Isolation and Characterization of Pectin from Pericarp of Citrus depressa

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Abstract: A polysaccharide was extracted from the pericarp of Citrus depressa which was collected Ogimi Village, Okinawa, Japan. The yield of purified polysaccharide was 2.6% (w/w) based on fresh material. The contents of total carbohydrate, uronic acid, ash and moisture of the polysaccharide were 88.0, 78.0, 4.7 and 7.2%, respectively. The degree of methoxylation of the polysaccharide was estimated to be 62.9%. The purified polysaccharide was composed of p-galacturonic acid, p-galactose, L-arabinose, L-rhamnose, p-glucose and p-mannose in the molar ratio of 100 : 9.20 : 1.02 : 0.88 : 0.78 respectively. The molecular mass of the polysaccharide was estimated to be approximately 6.8×10⁶ by gel chromatography. The specific rotation of the polysaccharide was +149° at 25°C, which indicated that the polysaccharide mainly had α-glycosidic linkages. The infrared spectra of the polysaccharide and the de-esterified polysaccharide were in agreement with those of standard pectin and de-esterified standard pectin over wide ranges of wave numbers. Chemical shifts of ¹H- and ¹³C-NMR spectra of the polysaccharide and the de-esterified polysaccharide were also consistent with those of standard pectin and de-esterified standard pectin. NOESY spectroscopy showed that the polysaccharide contained (1→4)-linked p-galacturonic acid residues. The polysaccharide and the de-esterified polysaccharide formed gels in the presence of sucrose under acidic conditions and of Ca²⁺ ions, respectively. These results indicated that the polysaccharide extracted from the pericarp of C. depressa was a pectin.

Key words: pectin, Citrus depressa, high methoxyl

Citrus depressa is a plant that grows naturally in Okinawa Prefecture, Japan. Its fruits have been eaten and used since old times as a stain remover and a cleaner for a local textile (Basyoju). Today the fruits are processed to juices, liquors and so on. They are special products in Okinawa. Furthermore, it was reported that they contained polymethoxyl flavonoids, particularly nobiletin, which carry side chains such as arabinan, galactan and arabinogalactan.

Pectins are a family of plant polysaccharides in the cell wall. Pectins are of great importance in the construction of primary cellular tissues and may be linked covalently to other polymers. Therefore they have great influence on growth, development and senescence, and affect the textural properties of plant tissues and fruits. Pectins are utilized as gelling agents and stabilizers of jams, jellies and other products in the food industry, and generally L-arabinose, D-galactose, L-rhamnose, D-xylose, D-mannose and D-glucose. The galacturonic acid residues are interspersed with (1→2) linked L-rhamnose residues, which carry side chains such as arabinan, galactan and arabinogalactan.

We have so far isolated and characterized a lot of polysaccharides from algae and plants growing in Okinawa, Japan. We have so far isolated and characterized a lot of polysaccharides from algae and plants growing in Okinawa, Japan. The polysaccharide from C. depressa has not been studied yet. We report here the isolation and characterization of the polysaccharide extracted from the pericarp of C. depressa.

MATERIALS AND METHODS

Materials. The fruits of C. depressa were collected in January 2001 from Ogimi Village, Okinawa, Japan. The pericarp was peeled from the fruit and then air-dried in an oven at 40°C for 24 h. The dried pericarp was crushed to powder by a mixer. A high-methoxyl (HM) pectin from Taiyo Kagaku Co., Ltd., Japan was used as a standard.

Extraction and purification. The crushed pericarp sample (5 g) was suspended in 0.05 m HCl and stirred at 85°C for 1 h since pectins are generally extracted under hot and acidic conditions in food industries. The suspension was filtered through a suction filter. The filtrate was adjusted to pH 4.5 with 0.5 m NaOH. The solution was filtered again and concentrated with an evaporator. EtOH
HPLC was performed at room temperature at a flow rate with 0.15M sodium chloride in 0.05 M sodium phosphate. The column was developed with 0.05 M CH₃COONa buffer (pH 4.8). The mixture was washed with 250 mL of the same buffer. The bound material was eluted with 600 mL of 1 M CH₃COONa buffer (pH 4.8). The eluate was dialyzed against distilled water and the dialyzed solution was freeze-dried.

De-esterification. The purified polysaccharide and a standard HM pectin were dissolved in distilled water and chilled to 4°C. Cold NaOH (0.1 M) was added slowly to a final concentration at 0.05 M. The mixture was kept at 4°C for 1 h with mild stirring at intervals. After 1 h, the pH of the mixture was brought to 4.5 with 1 M HCl and the mixture was dialyzed against distilled water, and then freeze-dried.

