Structure of a Pectic Polysaccharide Fraction from Zea Shoots

Yoji Kato and Donald J. Nevins

Laboratory of Food Science, Faculty of Education, Hirosaki University, Hirosaki 036 Japan (Y.K.), and Department of Vegetable Crops, University of California, Davis, California 95616 (D.J.N.)

ABSTRACT

A pectic fraction, accounting for about 0.3% of the total cell wall polysaccharide, was derived from the hot water extract of an insoluble fraction of the buffer-homogenate of Zea shoots. The pectic polysaccharide fraction was characterized by fragmentation analysis after hydrolysis with acid and Erwinia carotovora pectate lyase. The results suggest that the fraction consists of mostly a linear homopolymyxogalacturan with neutral sugar components or a homogalacturonan and a rhamnogalacturan with neutral sugar components.

To learn the nature of structural features of cell walls in elongating monocot tissues, we initiated a detailed study on Zea shoot cell-wall polysaccharides. In previous reports we reported on the structural characterization of the arabino-3,6-galactan obtained from a soluble fraction of the buffer-homogenate of Zea shoots (6), and of the (1→3),(1→4)-β-D-glucan and (1→3)-β-D-glucan, which were obtained by treatment of an insoluble fraction of the buffer-homogenate of Zea shoots with 3 M LiCl and hot water (8, 9). In the course of structural studies of polysaccharides obtained by treatment of an insoluble fraction of the buffer-homogenate of Zea shoots with hot water, we prepared a pectic polysaccharide fraction.

The pectic polysaccharides of the primary cell walls of monocots have not been studied extensively, although it is clear that monocot cell walls possess relatively small amounts of GalUA23 (3, 12).

This report is the fourth in a series of dealing with the water-soluble polysaccharides of Zea shoots and describes the structure of a pectic polysaccharide fraction from Zea shoots.

MATERIALS AND METHODS

Materials

Zea mays L. (B73 × Mo17) shoots (fresh weight, 3800 g), excised at the coleoptile node 9 h after imbibition, were homogenized with 10 mM Na-phosphate buffer (pH 6.5) and centrifuged. The precipitate (wet weight, 310 g) was treated successively with 3 M LiCl and hot water. After hydrolysis with α-amylase (Sigma, type 1A from porcine pancreas), the hot water extract (water-soluble polysaccharide fraction III, WSP-III) was resolved into seven fractions by (NH4)2SO4 precipitation. These procedures were described in a previous paper (9). One fraction, 20P-S (362 mL of aqueous solution), precipitated in 20% (NH4)2SO4 but soluble in water (see Table I in Ref. 9), was used for structural characterization.

Erwinia carotovora PL129, purified to the electrophoretically pure state, was a generous gift of Mr. Naganuma of Tohoku University (11).

General Methods

Concentration of carbohydrate solutions was performed under reduced pressure at 35 to 40°C. Paper chromatography was performed on Toyo No. 50 filter paper by the multiple ascending method using butanol:pyridine:water (6:4:3, v/v) (method A), or by the descending method using ethyl acetate:water:acetic acid:formic acid (18:4:3:1, v/v) (method B). Sugars on the chromatogram were detected with alkaline silver nitrate (13). Total carbohydrate and uronic acid were determined by the phenol-H2SO4 method (4) and the carbazole-H2SO4 method (1), respectively. GLC was conducted with a Yanagimoto model G-80 gas chromatograph equipped with a flame ionization detector at a nitrogen gas flow rate of 15 mL/min.

Analysis of Neutral Sugars in Poly- or Oligosaccharides

Polysaccharide (10–50 µg) was hydrolyzed with 2 M TFA for 5 to 6 h at 100°C. Oligosaccharide (5–10 µg) was hydrolyzed with 1 M TFA for 4 to 5 h at 100°C. Each hydrolysate was evaporated to dryness. Sugars were converted to alditol trifluoroacetates and analyzed by GLC on a column (0.4 × 200 cm) packed with 1.5% QF-1 on Chromosorb W at 140°C (5).

