Studies on Protopectinase-C Mode of Action: Analysis of the Chemical Structure of the Specific Substrate in Sugar Beet Protopectin and Characterization of the Enzyme Activity

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Chemical structures of pectic substances degraded by protopectinase-C (PPase-C) were characterized to identify the releasing mechanism of pectin from sugar beet protopectin by the action of that enzyme. The substrate of PPase-C was a polysaccharide isolated from sugar beet pulp by extraction with NaOH and sequential digestions with rhamnogalacturonase (PPase-T), β-1,4-d-galactanase, and α-L-arabinofuranosidase. The structure of this polysaccharide was analyzed by gas-liquid chromatography (GLC), NMR analysis, and gas chromatography-mass spectrometry (GC-MS), and it was identified as α-1,5-L-arabinin. According to our results, arabinan chains seemed to be connected to rhamnogalacturonan through a chain of β-1,4-d-galactan. PPase-C hydrolyzed both linear α-1,5-L-arabinin and ramified 1-arabinin in a random manner, producing 1-arabinose. From these results, PPase-C could be classified as arabinan endo-1,5-α-L-arabinase [EC 3.2.1.99]. Moreover, PPase-C seemed to split the 1-arabinin of the polysaccharides connecting the rhamnogalacturonan to the other constituents of the plant cell wall in sugar beet pulp, releasing water-soluble pectin.

Protopectin is a water-insoluble parent pectic substance found in plant tissues. The features of the insolubility of pectin in protopectin are complex and not totally known. Protopectin is solubilized by restricted hydrolysis, resulting in the liberation of water-soluble pectin. Enzymes that catalyze the solubilization of protopectin have tentatively been named protopectinases. Sakai and Okushima reported a protopectinase in a yeast strain in 1978. Since then, several other protopectinases have been described. We have isolated two different protopectinases from Bacillus subtilis IFO 3134 (PPase-C) and Trametes sanguinea IFO 6490 (PPase-T, unpublished results), which do not degrade the homogalacturonan chains in protopectin. PPase-T cleaved galactopyranosyluronic-rhamnopyranosyl linkages in protopectin (unpublished results). In a previous paper, it was reported that PPase-C specifically split α-1,5-L-arabinin, which was prepared from sugar beet pulp by the method of Tagawa and Kaji. In this case, PPase-C activity was only studied on that substrate, but nothing was mentioned about its activity when using high molecular weight substrates, which include the surrounding regions (i.e., galactan and rhamnogalacturan) in sugar beet protopectin. Furthermore, the relationship between arabinanase and protopectinase activities in PPase-C was not established.

On the other hand, it is necessary to characterize the chemical structures of pectic substances degraded by PPase-C to improve the knowledge on the mechanisms involved in the solubilization of sugar beet protopectin.

In this paper, we described the isolation of the neutral sugar side chains, called hairy regions, split from sugar beet pulp rhamnogalacturan by successive enzyme digestions, the identification of the polysaccharides degraded by PPase-C and also, the mode of action of this enzyme toward L-arabinan.

Materials and Methods

Chromatography. Sugar beet pulp was obtained from Tien Sugar Co. (Tienen, Belgium). Unless otherwise specified, other chemicals were from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and of reagent grade.

Enzyme preparations. α-L-Arabinofuranosidase was purified from a culture filtrate of Aspergillus niger K1 (kindly provided by Prof. A. Kaji, Kagawa University). Pure β-1,4-d-galactanase was kindly supplied by Dr. Y. Tominaga (Osaka Municipal Technical Research Institute). PPase-T was isolated from the culture filtrate of Trametes sanguinea IFO 6490.

Preparation of alkaline soluble polysaccharide (ASP). The substrate of PPase-C (rhamnogalacturan from sugar beet pulp containing the hairy regions) was prepared as follows: sugar beet pulp (50 g) was mixed with 0.1 N NaOH (3 liters), and the mixture was heated at 100°C for 1 h. The slurry was filtered and the filtrate was centrifuged. Then, three volumes of acetone were added to the supernatant. The precipitate formed was dissolved in distilled water. The solution was treated batchwise with Dowex-50 (H⁺-form) to remove cationic substances associated with pectin. The effluent was put on a DEAE-Cellulofine AH column (6 × 28 cm; Chisso Corp., Tokyo) and the bound polysaccharides were eluted with 1 M acetate buffer (pH 5.0). This eluate was concentrated, dialyzed against distilled water, precipitated by the addition of three volumes of ethanol, and finally lyophilized, giving ASP.

