Weakly Acidic Pectic Polysaccharides of Japanese Radish and Cabbage

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Pectic substances were extracted from the vegetables with oxalate buffer of pH 4.25 and, after saponification, fractionated into two components, weakly acidic pectic polysaccharide (WAP) and pectic acid, by DEAE-cellulose and Sephadex G-100 chromatographies. The galacturonic acid content (17.3 ~ 25.8%) of WAPs was much lower than that of pectic acids, though the neutral sugar compositions of both pectic substances were almost the same. The arabinose-galactose side chains were found to be very long or highly branched in WAPs compared with those in pectic acids.

All the WAPs were appreciably hydrolyzed by exo- and endopolygalacturonases. The limited-degradation products (the residual polysaccharides; i.e., the rhamnogalacturonan segments) obtained by endopolygalacturonase from both WAPs and pectic acids showed a similar behavior on Sephadex G-100 and Sepharose CL-4B gel filtrations; each of the rhamnogalacturonan segments was eluted in the void volume of the Sephadex G-100 column. From these results, we concluded that WAPs are probably an inherent pectic component of the cell walls of the vegetables.

Pectic substances of plant tissues comprise a group of acidic polysaccharides composed of galacturonic acid, arabinose, galactose, and other neutral sugars. Stoddart et al.1) reported that weakly acidic and strongly acidic pectinic acids were present in the pectic substances of the callus and the cambium tissues of sycamore, the former carrying large side chains of neutral sugars. Hatanaka and Ozawa2) also reported that the crude pectic acid preparations of mandarin peels and hemp bast contained a weakly acidic pectic polysaccharide (WAP) in addition to pectic acid; WAP was eluted from a DEAE-cellulose column with acetate buffer of pH 6.0, but pectic acid was eluted with 0.1 M sodium hydroxide solution. The galacturonic acid content of WAPs of mandarin peels and hemp bast was much lower than that of pectic acids.

At present, the structural relation between pectic acid and WAP are obscure. In this work, we compared the general chemical structures of WAPs from several vegetables with those of pectic acids from the same plant materials.

MATERIALS AND METHODS

Materials. DEAE-cellulose was a product of the Brown Co., Ltd. Sephadex G-100 and Sepharose CL-4B were obtained from Pharmacia Fine Chemicals, and ECNSS-M from Gasukuro Kogyo Co., Ltd. (Kyoto). Other chemicals, reagent grade, were purchased from Nakarai Chemicals (Kyoto).

Determination of sugars. Galacturonic acid was determined by the carbazole method, as modified by Bitter and Muir.3) Neutral sugars were determined by the gas chromatographic method of Kusakabe et al.4): samples were hydrolyzed in 0.2 N sulfuric acid at 120°C for 60 min and, after cooling, β-methylglucoside was added as an internal standard. The hydrolyzates were neutralized with barium carbonate and, after removing the precipitates formed by filtering, deionized with Amberlite IR 120. The neutral sugars obtained were converted into alditol acetates by the usual methods. The chromatographic conditions were as follows: column, 3% ECNSS-M 3 x 1500 mm glass column; column temperature, 180°C; carrier gas, N2, 30 ml/min; detector, FID. The content of neutral sugar was...
expressed as moles of the sugar per 100 mol of galacturonic acid residue in the sample.

Preparation of pectic substances. Japanese radish (Raphanus sativus L. var. macropodus Makino) roots and leaves, and cabbage (Brassica oleracea L. var. capitata Linne) leaves (200 g each) were homogenized in a blender with 0.035 M ammonium oxalate-oxalic acid buffer (500 ml) of pH 4.25. The homogenate was extracted with 300 ml of the same buffer at 25°C for 30 min or 75°C for 4 hr with occasional stirring. At 75°C, the pH of the extractant was maintained at about pH 4.3 by adding 0.035 M oxalic acid; the extraction was repeated five times in a similar manner.

Fractionation of pectic substances. Both extracts at 25°C and 75°C were dialyzed against water and the precipitates formed were removed by centrifugation. The pectic substances in the supernatant were saponified with 0.05 M sodium hydroxide at 0°C for 60 min. The resulting solutions after neutralization to pH 6.0 were put on a DEAE-cellulose column equilibrated with 0.05 M acetate buffer of pH 6.0 and eluted successively with a linear gradient of acetate buffer of pH 6.0 (0.05 M → 0.8 M) and with 0.1 M sodium hydroxide solution by the method of Hatanaka et al.2) The eluate with the acetate buffer and sodium hydroxide were dialyzed against water, the latter being previously neutralized to pH 6.0. After concentration the acetate buffer fraction was put on a Sephadex G-100 column (2 x 80 cm) equilibrated with 0.1 M acetate buffer of pH 6.0 and separated by elution with the same buffer into two components of high and low molecular weight. The high molecular weight one was designated weakly acidic pectic polysaccharide (WAP) because of its low content of galacturonate, and those from the pectic samples extracted at 25°C and 75°C are hereafter referred to as WAP-25 and WAP-75, respectively. As for the 0.1 M sodium hydroxide fraction (pectic acid), only those from the extracts with hot oxalate buffer at 75°C were used as pectic acid in the subsequent experiments.