Determination of the contents of total carbohydrate, uronic acid, moisture and ash, and degree of methylation. The content of total carbohydrate and uronic acid was determined by the phenol-sulfuric acid method and the carbazole-sulfuric acid method, respectively, using D-galacturonic acid as a standard. Moisture content was determined by drying at 110°C for 2 h. The content of ash was determined by incinerating overnight in a muffle furnace at 550°C. Degree of methoxylation (DM) was determined by the method of Inari et al.

Acid hydrolysis. The purified polysaccharide was dissolved in distilled water and sulfuric acid was added to a final concentration of 1.5 M. The mixture was heated at 100°C for 3 h. The hydrolyzate was neutralized with BaCO₃.

Liquid chromatography. The hydrolyzate was applied to a liquid chromatograph DX 500 (Dionex Co., Ltd., USA) fitted with a column of CarboPac PA1 (4×250 mm) and eluted with 15 mM NaOH for neutral sugars and with 100 mM NaOH/150 mM CH₃COONa for uronic acids. The chromatography was carried out at a flow rate of 1 mL/min at 35°C.

Molecular mass determination. The molecular mass of the purified polysaccharide was determined by high-performance liquid chromatography (HPLC) (LC-6A, Shimadzu Co., Ltd., Japan) on a column of TSK-gel GMPWxL (7.8×300 mm, Tosoh Co., Ltd., Japan). The HPLC was performed at room temperature at a flow rate of 0.3 mL/min with refractive index detection (RID-6A, Shimadzu Co., Ltd., Japan). The column was developed with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2) and elution was carried out with the same buffer. Standard pullulans (Shodex Standard P-82; Showa Denko Co., Ltd., Japan) including P-400 (molecular mass, 4.04×10⁴), P-100 (1.12×10⁴), P-20 (2.28×10⁴), and P-5 (5.9×10³) were used as molecular mass markers.

Specific rotation and infrared spectra. Specific rotation was measured at 589 nm on a polarimeter (DIP-180, Jasco Co., Ltd., Japan) for a 0.2% (w/v) solution in distilled water with a cell of 0.5 dm length.

Infrared spectra were recorded with an infrared spectrophotometer (FTS-3000, Bio-Rad Lab. Inc., USA) for the purified polysaccharide, the de-esterified polysaccharide, the HM pectin and the de-esterified HM pectin dispersed in KBr discs.

¹H- and ¹³C-nuclear magnetic resonance (NMR) spectroscopy. The ¹H- and ¹³C-NMR spectra were recorded on an FT-NMR spectrometer at 500.00 and 125.65 MHz (JNM α-500, JEOL Co., Ltd., Japan). The purified polysaccharide and de-esterified polysaccharide were dissolved in D₂O and recorded at 80°C. A sodium 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid (TSP, 0.00 ppm) was used as an internal standard.

Homonuclear NOE correlation (NOESY) spectroscopy. NOESY spectroscopy was performed in de-esterified polysaccharide in D₂O at 80°C. A 256(τ)×128(τ)×2 data matrix was used with a spectral width of 3.05×3.05 kHz, which gave a digital resolution of 11.92×11.92 Hz in f₁ and f₂, respectively. The mixing time of 100 ms was used in this experiment.

Preparation of the gels. The purified polysaccharide (1%) was dissolved in distilled water and sucrose (60%) was added to the solution. The mixture was heated at 100°C for 30 min with magnetic stirring. Then citric acid (0.5 M) was added slowly to reach 0.05 M in final concentration with stirring. The slurry was poured into a test tube and capped with a marble, and then was left overnight at ambient temperature. The de-esterified polysaccharide (1%) was dissolved in distilled water and CaCl₂ solution was added with heating to a final concentration of 3%.

RESULTS AND DISCUSSION

Preparation of the polysaccharide from pericarp of C. depressa.

The pericarp (1.0 kg) was air-dried in an oven at 40°C for 24 h and its weight was decreased to 340 g. The polysaccharide was extracted and then purified as described in MATERIALS AND METHODS, and a colorless, fibrous material was obtained. The yield was 2.6%, based on fresh material. Compared to other sources, this value was larger than those of apples (0.5–1.6%), apricots (0.7–1.3%) and the others (1.5%) except lemons (3.0–4.0%) and grapefruits (3.3–4.5%).

Chemical components of the purified polysaccharide.

The contents of total carbohydrate and uronic acid of the purified polysaccharide were determined to be 88.0 and 78.0% by the phenol-sulfuric acid method and the calbazol-sulfuric acid method, respectively. The moisture was estimated to be 7.2% and the ash 4.7%. Degree of methoxylation was determined to be 62.9%.