Resolution of Fraction 20P-S by DEAE-Sephadex A-25 Chromatography

Fraction 20P-S (475.6 mg as GalUA equivalent/350 mL of water) was mixed with 40 mL of 0.25 M Na-phosphate buffer (pH 6.0) containing 1% NaCl and the mixture was applied to a column (2 × 29.5 cm) of DEAE-Sephadex A-25 preequilibrated with 25 mM Na-phosphate buffer (pH 6.2) containing 1% NaCl. The column was washed with the same buffer (390 mL), then stepwise elution was carried out with buffers containing 0.1 M (523 mL), 0.2 M (400 mL), 0.5 M (362 mL), and

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Fraction (110 μg as Xyl equivalent/1 mL of water) was derived from a partial acid hydrolysate of Zea pectin (see "Materials and Methods") and applied to a column (1 × 60) of Bio-Gel P-2 operated at 45°C. One-half mL fractions were eluted with water and assayed for carbohydrate. Tubes 31 to 39, 40 to 44, 45 to 50, 51 to 55, 56 to 58, 59 to 64, 65 to 72, 73 to 82 were separately combined and concentrated to give fractions PAH-N-a to -h.

1.0 m NaCl (428 mL), 0.5 m NaOH (513 mL), and 0.5 m HCl (238 mL); 9.5 mL fractions were collected. Fractions were assayed for carbohydrate by the phenol-H2SO4 method. Unadsorbed fractions (20P-S-I) and fractions eluted with 0.5 m NaCl (20P-S-II) were separately combined and concentrated to about 100 mL. Methanol (400 mL) was added to the respective concentrates and mixed, the mixture was maintained at 4°C overnight and centrifuged. The precipitate was successively washed with 80% methanol, methanol and ether, and finally dried. The yields of fractions 20P-S-I and 20P-S-II were 376.2 mg and 23.7 mg GalUA equivalent respectively. 20P-S-I was designated as Zea pectin.

Chromatography on a Sepharose CL-6B column of fractions 20P-S-I and 20P-S-II.

Fraction 20P-S-I (1061 μg as GalUA equivalent/L of 20 mm Na-acetate buffer, pH 5.0) and fraction 20P-S-II (610 μg as GalUA equivalent/1 mL of 20 mm Na-acetate buffer, pH 5.0) were individually applied to a column (1.3 × 40 cm) of Sepharose CL-6B pre-equilibrated with 20 mm Na-acetate buffer (pH 5.0), followed by filtration through the column with the same buffer. Fractions of 0.5 mL each were collected and assayed for uronic acid. Leuconostoc dextran (10) Dextran 110, Dextran T-40, and glucose were used for column calibration.

Acid Hydrolysis of Zea Pectin

Zea pectin (about 60 mg as GalUA equivalent) was hydrolyzed with 20 mL of 1 M TFA for 5 h at 100°C and evaporated to dryness in the presence of methanol. The dried material was dissolved in 5 mL of water and applied to a column (1.8 × 8.5 cm) of Dowex-1 (acetate form). The column was washed with water (45 mL), then by stepwise elution with 0.1 M (145 mL), 0.2 M (220 mL), 0.5 M (160 mL), and 4 M (215 mL) acetic acid and 2 M NaOH (210 mL). Fractions of 5 mL were collected and assayed for carbohydrate. Tubes 4 to 10, 20 to 52, 53 to 115, 121 to 160, and 163 to 204 were separately combined and concentrated to give AH-1 through AH-5.

Partial Acid Hydrolysis of Zea Pectin

Zea pectin (about 32 mg as GalUA equivalent) was hydrolyzed sequentially with 0.1 m (6 mL), 0.5 m (4 mL), and 2 m (1 mL) TFA for 2 h at 100°C. After each hydrolysis, the residue was separated from the supernatant by centrifugation. The supernatant obtained in each step was evaporated to dryness, and then dissolved in 1 mL of water. Four mL of methanol was added to the solution, it was mixed and the mixture was maintained for 24 h at -20°C. The resulting