Preparation of polygalacturonase. A crude preparation of endopolygalacturonase (endo-PG, EC 3.2.1.15) of Kluyveromyces fragilis (IFO 0541) was prepared by the method of Ozawa et al.3) and purified by the affinity chromatography of Inoue et al.4) Exopolyp galacturonase (exo-PG, EC 3.2.1.67) was prepared from carrots in the manner described by Hatanaka et al.5)

Degradation of pectic substances by polygalacturonase. WAPs and pectic acids were degraded separately by endo-PG or exo-PG. The reaction mixtures containing pectic substance of 10 mg as galacturonic acid residue, 0.15 unit of endo-PG or 0.1 unit of exo-PG and 0.5 mmol acetate buffer of pH 4.5 in a total volume of 10 ml were incubated at 27°C for 7 days.

One unit of these polygalacturonases is the amount of the enzyme that releases 1 µmol of galacturonic acid from pectic acid per min.

Separation of the degradation products. The foregoing reaction mixtures were passed through a column of Amberlite CG-120 (2 × 3 cm, H⁺ form) and then the column was washed with water. The passed solution and washings were combined and concentrated. Followed by gel filtration with Sephadex G-100 in a manner similar to that described in Fractionation of pectic substances.

RESULTS AND DISCUSSION

Fractionation of pectic substances by DEAE-cellulose chromatography and gel filtration

All the pectic samples prepared from Japanese radish roots and leaves, and cabbage leaves were separated by DEAE-cellulose chromatography into two components, acetate buffer and sodium hydroxide fractions. Figure 1 shows typical profiles of the pectic samples from Japanese radish roots. For the pectic substances extracted at 75°C, the yield of the acetate buffer fraction from radish roots was 4.8 mg in the amount of galacturonic acid per g of the tissue on a dry weight basis. Those for radish leaves and cabbage were 7.7 and 4.8 mg, respectively. Similarly the yields of the sodium hydroxide fraction (pectic acid) were 144.2 mg for radish roots, 106.3 mg for radish leaves, and 84.9 mg for cabbage.

When the acetate buffer fractions were chromatographed on a Sephadex G-100 column, each of them was separated further into high and low molecular weight fractions (data not shown). The high molecular weight components were designated weakly acidic pectic polysaccharide (WAP) because of its low content of galacturonate, and those from the pectic samples extracted at 25°C and 75°C are hereafter referred to as WAP-25 and WAP-75, respectively. As for the 0.1 M sodium hydroxide fraction (pectic acid), only those from the extracts with hot oxalate buffer at 75°C were used as pectic acid in the subsequent experiments.

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Weakly Acidic Pectic Polysaccharides of Vegetables

Fig. 1. DEAE-Cellulose Chromatography of Pectic Substances of Japanese Radish Roots.

The pectic solution (about 0.1%, 10ml) was put on a DEAE-cellulose column (2x 5cm) in 0.05 m acetate buffer of pH 6.0. The pectic sample was extracted at 75°C from radish roots. A, eluted with a linear gradient of the acetate buffer (0.05->0.8m); B, eluted with 0.1 m sodium hydroxide solution; fraction size, 9.0 ml. ○, amount of galacturonic acid; ———, concentration of acetate buffer.