Analysis of sugar components. Five milligrams of polysaccharides were hydrolyzed with 5 ml of 250 mM H₂SO₄ at 100°C for 8 h, neutralized with BaCO₃, and filtered. The filtrate was deionized by using the resin Dowex-50W (X8, H⁺-form) and Dowex-1 (X8, OH⁻-form). The deionized solution was filtered through a 0.45 μm membrane, concentrated under reduced pressure at room temperature, and analyzed by HPLC.

High performance liquid chromatography (HPLC). For the analysis of sugar composition, HPLC was done with a Shodex KS-801 and KS-802 columns (Showa Denko, Tokyo) with distilled water as solvent at the flow rate of 0.6 ml/min at 80°C. The effluent was monitored with a refractive index detector (Showa Denko). Sugars, whose reducing groups were previously labeled with tritium, were chromatographed with a KS-802.
column with distilled water as solvent at a flow rate of 0.7 ml/min at room temperature. The radioactivity was detected by a radiisotope detector Model 171 (Beckman Instruments Inc., Fullerton, Calif.).

**Gel permeation chromatography.** Gel permeation chromatography was done in a FPLC apparatus by injecting 500 µl of the pectic solution into a Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology AB Uppsala, Sweden), equilibrated with 20 mM AcB, pH 5.0, containing 100 mM NaCl. Fractions of 1 ml were collected at the flow rate of 0.75 ml/min.

**Iodexchange chromatography.** The pectic solution was put on a DEAE-Toyopearl 650M column (2 × 16 cm; Tosoh Corp., Tokyo) equilibrated with 50 mM AcB, pH 5.0. The unbound materials were washed with 100 ml of the same buffer, the bound pectic substances were eluted by a linear gradient (200 ml, 50 to 1000 mM, pH 5.0) of AcB at a flow rate of 30 ml/h, and 5-ml fractions were collected.

**Methylation analysis.** Polysaccharides were methylated by the use of methylsulfonyl carbamion (MeSC) in dry MeSO and CHCl₃ by the Hakomori procedure. MeSC was prepared by the method of Rauvala. The permethylated products were hydrolyzed, and the released sugars were converted into their alditol acetates and analyzed by GLC and GC-MS.

**Conditions of GLC.** GLC was done with a Hewlett Packard Component HP 5890A chromatograph with a flame ionization detector on a capillary column (SP 2330, 0.32 mm × 30 m; Supelco Japan, Ltd., Tokyo). The column temperature was programmed to increase from 160°C to 220°C at the rate of 4°C/min, and the injector and detector temperatures were both 180°C. The flow rate of the carrier gas (He) was 25 ml/min.

**Conditions of GC-MS.** GC-MS was done with a JMS-AX500 (JEOL, Tokyo) chromatograph with a mass spectrometer detector by using a glass column (OV-1, 5 mm × 2 m). The column temperature was programmed to increase from 160°C to 220°C at the rate of 4°C/min, and was then held at 220°C. The injector and detector temperatures were both 250°C. The flow rate of the carrier gas (He) was 10 ml/min. The ion source temperature of the mass spectrometer was 200°C and the ionizing energy was 70 eV.

**NMR spectroscopy.** NMR spectra of polysaccharides were measured (P0; 5 mg) in (CD₃)₂SO (2 ml) in a 5-mm o.d. NMR tube at 30°C. Acquisition and processing were done on a Bruker AM500 instrument equipped with an Aspect 3000 computer running DISR871 (Bruker Japan Co., Ltd.). For spin-lock experiments, a Bruker correlation unit was used, giving a 2.5-KHz radio frequency field. All spectra were measured with a chemical shift spectral window of 2717 Hz.

**Enzyme assay.** Enzyme activities toward ASP-1a and PO (described in Results) were assayed by measuring the release of reducing groups by the method of Somogyi. One unit of activity was defined as the activity that liberates reducing groups corresponding to 1 µmol of L-arabinose per milliliter of reaction mixture at 37°C in 60 min.

**Analytical methods.** Galacturonic acid was assayed by the method of Blumenkrantz and Asboe-Hansen. Total neutral sugars were measured by the phenol-sulfuric acid method (17) (using D-galacturonic acid and L-arabinose as the standards) as the difference between the total absorbance and the one corresponding to galacturonic acid.

**Results**

**Isolation and characterization of polysaccharides able to be degraded by PPass-C**

In preliminary experiments, PPase-C activity was higher when using the unbound fraction (in the DEAE-Cellulofine AH column during the preparation of ASP) than when using the bound fraction as substrate. The former had a lower galacturonic acid content than the latter. Nevertheless, the bound fraction was also collected to investigate the relationship between PPase-C activity and the chemical structure of the pectin.

