) that had been equilibrated with the same buffer. The bound enzyme was eluted with a linear gradient of NaCl from 0.0 M (270 ml) to 0.4 M (270 ml) in the same buffer. The flow rate was 20 ml/h, and 5.0-ml fractions were collected. Galase was eluted with 0.10 M NaCl (E1), 0.18 M NaCl (E3), and 0.20 M NaCl (E4). The E1 fraction was pooled and concentrated to 1.6 ml by an Amicon ultrafiltration system.

Step 5. The concentrated fraction of E1 was loaded into a Sephacryl S-300 column (1.5 cm × 95 cm), and filtered under the conditions just described. The active fractions were pooled and dialyzed against a 20 mM sodium acetate buffer (pH 5.0) for 1 day at 4°C.

Step 6. The dialysate of E1 was re-chromatographed in a CM-Toyopearl 650M column (1.0 cm × 6.5 cm) which had been pre-equilibrated with a
20 mm sodium acetate buffer (pH 5.0). The bound enzymes were eluted with a linear gradient of NaCl from 0.0 M (270 ml) to 0.2 M (270 ml) in the same buffer (pH 5.0). The flow rate was 20 ml/h, and 5.0-ml fractions were collected. The galases were eluted at around 0.10 M and 0.14 M NaCl, and are designated as β-galactosidase-II (galase-II) and β-galactosidase-III (galase-III). They were respectively pooled and dialyzed against a 20 mm sodium acetate buffer (pH 5.0).

Polyacrylamide gel electrophoresis. The intact molecular weight of the galase isoforms was examined by active staining after performing PAGE with 10% acrylamide for the separating gel.

Extraction of soluble polysaccharides. Soluble polysaccharides such as pectin and hemicellulose were extracted from the alcohol-insoluble substance (AIS) by the method of Lazan et al. AIS (200 mg) was treated four times with 30 ml of deionized water for 24 h at 20°C while occasionally stirring. The extracted mixture was squeezed through nylon cloth and centrifuged at 9,000 × g for 40 min, the resulting supernatant being pooled as water-soluble polysaccharide (WSS). The residual cell wall material was treated with 30 ml of a 4% sodium-hexametaphosphate solution of pH 4 for 2 h at 80°C, and the resulting extract was pooled as hexametaphosphate-soluble polysaccharide (HPS). The residual cell wall material was further treated with 30 ml of 0.05 N HCl for 1 h at 100°C, and the extract was neutralized by adding 1 N NaOH, before the extract was pooled as HCl-soluble polysaccharide (ASS). The extracted polysaccharides were concentrated by a rotary evaporator to about 3.0 ml and stored at −20°C.

Gel-filtration chromatography of the polysaccharides. Each cell wall component (500 μl each) was applied to a column of Sepharose CL-4B (1.0 cm × 48.0 cm), which had been equilibrated with a 50 mm sodium acetate buffer (pH 5.0), and eluted with the same buffer. The total sugar content of each fraction was measured by the phenol-sulfuric acid method, and the uronic acid content was measured by the carbazole-sulfuric acid method. The polysaccharide in each fraction was concentrated to about 3.0 ml and stored at −20°C. Pullulans (Showa Denko Co. Ltd., Japan) were used as standards for the molecular weight estimation.

Determination of the sugar composition. To determine the neutral sugar and uronic acid contents, the polysaccharides (0.1%) were hydrolyzed at 121°C for 2 h by 2 N trifluoroacetic acid. After removing the acid by evaporation, the hydrolysate was passed through a Millipore Mocut II GC filter. The uronic acid content of the filtrate was directly analyzed by HPLC in a Shodex Sugar SH-1821 column by the method of Matsushashi et al. The neutral sugar content of the filtrate was analyzed as the borate complex by HPLC in a TSKgel Sugar AXI column in the presence of ethanolamine.

Results

Color, firmness, and glycosidase activity in ripening persimmon

The firmness of the persimmon pulp decreased rapidly in parallel with the change in color of the fruit. After the color had changed from green to yellow (stages 1 to 3), the firmness had decreased to 1/8 that of the immature green fruit (stage 1). The firmness changed little from stages 3 to 7. This result indicates that softening of the persimmon fruit tissue occurred in the early stages of ripening (Fig. 1(A)).