Identification of sugar components of the purified polysaccharide.

The liquid chromatography of the hydrolyzate of the purified polysaccharide showed five peaks, which were identical to those of L-rhamnose, L-arabinose, D-galactose,
D-glucose and D-mannose, respectively. By the liquid chromatography for uronic acids, the uronic acid was determined to be D-galacturonic acid. The molar ratio of D-galacturonic acid, D-galactose, L-arabinose, L-rhamnose, D-glucose and D-mannose was estimated to be 100 : 9.20 : 1.34 : 1.02 : 0.88 : 0.78 from the areas of the peaks and the ratio of neutral sugars to galacturonic acid determined by calculating the content of neutral sugars (as galactose) by the phenol-sulfuric acid method.

**Determination of molecular mass.**

The molecular mass of the purified polysaccharide was measured by gel chromatography on a column of TSK-gel GMPWXL. According to the standard calibration curve obtained from the definite molecular mass pullulans, the molecular mass of the purified polysaccharide was estimated to be approximately $6.8 \times 10^4$.

**Specific rotation and infrared spectra.**

The specific rotation of the purified polysaccharide at various temperatures is shown in Table 1. It showed the value of $+138^\circ$ at 60°C. The same value was maintained with a decrease in temperature to 30°C, and then gradually increased with the decreasing temperature. This value indicated that the purified polysaccharide mainly had $\alpha$-glycosidic linkages.

The infrared spectra of the purified polysaccharide, the de-esterified polysaccharide, the standard HM pectin and the de-esterified standard pectin are shown in Fig. 1. The spectra indicated a major absorption at around 3400 cm$^{-1}$ due to stretching of the hydroxyl groups and an absorption at about 2900 cm$^{-1}$ corresponding to the C-H stretching of the CH$_3$ groups. An absorption at 1610 cm$^{-1}$ was caused by the C=O stretching vibration of ionic carboxyl groups, and an absorption at 1740 cm$^{-1}$ by the C=O stretching vibration of methyl-esterified carboxyl groups, of which the absorption disappeared in the de-esterified polysaccharide. An absorption at 830 cm$^{-1}$ indicated that the extracted polysaccharide had $\alpha$-glycosidic linkages. These data for the extracted polysaccharide and the de-esterified polysaccharide were adequately consistent with those of standard HM pectin and the de-esterified standard pectin over wide ranges of wave number, respectively.

**$^1$H- and $^{13}$C-NMR spectra.**

The $^1$H-NMR spectra of the purified polysaccharide and the de-esterified polysaccharide are shown in Fig. 2. In the spectrum of the purified polysaccharide, the signals at 1.25 and 1.31 ppm were attributed to the methyl groups of L-rhamnose. These methyl groups appeared due to the presence of two different rhamnose residues. The signals at 1.25 and 1.31 ppm were respectively assigned to the rhamnose linked only at O-2 and to the rhamnose linked both at O-2 and O-4. The signals at 2.09 and 2.17 ppm were attributed to the acetyl groups attached to D-galacturonic acid. According to the reports of Renard et al., and Perrone et al., the former was assigned to 3-O-acetyl groups and the latter to 2-O-acetyl groups. This indicated that the position of acetyl groups was predominantly linked to O-3 of D-galacturonic acid in the polysaccharide from *C. depressa*. The area of the acetyl groups being compared with that of methyl groups of rhamnose, the degree of acetylation (DA) was estimated to be 1.0%.

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**Table 1.** Specific rotation of the polysaccharide extracted from *Citrus depressa*.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>10</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\alpha]_D$ (degree)</td>
<td>+198</td>
<td>+176</td>
<td>+149</td>
<td>+138</td>
<td>+138</td>
<td>+138</td>
<td>+138</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Infrared spectra of the polysaccharide extracted from *Citrus depressa*, de-esterified polysaccharide, standard pectin and de-esterified standard pectin.

(1), extracted polysaccharide; (2), standard pectin; (3), de-esterified polysaccharide; (4), de-esterified standard pectin.
from the content of rhamnose. A large signal at 3.8 ppm was estimated to be the ester-linked methyl groups of carboxyl groups in galacturonic acids. As the purified polysaccharide was de-esterified, the spectrum was simplified. Two methyl group signals of acetyl groups and methyl-esterified galacturonic acids disappeared. Furthermore, chemical shifts of H1, H4 and H5 converged on sharp signals. According to the results of Grasdalen et al.,30 it was reported that chemical shifts of H1, H4 and H5 depended on whether the nearest neighbor was esterified or not esterified, and therefore they were split. Further it was also reported that signals of H5 of esterified units were near H1, and those of non-esterified units were near H4. As a result, the spectrum of the purified polysaccharide was complicated. The spectrum of the de-esterified polysaccharide, however, showed five major signals and they were assigned as D-galacturonic acid to H1, 5.09 ppm; H2, 3.76; H3, 3.97; H4, 4.42; H5, 4.69, respectively (Table 2).33 These chemical shifts were in agreement with those of the de-esterified standard pectin.