Table I. Yields and Neutral Sugar Composition of Fractions Obtained from a Low Mol Wt Neutral Fraction Prepared from the Partial Acid Hydrolysate of Zea Pectin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube Nos. in Figure 3</th>
<th>Yield*%</th>
<th>Neutral Sugar Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rha</td>
</tr>
<tr>
<td>PAH-N-a</td>
<td>31-39</td>
<td>23.0</td>
<td>9.1</td>
</tr>
<tr>
<td>-b</td>
<td>40-44</td>
<td>7.8</td>
<td>6.6</td>
</tr>
<tr>
<td>-c</td>
<td>45-50</td>
<td>5.6</td>
<td>3.1</td>
</tr>
<tr>
<td>-d</td>
<td>51-55</td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td>-e</td>
<td>56-58</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>-f</td>
<td>59-64</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>-g</td>
<td>65-72</td>
<td>12.5</td>
<td>15.0</td>
</tr>
<tr>
<td>-h</td>
<td>73-82</td>
<td>44.7</td>
<td>14.6</td>
</tr>
</tbody>
</table>

* The amount of carbohydrate material in each peak, expressed as percent of the total recovered carbohydrate, was determined by the phenol-H2SO4 method.
mixture was centrifuged to remove the methanol-precipitated materials. The supernatant (low mol wt fraction) was evaporated to dryness. The dried material was dissolved in 1 mL of water and passed through a column (0.4 x 6 cm) of Dowex-1 (acetate form), yielding the neutral fraction. Acidic sugars were eluted from the column with 8.5 M acetic acid.

The neutral fraction obtained from the low mol wt fraction of 0.1 M TFA hydrolysate was designated as a low mol wt neutral fraction (yield: 110 μg as Xyl equivalent). The acidic fractions obtained from the low mol wt fractions in the 0.1, 0.5, and 2 M TFA hydrolysates were combined to give a low mol wt acidic fraction (yield: 788 μg as GalUA equivalent).

Enzymic Hydrolysis of Zea Pectin

Zea pectin (5.3 mg as GalUA equivalent) was dissolved in 2 mL of 50 mM Tris-HCl buffer (pH 8.2) and incubated with 20 μL of Erwinia carotovora pectate lyase (PL, 1 mg/50 mM Tris-HCl buffer, pH 8.2) at room temperature. At intervals, the incubation mixture was monitored for 4,5-unsaturated galacturonic acid by measuring absorbance at 235 nm. After 1 h incubation, a portion (0.4 mL) of the incubation mixture was subjected to gel-filtration on Sepharose CL-6B, and another portion (1.5 mL) was subjected to DEAE-Sephadex A-25 chromatography.

RESULTS

Isolation and Purification of a Pectic Polysaccharide Fraction

Carbohydrate (as Glc equivalent), uronic acid (as GalUA equivalent), and protein content in WSP-III in 2.6 L of Na-phosphate buffer obtained from Zea shoots (fresh weight, 3800 g) were 2444, 906, and 20.8 mg, respectively. Sugar composition analysis showed that WSP-III consisted of Rha, Rib, Ara, Xyl, Man, Gal, Glc, and uronic acid in the molar proportions of 0.3: 2.2: 3.8: 2.2: trace: 4.6: 39.9: 47.0. WSP-III was fractionated into seven fractions by the graded (NH₄)₂SO₄ precipitation method as described previously (9). Studies on certain fractions reveal that most of the glucose residues are derived from (1→3), (1→4)-β-D-glucan and (1→3)β-D-glucan (8, 9).

Uronic acid content in fraction 20P-S (362 mL of aqueous solution), obtained after resolution of WSP-III by the graded (NH₄)₂SO₄ precipitation method, was 492.3 mg. About 55% of the total uronic acid in WSP-III was recovered as fraction 20P-S. Sugar composition analysis of fraction 20P-S showed that it also consisted of Rha, Ara, Xyl, Gal, Glc, and uronic acid in the molar proportions of 0.4: 0.5: 0.4: 2.0: 1.1: 95.6. When fraction 20P-S (350 mL of the sample) was chromatographed on DEAE-Sephadex A-25 (phosphate form), it was separated into two fractions: one that was not retained by the column (fraction 20P-S-I) and another that eluted from DEAE-Sephadex at 0.5 M NaCl (fraction 20P-S-II) when subjected to elution by stepwise increments of salt solutions. Polysaccharides from both fractions were recovered by centrifugation after precipitation with four volumes of methanol. Yields of fractions 20P-S-I and -II were 376.2 and 23.7 mg as GalUA equivalent. The total yield accounted for approximately 10% of the total WSP-III. Previous studies have shown that WSP-III accounts for approximately 2.5% of the total Zea cell wall polysaccharides (6, 7). Therefore, one may estimate that the pectic polysaccharide recovered in these fractions accounts for about 0.3% of the total Zea cell wall polysaccharides.