**Fig. 1.** Chromatography of Pectic Substances Degraded by PPase-T on DEAE-Toyopearl for the Preparation of Hairy Fragments.

Chromatography was done as described in the text. (A), ASP before the digestion; (B), after the digestion. — ○ —, neutral sugar; — - - - , uronic acid.

To isolate the hairy region connecting with rhamnose in rhamnogalacturonan of sugar beet pectin, ASP was partially hydrolyzed with PPase-T, which splits galactopyranosyl-uronic-rhamnopyranosyl linkages in rhamnogalacturonan, in 20 mM AcB, pH 5.0, at 45°C. After the enzyme was denatured by heating for 5 min, the reaction products were put on a DEAE-Toyopearl 650M column and three fractions, which were called ASP-1, -2, and -3, were collected. (Fig. 1). These fractions contained different proportions of galacturonic acid and consequently, they were eluted by using an ion strength gradient in the anion-exchange chromatography. PPase-C showed more activity on ASP-1. This fraction, which was unbound on DEAE-Toyopearl chromatography, contained fewer galacturonic acid than the other two fractions (Table I) and seemed to be the hairy regions split from rhamnogalacturonan by PPase-T. ASP-3 might be the rhamnogalacturonan region that was not degraded by PPase-T or it might contain low molecular weight hairy regions.

To isolate the specific region in the substrate that was split by PPase-C, ASP-1 was completely digested by β-1,4-d-galactanase in 20 mM AcB, pH 5.0, at 40°C for 24 h and then chromatographed with a Superose 12 column (Fig. 2). Three fractions were obtained in this step and they were termed ASP-1a, -1b, and -1c, respectively. Their sugar compositions are summarized in Table I. PPase-C hydrolyzed ASP-1a, which was composed of 93%
Table I. Sugar Composition of Sugar Beet Pectin Fractions Treated with PPase-T and β-1,4-d-Galactanase

<table>
<thead>
<tr>
<th>Pectins</th>
<th>Gal/A</th>
<th>Rha (mol%)</th>
<th>Gal</th>
<th>Ara</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>8</td>
<td>5</td>
<td>15</td>
<td>72</td>
</tr>
<tr>
<td>ASPT-1</td>
<td>4</td>
<td>3</td>
<td>14</td>
<td>79</td>
</tr>
<tr>
<td>ASPT-2</td>
<td>15</td>
<td>12</td>
<td>18</td>
<td>55</td>
</tr>
<tr>
<td>ASPT-3</td>
<td>19</td>
<td>15</td>
<td>21</td>
<td>45</td>
</tr>
<tr>
<td>ASPT-1a</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>ASPT-1b</td>
<td>5</td>
<td>4</td>
<td>23</td>
<td>68</td>
</tr>
<tr>
<td>ASPT-1c</td>
<td>6</td>
<td>7</td>
<td>75</td>
<td>12</td>
</tr>
</tbody>
</table>

* Small amounts of sugars were neglected. Detailed descriptions of the different pectin fractions are in the text.

arabinose. ASPT-1c had high galactose and reducing sugar contents, which indicated that galactose was separated from ASPT-1 by the enzyme digestion. The ratio of galacturonic acid to neutral sugar content was increased in ASPT-1b and -1c, which suggested that rhamnogalacturonan was connected to galactose residues.

The following procedure was done by the method of Tagawa and Kaji. ASPT-1a was digested with purified α-L-arabinofuranosidase from Aspergillus niger K1 in 20 mM citrate-phosphate buffer, pH 4.0, at 40°C. The reaction was monitored by the increase of reducing ends of ASPT-1a. When about 30% of the ASPT-1a was hydrolyzed, the rate of hydrolysis decreased and the mixture was cooled to 20°C and incubated at this temperature for 12 h. The reaction mixture was then heated for 5 min at 100°C to inactivate the enzyme, and ethanol was added to a final concentration of 80%. The precipitate was collected and dissolved in hot water, and the solution was left at 2°C for 24 h for reprecipitation. After centrifugation the supernatant was discharged and the solids were lyophilized yielding the polysaccharide (PO). PO showed almost the same characteristics that those described by Tagawa and Kaji and, also it was partially degraded by PPase-C.

Fig. 2. Elution Pattern of Reaction Products of ASPT-1 with β-galactanase on Superoxide 12 Column Chromatography.