Table I. Sugar Compositions of WAPs and Pectic Acids

<table>
<thead>
<tr>
<th>RAD-R</th>
<th>WAP-25</th>
<th>3.1</th>
<th>ND</th>
<th>159.9</th>
<th>6.2</th>
<th>9.3</th>
<th>298.1</th>
<th>+</th>
<th>17.3</th>
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<tr>
<td>WAP-75</td>
<td>5.8</td>
<td>3.3</td>
<td>175.9</td>
<td>10.4</td>
<td>4.1</td>
<td>182.1</td>
<td>+</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>Pectic acid</td>
<td>1.0</td>
<td>ND</td>
<td>10.1</td>
<td>0.4</td>
<td>0.3</td>
<td>7.8</td>
<td>ND</td>
<td>83.6</td>
<td></td>
</tr>
<tr>
<td>WAP-25</td>
<td>7.4</td>
<td>10.4</td>
<td>118.8</td>
<td>3.1</td>
<td>4.9</td>
<td>143.4</td>
<td>+</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>WAP-75</td>
<td>3.4</td>
<td>21.8</td>
<td>166.6</td>
<td>2.3</td>
<td>9.9</td>
<td>256.9</td>
<td>ND</td>
<td>17.8</td>
<td></td>
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<tr>
<td>Pectic acid</td>
<td>1.9</td>
<td>ND</td>
<td>7.2</td>
<td>0.8</td>
<td>1.1</td>
<td>3.4</td>
<td>ND</td>
<td>88.0</td>
<td></td>
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<tr>
<td>WAP-25</td>
<td>9.6</td>
<td>ND</td>
<td>175.9</td>
<td>10.4</td>
<td>+</td>
<td>182.1</td>
<td>+</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>WAP-75</td>
<td>3.5</td>
<td>3.1</td>
<td>208.3</td>
<td>3.5</td>
<td>10.3</td>
<td>343.1</td>
<td>ND</td>
<td>14.9</td>
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<tr>
<td>Pectic acid</td>
<td>2.1</td>
<td>0.5</td>
<td>18.4</td>
<td>2.2</td>
<td>0.9</td>
<td>13.0</td>
<td>ND</td>
<td>72.9</td>
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<tr>
<td>CAB-L</td>
<td>WAP-25</td>
<td>9.6</td>
<td>ND</td>
<td>175.9</td>
<td>10.4</td>
<td>+</td>
<td>182.1</td>
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<td>20.9</td>
</tr>
<tr>
<td>WAP-75</td>
<td>3.5</td>
<td>3.1</td>
<td>208.3</td>
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<td>10.3</td>
<td>343.1</td>
<td>ND</td>
<td>14.9</td>
<td></td>
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<tr>
<td>Pectic acid</td>
<td>2.1</td>
<td>0.5</td>
<td>18.4</td>
<td>2.2</td>
<td>0.9</td>
<td>13.0</td>
<td>ND</td>
<td>72.9</td>
<td></td>
</tr>
</tbody>
</table>

RAD-R, radish roots; RAD-L, radish leaves; CAB-L, cabbage leaves. The pectic acids were prepared from the pectic samples extracted at 75°C. +, trace; ND, not detected. The values of neutral sugars were expressed as moles per 100 mol of galacturonic acid residue and those of galacturonic acid expressed as molar per cent.

Table I shows the neutral sugar compositions of WAPs-25, WAPs-75, and pectic acids. All the WAPs contained large amounts of neutral sugars, particularly arabinose and galactose; the galacturonic acid content of WAPs was about one fourth that of the pectic acids. The kind of neutral sugar of WAPs was almost the same as that of the pectic acids.

Pectic substances commonly have two distinct backbones, galacturonan and rhamnogalacturonan, also segments called 'smooth' and 'hairy' regions. In the latter the α-1,4-linked galacturonan chain is interrupted by the insertion of 1-rhamnose residues, to which neutral sugar side chains are attached blockwise.
Enzymic degradation of WAPs and pectic acids

All the WAPs were appreciably hydrolyzed by exo- and endo-PGs. By gel filtration using a Sephadex G-100 column, the degradation products of both WAPs and pectic acids were separated into two fractions of high and low molecular weight (Fig. 2); the high molecular weight components correspond to the rhamnogalacturonan segments of parental molecules. Table II shows the distribution of galacturonic acid in the two fractions. Although the extent of the degradation of WAPs by endo-PG was much lower than that of the pectic acids, the results indicate that like pectic acids they have certain homogalacturonan regions in the molecules. From the susceptibility of WAPs to the action of exo-PG, it is very probable that a part of the homogalacturonan regions is attached to the non-reducing end, probably also to the reducing end, of the molecule.