**NOESY spectrum.**

NOESY spectroscopy aids the determination of the sequence of residues by observing n.O.e. between anomeric and aglyconic protons, and in some cases can be used to provide evidence concerning solution conformation.36,39 In the NOESY spectrum of the de-esterified polysaccharide, a cross peak between H1 and H4 in D-galacturonic acid residues was observed though a lower cross peak between H1 and H2 was also observed (Fig. 4). Therefore it indicated that the purified polysaccharide contained (1→4)-linked D-galacturonic acid residues.

**Gelation.**

It is known that high methoxyl pectins (Methoxyl con-
tent (7%; DM ≤ 43%) can form gels at pH 2.2–3.5 if a cosolute such as sucrose is present at a concentration of ≥55% by weight. Low-methoxyl pectins (Methoxyl content < 7%; DM < 43%) can form gels in the presence of an adequate concentration of a divalent cation such as calcium in a wide range of soluble-solid content. The solution of the purified polysaccharide became a gel in the presence of sucrose and citric acid. The solution of the de-esterified polysaccharide became a gel (Fig. 5), which might be formed by Ca bridges through carboxyl groups of D-galacturonic acid with different molecules with ionic bonding.

In conclusion, these results indicated that the polysaccharide extracted from the pericarp of C. depressa was a pectin. We obtained a pectin with the relatively high yield of 2.6%. The pectin has a high proportion of galactose

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**Table 3.** Chemical shifts of the $^{13}$C-NMR spectrum of the de-esterified polysaccharide extracted from *Citrus depressa* and the de-esterified standard pectin.

<table>
<thead>
<tr>
<th>Solvent: D$_2$O</th>
<th>$^{13}$C Chemical shifts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1</td>
</tr>
<tr>
<td>De-esterified</td>
<td>102.1</td>
</tr>
<tr>
<td>polysaccharide</td>
<td>107.2</td>
</tr>
<tr>
<td>Galacturonic acid (GA)</td>
<td></td>
</tr>
<tr>
<td>Galactose (G)</td>
<td></td>
</tr>
<tr>
<td>De-esterified</td>
<td>101.9</td>
</tr>
<tr>
<td>standard pectin</td>
<td>107.2</td>
</tr>
<tr>
<td>Galacturonic acid (GA)</td>
<td></td>
</tr>
<tr>
<td>Galactose (G)</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 3.** $^{13}$C-NMR spectra of the polysaccharide extracted from *Citrus depressa* and de-esterified polysaccharide in D$_2$O at 80°C.

(1), extracted polysaccharide; (2), de-esterified polysaccharide. GA, galacturonic acid; G, galactose.

**Fig. 4.** NOESY spectrum of the de-esterified polysaccharide from *Citrus depressa*.

**Fig. 5.** Photographs of the gels of the polysaccharide extracted from *Citrus depressa* and de-esterified polysaccharide.

(1), extracted polysaccharide (polysaccharide extracted from *Citrus depressa*, with 0.05 M HCl at 85°C for 1 h, polysaccharide, 1%; sucrose, 60% (w/w)); (2), de-esterified polysaccharide (de-esterified polysaccharide treated with 0.05 M NaOH at 4°C for 1 h, polysaccharide, 1%; CaCl$_2$, 3% (w/w)).
(69.6%) in neutral sugars in comparison with other citrus plants (15.7–55.0%) and doesn’t contain any xylose which almost all citrus plants except grapefruits do. The degree of methoxylation and degree of acetylation are relatively lower than those of other citrus plants (DM, 70–90%; DA, 3–5%). Since the gel was formed under sucrose and citric acid, and the degree of methoxylation in the polysaccharide was found to be over 43%, it was indicated that the purified polysaccharide was a high methoxyl pectin.

The cultivation of *C. depressa* is doing very well in the northern area in Okinawa and the fruit attracts considerable attention for its effects on diabetes and so on. However these days, almost all the peel of *C. depressa* used for juice, etc., is thrown out. Pectins are very important materials in food industries today, particularly for jams, jellies and some foods. We can extract a pectin from pericarp of *C. depressa* with high yield and a simple extraction procedure. Thus it is hoped that the peel of *C. depressa* will be utilized effectively in Okinawa, Japan.

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シークワーサー果皮からペクチンの分離・同定

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