Table II. Relative Retention Times on GC of Partially Methyalted Alditol Acetate

<table>
<thead>
<tr>
<th>Standard sample</th>
<th>Relative retention timea</th>
</tr>
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<tbody>
<tr>
<td>1,4-Ac-2,3,5-Me-t-Arabinitol</td>
<td>0.69</td>
</tr>
<tr>
<td>1,5-Ac-2,3,4-Me-t-Arabinitol</td>
<td>0.79</td>
</tr>
<tr>
<td>1,4,5-Ac-2,3-Me-t-Arabinitol</td>
<td>1.13</td>
</tr>
<tr>
<td>Major peak</td>
<td>1.13</td>
</tr>
<tr>
<td>Minor peak</td>
<td>0.69</td>
</tr>
</tbody>
</table>

* Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol.

b Authentic samples were synthesized chemically. Ac, acetyl; Me, methyl.

Table III. 1H- (A) and 13C- (B) NMR Chemical Shift Data for PO

<table>
<thead>
<tr>
<th>(A)</th>
<th>δ (ppm)</th>
<th>(B)</th>
<th>δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>109.46</td>
<td>C-1</td>
<td>109.46</td>
</tr>
<tr>
<td>C-2</td>
<td>83.02</td>
<td>C-2</td>
<td>83.02</td>
</tr>
<tr>
<td>C-4</td>
<td>82.79</td>
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<td>82.79</td>
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<tr>
<td>C-3</td>
<td>78.60</td>
<td>C-3</td>
<td>78.60</td>
</tr>
<tr>
<td>C-5</td>
<td>68.38</td>
<td>C-5</td>
<td>68.38</td>
</tr>
</tbody>
</table>

Identification of PO

The acid hydrolyzate of PO was identified as arabinose by HPLC analysis, and PO was found to be an arabinan. In methylation experiments with GLC, two sharp peaks were detected around the retention times of 8 and 12.5 min. The relative retention times of these compounds and authentic samples to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol were calculated (Table II). From the results, the minor peak in GLC was identified as the authentic 1,4-di-O-acetyl-2,3,5-tri-O-methyl-t-arabinitol, and the major peak corresponded to authentic 1,4,5-tri-O-acetyl-2,3,5-di-O-methyl-t-arabinitol. Analysis of these peaks by GC-MS supported the above identification (data not shown). The major compound might be formed from the 1,5-linkage of L-arabinose, and the minor compound might be derived from the non-reducing terminal arabinose of PO. The molar ratio of these compounds was calculated to be about 1:25. From these results, PO was found to be a homopolymer composed of about 25 residues of l-arabinofuranose (1,5-L-arabinanin).

The spectral data of 1H- and 13C-NMR of PO are given in Table III. The signal for H-1 was assigned to δ 4.76 ppm on account of the position of the resonance of H-2 and H-4 (at δ 3.90-3.78 ppm), H-3 (at 3.70-3.56 ppm), and H-5 (at δ 3.56-3.44 ppm). This was confirmed by 1H-13C correlation spectroscopy (COSY) (Fig. 3). The coupling constant of the doublet for H-1 (J = 1.9 Hz) suggested that the glycosidic linkage was in the α-configuration. The assignment of the resonances for H-1, -2, -3, -4, and -5 was obtained in a straightforward way from the 1H-1H COSY spectrum (Fig. 4). There was only one cross-peak of H-1 with H-2 at δ 3.8 ppm, and no additional peak of H-1 was observed. Doublet signals at δ 5.38 and 5.21 ppm made cross-peaks with H-2 and H-3, respectively. These findings might indicate that the doublet signals at δ 5.38 and 5.21 ppm were for the -OH of H-2 and H-3. From data
obtained by $^{13}$C-NMR, C₂ did not seem to have any –OH groups. From these results PO could be identified as α-1,5-L-arabinan. ASPT-1a, which was the polysaccharide before digestion with α-L-arabinofuranosidase, seemed to be L-arabinan. It has a main structure consisting of α-1,5-L-arabinan to which L-arabinofuranose units were attached at position 3 in the α-configuration to form one-unit side chains, according to the data of Tagawa and Kaji.¹⁰

**Reaction of PPase-C with L-arabinan**

PPase-C seemed to split L-arabinan in sugar beet protopectin. The reaction pattern of the enzyme with L-arabinan was studied in a reaction mixture containing 1 mg of substrate, 5 μl of enzyme solution, and 150 μl of 100 mM acetate buffer, pH 6.0. Ten microliter samples were withdrawn for the identification of intermediate and end products by HPLC from 1 min to 4 h of incubation at 60°C. The reaction was stopped in each sample by the addition of 50 μl of 25 mM NaOH, and the reducing sugars produced were labeled with tritium by treatment with NaB³H₄ by the method of Takasaki and Kobata.¹⁸ Figure 5 showed the HPLC chromatograms of the different samples. It was seen that saccharides with high molecular mass appeared in the early stages of the reaction, which indicated that the enzyme hydrolyzed L-arabinan in a random manner. The concentra-
tion of arabinose increased, reaching the maximum one at around 2 h of reaction time. Afterwards, arabinose concentration diminished, and arabino- triose concentrations increased continuously.