Comparison of the size of pectic acids, WAPs, and their rhamnogalacturonan segments

As shown in Fig. 2 and Table II, most of the degradation products of WAPs by endo-PG

\[\text{Table II. Distribution of Galacturonic Acid Residues in the High and Low Molecular Weight Fractions of the Degradation Products of WAPs and Pectic Acids by Exo- and Endo-PGs}\]

<table>
<thead>
<tr>
<th></th>
<th>Exo-PG</th>
<th>Endo-PG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>RAD-R</td>
<td>WAP-25</td>
<td>89.5</td>
</tr>
<tr>
<td></td>
<td>WAP-75</td>
<td>63.7</td>
</tr>
<tr>
<td></td>
<td>Pectic acid</td>
<td>62.7</td>
</tr>
<tr>
<td>RAD-L</td>
<td>WAP-25</td>
<td>91.5</td>
</tr>
<tr>
<td></td>
<td>WAP-75</td>
<td>55.1</td>
</tr>
<tr>
<td></td>
<td>Pectic acid</td>
<td>62.7</td>
</tr>
<tr>
<td>CAB-L</td>
<td>WAP-25</td>
<td>88.5</td>
</tr>
<tr>
<td></td>
<td>WAP-75</td>
<td>85.9</td>
</tr>
<tr>
<td></td>
<td>Pectic acid</td>
<td>63.6</td>
</tr>
</tbody>
</table>

H and L represent respectively the high and low molecular weight fractions of the degradation products on Sephadex G-100 gel filtrations. The values were expressed as the percentage of total galacturonic acid residues in the degradation products. For other details see Table I and the text.
were eluted in the void volume of the Sephadex G-100 column, the exclusion limit of Sephadex G-100 being $1 \times 10^5$ D for dextrans and $1.5 \times 10^5$ D for globular proteins. Accordingly the endo-PG limit-products (the rhamnogalacturonan segments) should have very high molecular weights. These results strongly indicate the presence of a large 'hairy' region in the molecule of WAPs irrespective of their origin. Probably the same is true in the pectic acids because their limit-products by endo-PG also appeared in the void volume (Fig. 2). Furthermore, comparison of the size of WAPs, pectic acids, and their rhamnogalacturonan segments by Sepharose CL-4B gel filtration gave additional evidence to support the presence of a large 'hairy' region in both WAP and pectic acid molecules (Fig. 3). Though the molecular size of each WAP was much smaller than those of the pectic acids, the rhamnogalacturonan segments obtained from WAPs and even those from pectic acids were similar in size to the undegraded WAPs, the fractionation range of Sepharose CL-4B being $3 \times 10^4$ D~$5 \times 10^6$ D for dextrans and $6 \times 10^4$~$2 \times 10^7$ D for globular proteins. The difference of molecular size between WAPs and pectic acids is probably ascribable to the difference of their homogalacturonan contents.

**Structural relation between WAPs and pectic acids**

Most pectic substances have a substantial amount of neutral sugars such as arabinose and galactose and; they form side chains in the molecules of pectic substances. Glycosidic linkages of these neutral sugars are labile compared with those of galacturonic acids. Accordingly, under mild extraction conditions like those of this work (at 75°C, pH 4.3), the possibility still exists that solubilization of pectic substances is accompanied by degradation of their labile linkages to some extent. For this reason, the homogenate of the same plant materials were separately extracted with oxalate buffer under a milder conditions (at 25°C for 30 min).

As shown in Table I, the content of neutral sugars, in particular arabinose and galactose, of WAPs very high compared with that of pectic acids. Most of the neutral sugar side chains are thought to be attached to the arabinogalacturonic residues in 'hairy' regions, Accordingly the arabinose-galactose side chains in WAPs, particularly in WAPs-75, must be very long or highly branched compared with those in pectic acids. The crude pectic samples for WAPs-75 were extracted at higher temperature than those for WAPs-25. Nevertheless, WAPs-75 had higher contents of labile neutral sugar residues such as arabinose than WAPs-25. The results practically eliminate the possibility of degradation of the labile linkages in pectic substances under these extraction conditions (at 75°C, pH 4.3). These findings also provide evidence for the presence of substantial amounts of weakly acidic pectic substances similar to WAPs-75 or WAPs-25 along with ordinary pectic acids in situ at least in the vegetables used in this study.

Recently, de Vries et al. presented five simple models (type A~E) for the pectin molecules of ripe and unripe apples. They constructed the models by placing 'hairy' regions at regular intervals and close to the ends of homogalacturonan chains. One of these, type E, a low molecular weight pectin fraction, had ten times the neutral sugar content of type B which was the dominant molecule of apple pectin and had one 'hairy' region at the end of the homogalacturonan. Type E and WAPs in this work resemble each other in neutral sugar content and molecular size. Although type E pectin was regarded as a degradation product, Stoddart et al. suggested that the two different components, the weakly and strongly acidic pectinic acids, of sycamore pectic substances have distinct functional roles in situ in the growing cell walls. The results obtained in this work also suggest that WAP is an inherent component of the vegetable pectic substances and that both WAPs and pectic acids are probably functionally distinct in the cell walls of vegetables.
REFERENCES