The fruit extract contained many glycosidases, with β-D-xylosidase, α-D-mannosidase, α-D-galactosidase and galase being particularly detected in persimmon fruit (Fig. 1(A)), although α-D-glucosidase, β-D-glucosidase, α-L-arabinosidase, α-D-xylosidase, α-D-fucosidase, and α-D-rhamnosidase were scarcely detected (data not shown). Galase was considered to be the predominant enzyme among them.
The digestion products of citrus pectate by the persimmon extracts were analyzed by HPLC in a Shodex Sugar SH-1821 column. A solution containing 0.2% of citrus pectate and 0.1% of glycerol in a 20 mM sodium-acetate buffer at pH 5.0 was incubated at 30°C for 24 h with 0.2 g of an extract prepared from 100 g of fresh fruit. After passing the reaction solution through a Millipore Moltex II GC filter, 50 μl of the filtrate was analyzed under the following conditions: precolumn, Shodex Sugar SG-1011P; main column, Shodex Sugar SH-1821 (8.0 mm × 300 mm); column temperature, 30°C; mobile phase, 1/1000 n H₂SO₄; flow rate, 1.0 ml/min; pump, 880-PU (Jasco Co. Ltd., Japan); detector, 930-RJ (range 1E-5, Jasco Co. Ltd., Japan); recorder, Chromatocorder 14 (Jasco Co. Ltd., Japan).

(a) Reaction mixture without an extract (substrate control). (b) Reaction mixture without citrus pectate (enzyme control). (c) Reaction mixture: GalAc, galacturonic acid; GalAc₂, digalacturonic acid; GalAc₃, trigalacturonic acid; Gly, glycerol; Ac, acetate; Na, sodium; NS, neutral sugars.

Glycanase activity in ripening persimmon
The activities of galactanase and PGase were also measured (Fig. 1(B)). Both enzyme activities showed two maxima during fruit ripening. The galactanase activity showed maxima at stages 2 and 6, the firmness of the pulp tissue decreasing markedly at the first maximum peak. At the second maximum, the color of the pulp changed most dramatically. The peak for the second maximum is considered to have been composed of not only galactanase, but also of exo-type galase, because galase activity was also detected at these stages. The PGase activity showed maxima at stages 2, 3, and 6. As the activity of PGase appeared in two peaks, their hydrolytic types were examined. Figure 2 shows HPLC chromatograms of the digested products of citrus pectate at each stage. PGase in stages 2 and 3 is considered to have been endo-PG, because this enzyme liberated mono-, di-, and trigalacturonic acid from citrus pectate and could not digest trigalacturonic acid into monogalacturonic acid in spite of a 24 h reaction time. On the other hand, PGase in the later stage is considered to have been exo-PG, because this enzyme only liberated monogalacturonic acid from citrus pectate.

These results indicate that the activity of galase was greater than that of other glycosidases and glycanases in persimmon fruit, and had a close relationship with the fruit’s ripening. We therefore extracted and purified this enzyme from mature persimmon fruit, and investigated its properties.

Purification and some properties of Galase
In the initial steps prior to enzyme fractionation, the supernatant from a crude extract upon standing and dialysis against an acidic buffer became very viscous and could not be used in the subsequent chromatographic steps. Changing the extraction buffer from 50 mM sodium acetate containing 0.5 mM NaCl (pH 5.0) to 10 mM sodium phosphate containing 0.5 mM NaCl (pH 7.2) decreased the viscosity of the sample without affecting the extractability of the enzyme. The separation of galase was commenced by anion-exchange chromatography in a DEAPE-Sepharose column. In this step, galase was resolved into at least two major fractions (Fig. 3(A)). The bound galase fraction (E2) was loaded into a
was loaded into a Sephacryl S-300 column, and the active fraction was eluted as a single peak. This active fraction (E1') was re-chromatographed in the CM-Toyopearl 650M column, and resolved into the two active fractions of E1' fr. 1 (galase-II) and E1' fr. 2 (galase-III) (Fig. 3(B)). The galase isoforms were purified by up to 161.8-fold (galase-I), 450.0-fold (galase-II), and 408.8-fold (galase-III), with recovery of 22.5%, 1.3%, and 5.4% from the crude extract, respectively (Table 1).

Table 2 shows the properties of the purified galase isoforms. Active-staining PAGE showed that the isoforms had similar molecular weights of 99.0 kDa for galase-I and 110 kDa for galase-II and galase-III. The isoelectric point was estimated to be pH 4.88 for galase-I, pH 6.76 for galase-II, and pH 7.05 for galase-III (Fig. 4). The $K_m$ values of Gal-I, Gal-II, and Gal-III to $p$-nitrophenyl-$\beta$-D-galactopyranoside were 1.0, 1.1, and 0.6 mM, respectively. Each galase was slightly activated by CaCl$_2$, while the activity was reduced by CuCl$_2$.