**Extraction of pectin from propectein of sugar beet pulp with PPase-C**

To confirm the fact that PPase-C splits l-arabinan in sugar beet pulp and releases water-soluble pectin, extraction of pectin from the pulp with the enzyme was done. One hundred grams of dried sugar beet pulp previously washed with 50 mM EDTA solution and water, were incubated with 10,000 U of PPase-C at 37°C for 24 h. After the reaction, the mixture was filtered and the filtrate was put on DEAE-Toyopearl for purification of pectin. The elution pattern is shown in Fig. 6. A high proportion of neutral sugars, especially arabinose, was found in the unbound fraction, which indicated that PPase-C cleaves arabinan in sugar beet pulp. The bound sugars were eluted with 1 M acetate buffer, pH 6.0. Both fractions were concentrated, dialyzed against water, and precipitated by the addition of 3 volumes of ethanol yielding C1 and C2. Their properties are summarized in Table IV.

**Discussion**

Protopectin is composed of several neutral sugar chains such as arabino-galactan and xyloglucan, besides polysaccharides that contain galacturonic acid, partially esterified. PPase-C is one of the enzymes that catalyze the solubilization of protoplast in without degrading homogalacturonan.

In this paper, we described the characterization of the chemical structures of pectic substances degraded by PPase-C, to elucidate the releasing mechanism of pectin from sugar beet protoplastin. ASP, which contains homogalacturonan connecting with hairy regions and can be degraded by PPase-C, were prepared from sugar beet pulp. Hairy regions in ASP were sequentially split from homogalacturonan by using first PPase-T, then β-1,4-d-galactanase, and finally α-l-arabinofuranosidase. The structure of the polysaccharide that resists these enzymes was identified as α-1,5-l-arabinan by using GLC, GC-MS, and NMR analysis. From these results, it was concluded that arabinan was one of the polysaccharides composing hairy regions.

Albersheim ²¹ has mentioned that rhamnogalacturonan in sycamore cells was attacked at the reducing end of the arabinogalactan molecule, and also that arabinogalactan seemed to consist of a chain of arabinose coupled to another chain of galactose. We have demonstrated that rhamnogalacturonan connected to hairy regions in sugar beet pectin was degraded by β-1,4-d-galactanase and formed rhamnogalacturonan, arababin, and galactooligosomers. This indicated that the structure of the hairy regions in sugar beet protoplastin was substantially the same as in sycamore cells, with rhamnogalacturonan attached to other cell wall constituents by chains of arabino-galactan.

PPase-C catalyzed the degradation of l-arabinan as well as α-1,5-l-arabinan from sugar beet pulp. When sugar beet pulp was incubated with the enzyme, a pectic substance was released together with a high proportion of arabinooligomer. Therefore, l-arabinan seemed to be one of the types of polysaccharide chains connecting pectin to other cell wall substances, resulting in the insolubility of pectin. PPase-C splits α-1,5-l-arabinose linkages, so it was possible to classify it as belonging to the type of arabian endo-1,5-α-l-arabinase [EC 3.2.1.99].

The different HPLC chromatograms of the PPase-C reaction with l-arabinan could be explained assuming that arabinose was converted to arabinobiose and arabino-triase upon further reaction, but it was not clear which was the arabinose acceptor. Knowledge of arabinosyltransferase activity in PPase-C is not clear, and research on this mechanism is being undertaken.

On the other hand, the PPase activities of endo-1,5-α-l-arabinase (the enzyme that catalyzes the hydrolysis of arabinooligomer to form l-arabinose and arabinobiose) and α-l-arabinofuranosidase were assayed by using the enzymes from B. subtilis ²², ²³ and A. niger, ¹ ¹ respectively. The ratios of PPase to arabinase activities for both enzymes were low compared with the same ratio for PPase-C. Thus, PPase-C could be consider a novel arabinase because it also showed a potent propectinase activity.

Among the properties of the pectic fractions (C1 and C2) released by PPase-C from sugar beet protoplastin, it was remarkable that they showed a lower molecular weight than the usual ones of apple or citrus pectins.

Finally, we concluded that PPase-C splits α-1,5-l-arabinofuranoside linkages of the arabian region connecting homogalacturonan with cell wall constituents in sugar
beet pulp releasing water-soluble pectin.

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References