To attempt purification, fraction 20P-S-I was chromatographed on DEAE-Sephadex A-25 (acetate form). However, the fraction was strongly absorbed on the gel and no carbohydrate eluted during stepwise elution with 0 to 1, 1, and 2 M NaCl in the same buffer or with 0.1, 0.5, 2 M NaOH. The results suggest that fraction 20P-S-I does not contain neutral polysaccharide(s). Fraction 20P-S-I was insoluble in cold water but became soluble after heating in a boiling water bath for 10 min. The aqueous solution exhibited a high viscosity and formed a gel upon addition of acid. When fractions 20P-S-I and -II were separately subjected to gel-filtration chromatography on Sepharose CL-6B, they both eluted near the void volume of the column. Sugar composition analysis showed...
that fractions 20P-S-I and -II consisted of Rha, Fuc; Ara, Xyl, Man, Glc, Gal, and GalUA in the molar proportions of 0.7: 0.2: 1.1: 1.23: 0.2: 1.3: 1.5: 93.9 and of 0.6: 0.1: 0.1: 0.1: 0.9: 1.2: 4.2: 92.7, respectively. Both fractions contained a small amount of unidentified sugars (about 0.5%). Fraction 20P-S-I is hereafter designated as Zea pectin.

Acid Hydrolysis of Zea Pectin and Analysis of the Hydrolysate

Zea pectin (about 60 mg as GalUA equivalent) was hydrolyzed with 1 M TFA. The hydrolysate was chromatographed on a Dowex-1 column, and five carbohydrate fractions AH-1 (unadsorbed fractions), AH-2 (eluted with 0.1 M CH₃COOH), AH-3 (eluted with 0.2 and 0.5 M CH₃COOH), AH-4 (eluted with 4.0 M CH₃COOH) and AH-5 (eluted with 2.0 M NaOH) were obtained at yields of 8.3, 33.5, 2.8, 3.1, and 2.0 mg as GalUA equivalents, respectively.

The major fraction, AH-2, was indistinguishable from authentic galacturonic acid upon paper chromatographic analysis (method B). AH-3 resolved into two components (R$_{GalUA}$ 0.14 and 0.04, method B), and the major compound, AH-3-i (R$_{GalUA}$ 0.14, about 70% of AH-3) was isolated by preparative paper chromatography. Neutral sugar analysis of the acid hydrolysate by GLC and paper chromatography (method A) indicated that rhamnose is the sole neutral sugar in AH-3-i, and paper chromatographic analysis (method B) showed that galacturonic acid is a sole acidic sugar. The ratio of galacturonic acid and rhamnose of AH-3-i was approximately 5:1.

The unadsorbed fraction, AH-1, was chromatographed on a column (1.0 × 60 cm) of Bio-Gel P-2, and fractions corre-
GalUA-(1→4)-GalUA

Figure 4. Fractionation of fraction EH-e. A concentrate of fraction EH-e obtained in Figure 3 was applied to a column (1 × 55 cm) of Bio-Gel P-2 pre-equilibrated with 1 M ammonium acetate and operated at 45°C. The column was eluted with 1 M ammonium acetate. Fractions of 0.5 mL were collected and assayed for carbohydrate (Amon) and 4.5-unsaturated galacturonic acid (Aga). The arrows on the figure index the elution positions of apple pectin (V₀) and GalUA used for column calibration. Tubes 39 to 46 (fraction EH-e-1), 47 to 53 (EH-e-2), and 54 to 59 (EH-e-3) were separately combined, concentrated, and refined by rechromatography on the same column.

Partial Acid Hydrolysis of Zea Pectin and Analysis of the Hydrolysate

Zea pectin (about 32 mg as GalUA equivalent) was hydrolyzed sequentially with 0.1, 0.5, and 2 M TFA. A low mol wt acidic fraction, and a low mol wt neutral fraction were prepared from the hydrolysates as described in "Materials and Methods."

The low mol wt acidic fraction was analyzed by paper chromatography (method B). Three components (R₀GalUA 1.0, 0.28, and 0.11) were detected; the R₀GalUA values were identical with those of authentic GalUA, GalUA-(1→4)-GalUA, and GalUA-(1→4)-GalUA-(1→4)-GalUA.