A study on the substrate specificity showed that galase-II and galase-III could only digest $p$-nitrophenyl-$\beta$-D-galactopyranoside, and not other $p$-nitrophenol substrates (0.5 mm, pH 4.0). Galase-I was able to digest $p$-nitrophenyl-$\alpha$-L-arabinofuranoside, but could not digest pectic arabinan (data not shown). To further characterize these enzymes, each was incubated with several galactose-containing substrates (Table 2). Galase-I digested $\beta$-D-galactosyl-disaccharides, but not galactosyl-trisaccharides and polysaccharides. Galase-II was specific for the $\beta$-D-galactose residues of $\beta$-D-Gal-(1$\rightarrow$6)$\rightarrow$D-Gal, $\beta$-D-Gal-(1$\rightarrow$6)$\rightarrow$D-Man, and $\beta$-D-Gal-(1$\rightarrow$4)$\rightarrow$L-Ara. On the other hand, galase-III was specific for the $\beta$-1,4-linked galactosyl trisaccharides and polysaccharides.

**Cell wall polysaccharide modification in ripening persimmon**

In order to understand the state of the cell wall polysaccharides during fruit ripening, we prepared...
Table 2. Properties of Galases from Japanese Persimmon Saijo

<table>
<thead>
<tr>
<th>Property</th>
<th>Galase-I</th>
<th>Galase-II</th>
<th>Galase-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>100,000</td>
<td>115,000</td>
<td>115,000</td>
</tr>
<tr>
<td>Gel-filtration</td>
<td>99,000</td>
<td>110,000</td>
<td>110,000</td>
</tr>
<tr>
<td>PAGE (native)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>4.88</td>
<td>6.76</td>
<td>7.05</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>3.0</td>
<td>4.0</td>
<td>4.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>4.5–7.5</td>
<td>4.3–7.5</td>
<td>4.0–7.0</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>60.0</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Thermal stability (°C)</td>
<td>&lt;54.0</td>
<td>&lt;48.0</td>
<td>&lt;47.0</td>
</tr>
<tr>
<td>Effect of cations (%)</td>
<td>Ca(162), Cu(73)</td>
<td>Ca(168), Cu(77)</td>
<td>Ca(177), Cu(68)</td>
</tr>
<tr>
<td>( K_m ) (mM)(^{2})</td>
<td>1.0</td>
<td>1.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

(A) \( p \)-Nitrophenyl (pnP)-glycosides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Galase-I</th>
<th>Galase-II</th>
<th>Galase-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{pnP-}\beta-\text{d-Glc} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \text{pnP-}\beta-\text{d-Gal} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \text{pnP-}\beta-\text{d-Man} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \text{pnP-}\beta-\text{d-Xyl} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \text{pnP-}\beta-\text{d-Fuc} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \text{pnP-}\beta-\text{d-Rha} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

(B) Galactose-containing substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Galase-I</th>
<th>Galase-II</th>
<th>Galase-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta-\text{d-Gal-[1 \rightarrow 6]-d-Gal} )</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>( \beta-\text{d-Gal-[1 \rightarrow 6]-p-Man} )</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>( \beta-\text{d-Gal-[1 \rightarrow 4]-L-Ara} )</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>( \beta-\text{d-Gal-[1 \rightarrow 4]-d-Glc} )</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha-\text{d-Gal-[1 \rightarrow 4]-d-Gal} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \beta-\text{d-Gal-[1 \rightarrow 4]-d-Gal-[1 \rightarrow 4]-d-Glc} )</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pectate (from Citrus limon)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>


Notes:

1) Galase was gel-filtered in a Sephacryl S-300 column by the method already described, and the molecular weight was estimated by a gel-filtration standard (Bio-Rad Lab., USA).

2) To investigate the isoelectric point of galase, analytical isoelectric focusing was carried out on an Ampholine PAG plate (Pharmacia LKB, Sweden). The voltage of 200 V was run for 4 h at 4°C. After isoelectric focusing had been carried out, the Ampholine PAG plate was dialyzed against a 0.1 mM acetate buffer (pH 4.0) for 1 h at 4°C to extract ampholine from the gel. The gel was then incubated at 35°C for 20 min with 4-MeUmβ-p-d-galactoside, and the formed 4-MeUmb was detected under UV-radiation conditions. The isoelectric point was calibrated by an IEF pl calibration kit (Pharmacia LKB, Sweden).

3) Galase (1 mM unit) was exposed to a range of pH (2.2–8.5) at 30°C for 6 h prior to the enzyme assay, and the residual activity was measured under the standard assay conditions. A 0.1 mM Macilvaine buffer (pH 2.2–3.0), 0.1 mM sodium acetate buffer (pH 3.0–5.5), 0.1 mM sodium phosphate buffer (pH 5.5–7.5), and 0.1 mM Tris-HCl buffer (pH 7.5–8.5) were used for adjusting the pH.

4) Galase (0.1–0.6 μM of protein) was exposed to a range of temperatures (20–90°C) for 2 h prior to the enzyme assay, and the residual activity was measured under the standard assay conditions.

5) The effects of cations on the activity of galase (1 mM unit) were examined by adding CaCl\(_2\) and CuCl\(_2\) (final concentrations of 1.0 mM) to the assay mixture.

6) Galase (1 mM unit) was assayed in the presence of 1–20 mM \( p \)-nitrophenol-β-d-galactopyranoside, and \( K_m \) was calculated from Lineweaver-Burk plots.

7) Galase (1 mM unit) was incubated in the presence of 1–20 mM \( p \)-nitrophenyl glycosides, and \( p \)-nitrophenol formed was determined from the absorbance at \( A_{405} \). In the case of oligosaccharides or pectate being used as the substrate, the generated products were analyzed by HPLC according to the method described in Fig. 2. Reaction mixtures containing 0.1% (w/v) of a sugar sample, 0.1% glycerol (internal standard), galase (1 mM unit), and a 50 mM sodium acetate buffer (pH 4.0) were incubated at 30°C for 24 h, and then filtered with a Millipore Membrane II GC. The filtrate was then analyzed. +, activity visible after 24 h of incubation; –, activity not visible after 24 h of incubation.

WSS, HPS, and ASS from persimmon fruit at stages 1, 2, 4, and 7, respectively. Data of the sugar content at each stage showed that the quantity of WSS increased, but that of HPS and ASS respectively decreased during ripening (Table 3). This result suggested that HPS and ASS might have been digested and converted into WSS during softening of the fruit.

Figure 5 shows the gel-filtration chromatograms of the WSS fractions extracted at each stage. In immature green persimmon (stage 1), the content of the WSS fraction was very low, but the molecular weight was very high and estimated to be about 700 kDa (Fig. 5). At stage 2, the high molecular weight of the main fraction was slightly degraded to about 400 kDa. At stage 4, the change in molecular weight was most marked, the high molecular weight fraction being further degraded to about 150 kDa.
The sugar composition of each extract is summarized in Table 4. The galactose content of the high molecular weight fraction of WSS (WSS-HW, >100 kDa) decreased with ripening (most marked from stages 4 to 7), in contrast to that of the low molecular weight fraction (WSS-LW, <100 kDa) which remained unchanged. In respect of HPS, the galactose content decreased dramatically from stages 4 to 7, similar to that of WSS-HW. On the other hand, the galactose content of ASS was lower than that of the other fractions and remained unchanged during ripening. Furthermore, ASS was rich in galacturonic acid, and seems to have consisted of pectin mainly composed of homogalacturonan.

**Discussion**

We investigated the macerating mechanism of persimmon "Saijyo" fruit from the activities of the cell wall degradation enzymes and states of the cell wall polysaccharides. Persimmon fruit showed substantial changes in the glycanase and glycosidase activities, cell wall components and polyuronide solubilization during ripening.

The change in firmness of persimmon pulp decreased rapidly in the early stages of ripening (Fig. 1(A)). Yoshioka et al.\(^\text{10}\) have studied the relationship between the firmness and ripening of apple and pear fruits, and reported that the firmness of apple fruit decreased slowly during ripening, and that of pear fruit decreased rapidly during the early stages of ripening. Zhang et al.\(^\text{20}\) have also reported that the tissue firmness decreased rapidly during the early stages of ripening of mature green mume. The change in firmness of persimmon pulp tissue was similar to that of pear and mature green mume that they reported.