The low mol wt neutral fraction was chromatographed on a column (1.0 × 60 cm) of Bio-Gel P-2 (Fig. 1). Neutral sugar composition of fractions PAH-N-a to h (Fig. 1) were analyzed by GLC, and the results are summarized in Table I. All of the fractions consisted of Rha, Fuc, Ara, Xyl, Man, Glc, and Gal.

Enzymic Hydrolysis of Zea Pectin and Analysis of the Hydrolysate

Zea Pectin (about 5.3 mg as GalUA equivalent) was hydrolyzed with Erwinia carotovora PL. A portion of the enzymic hydrolysate was chromatographed on Sepharose CL-6B. Figure 2 reveals that the enzymic hydrolysate of Zea pectin exhibits a much lower degree of polymerization compared with that of the original Zea pectin which eluted near the V₀ with a peak at tube No. 45.

Another portion of the enzymic hydrolysate of Zea pectin was chromatographed on DEAE-Sephadex A-25 (Fig. 3), and eight fractions, EH-a to EH-h were obtained. The elution pattern of a substance with absorption at 235 nm, corresponding to the 4.5-unsaturated GalUA, is associated with that of carbohydrate (A₄₅₀) (Fig. 3). Yields, ratios, of neutral and acidic sugars and neutral sugar compositions of the fractions are summarized in Table II. All except for fraction EH-e consist of neutral and acidic sugars and their neutral sugar composition is nearly the same.

The major fraction, EH-e, was chromatographed on Bio-Gel P-2 (Fig. 4). Three fractions, EH-e-1, -2, and -3 obtained were rechromatographed on the same column. When each of the fractions was subjected to paper chromatography (method B), a single spot was observed; R₀GalUA values were 0.06 for EH-e-1, 0.21 for EH-e-2, and 0.79 for EH-e-3. Paper chromatographic analysis (method B) of the acid hydrolsates of EH-e-1 to -3 showed that these three fractions contained GalUA. Sugiura (15) reported that E. carotovora PL hydrolyzed pectic acid into mono-, di-, tri-, tetra-, pentasaccharides and higher oligomers. EH-e-1, -2, and -3 probably represent tetra-, tri-, and disaccharides of GalUA, the nonreducing terminals of which are 4.5-unsaturated GalUA.

DISCUSSION

Ray and Rottenberg (12) isolated a GalUA disaccharide from oat coleoptile cell walls, and Wada and Ray (16) extracted polygalacturonic acid from oat coleoptile cell walls by ammonium oxalate. Methylation analyses of maize coleoptile cell walls have demonstrated the presence of 4-linked galacturonosyl residues (2). The results of these studies suggest that monocot cell walls contain small amounts of 4-linked galacturonans. More recently, Shibuya and Nakane (14) isolated and characterized pectic polysaccharide of rice endosperm cell walls.

The isolation of rhamnose-containing oligagalacturonic acid from the acid-hydrolysate of Zea pectin clearly indicates that the Zea pectin contains rhamnogalacturonan. However, it is most likely that the Zea pectin preparation obtained here is a mixture of rhamnogalacturonan and polygalacturonic acid.

Zea pectin was hydrolyzed with pectate lyase. Fractions EH-a, -b, -c, -d, -e, -f, -g, and -h obtained from the hydrolysate contained GalUA, Rha, Fuc, Ara, Xyl, Man, Glc, and Gal in similar concentrations (Table II). Undoubtedly, these fractions were derived from the Zea pectin preparation. The fractions exhibit absorption at 235 nm because of the presence of 4.5-unsaturated GalUA (Fig. 3). Moreover, fractions PAH-N-a to h obtained by Bio-Gel P-2 chromatography after partial acid hydrolysis of Zea pectin revealed oligosaccharides comprised of Rha, Fuc, Ara, Xyl, Man, Glc, and Gal (Table I). Although detailed structural studies of fractions EH-a to -h and PAH-N-a to -h have not been completed, the results suggest glycosidic bonds between other cell wall polysaccha-
rizes (including pectic neutral oligo- and polysaccharides) and rhamnogalacturonan in *Zea* shoot cell walls.

**LITERATURE CITED**