Many cell wall degradation enzymes were found in the pulp tissue of persimmon. The activation of glycanase (galactanase and endo-PG) and glycosidases was related to the ripening stage of the fruit, and is considered to have been activated in the early stages and later stages, respectively. A similar result has been reported for pear fruit,\(^\text{31}\) and it seems that ripening of persimmon was consecutively caused by solubilization of the pectic polysaccharides by glycanases and digestion of the soluble pectin by glycosidases.

Among the cell wall degradation enzymes, the activity of galase was the most significant, and was presented in at least three isoforms having different pI values and substrate specificity. Galase and its isoforms have been found in some plants.\(^\text{10,18,29-33}\) Yoshioka et al.\(^\text{10}\) found four types of galase in apple.

**Fig. 4.** Isoelectric Focusing of Galases Isolated from Japanese Persimmon "Saijyo."

IEF was carried out on the three separated types of galases. The zymograms were visualized by a reaction at pH 4.0 with 4-MeUmb-β-galactoside.

**Table 3.** Sugar Content of the Cell Wall Polysaccharides Extracted from Persimmon Pulp during Ripening

<table>
<thead>
<tr>
<th>Stage</th>
<th>Sugar content (mg/100 g F.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSS</td>
</tr>
<tr>
<td>1</td>
<td>50.8</td>
</tr>
<tr>
<td>2</td>
<td>290.6</td>
</tr>
<tr>
<td>4</td>
<td>679.5</td>
</tr>
<tr>
<td>7</td>
<td>752.1</td>
</tr>
</tbody>
</table>

**Fig. 5.** Gel-filtration Chromatography of WSS in a Sepharose CL-4B Column.

WSS extracted at each stage was applied to a Sepharose CL-4B column that had been equilibrated with a 50 mM sodium acetate buffer at pH 5.0. The flow rate was 12.0 ml/h, and 5.0-ml fractions were collected. •, total sugar; ○, uronic acid. Molecular weight was calibrated against standard pullulans of 1600 kDa (11), 400 kDa (12), 50 kDa (13), and 20 kDa (14). Fraction numbers 25-46 and 55-80 were pooled and designated as WSS-HW and WSS-LW, respectively.
fruit, and reported that one, which could digest pectic galactan, was gradually activated and that others were gradually inactivated during ripening. This result suggests that galase was related to the ripening of fruit, but that some galases might play an other role in fruit.

Galase purified from apple, which could digest pectic galactan, had a 77.5-kDa molecular mass, which was estimated by gelfiltration, and was smaller than the 115 kDa of galase-III which we obtained from persimmon. Other properties, \( K_m \) and substrate specificity were similar to those of the galases from pear, musk melon, and barley.

The loss of galactose from cell wall of ripening fruit has been documented. Total sugars and extractable pectic polysaccharides, which we prepared from AIS of persimmon fruit at ripening stages 1, 2, 4, and 7, were similar to those of mature green mume. HPS and ASS might have been solubilized and converted into WSS during ripening. The total sugar content of the extracts decreased slightly in the latter stage because low-molecular-weight polysaccharides were possibly lost in the preparation of AIS.

The galactose content of WSS and HPS changed according to the ripening stage, this being caused by the digestion of galactan chains in WSS-HW by galase-III which was activated from stage 4 to stage 7 (Table 4).

Pectic polysaccharides are generally composed of two specific structures, homogalacturonan consisting of only -4\( \alpha \)-d-galacturonic acid (GalAc)-(1-4) and rhamnogalacturonan consisting of the diglycosyl repeating unit, -4\( \alpha \)-d-GalAc-(1 \rightarrow 2)-\( \alpha \)-L-Rha-(1-4), which had been found in a pectic polysaccharide from sycamore cells. The molar ratio of Rha/GalAc in WSS-HW was 1:4.9 (stage 1), 1:4.6 (stage 2), 1:3.1 (stage 4), and 1:2.2 (stage 7). The GalAc content against Rha remained unchanged during stages 1 and 2, but changed dramatically from stage 2 to stage 7. This result indicates that homogalacturonan was successively digested by endo-PG (in the early stages) and exo-PG (in the later stages).

These results indicate that tissue ripening of the persimmon fruit took place by two steps of enzymatic digestion. In the first step, high-molecular-weight soluble or insoluble cell wall polysaccharides were digested by endo-type glycanase and PGase, and the tissue firmness decreased most markedly. In the second step, partially digested high-molecular-weight polysaccharides were further digested by exo-type glycosidase, especially galase and exo-PG, and then tissue softening was completed with the weakened binding power between cell walls.

### References


