Characterization of an Acidic Polysaccharide with Immunological Activities from the Tuber of Pinellia ternata

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An acidic polysaccharide, called pinellian PA, was isolated from the tuber of Pinellia ternata BREIT. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 11.8 × 10⁴. Pinellian PA is composed of L-arabinose: D-galactose: L-rhamnose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 5:15:1:3:3, in addition to small amounts of D-acetyl groups and peptide moieties. Reduction of carboxyl groups, methylation analysis and nuclear magnetic resonance studies show that the core structural features include a backbone chain composed of β-1,3-linked D-galactose units. Some of the galactose units in the backbone carry β-1,6-linked α-galactosyl side-chains at position 6. Pinellian PA produces significant potentiation of the reticuloendothelial system, as shown by a carbon clearance test, and also exhibits potent anti-complementary activity.

Keywords: polysaccharide structure; immunological activity; acidic arabinogalactan; Pinellia ternata; tuber; pinellian PA

The tuber of Pinellis ternata BREIT. is a well-known traditional crude drug in China and Japan. It is used as antiemetic, anti-inflammatory, sedative, antitussive and expectorant. Recently, a heteropolysaccharide having an arabinan backbone was isolated as an antiemetic principle.¹ We very recently obtained a glucan, called pinellian G, as a major polysaccharide from this material, and its structural features were elucidated.² This glucan exhibited significant potentiation of the reticuloendothelial system (RES) and had anti-complementary activity; it is the first example of a compound with immunological properties which is a constituent of this crude drug.³ The present paper describes the isolation, structural features and immunological activities of a novel acidic polysaccharide from the aqueous extract of the tuber of Pinellia ternata. This acidic polysaccharide exhibited higher RES-stimulating and anti-complementary activities than pinellian G.

MATERIALS AND METHODS

Isolation of the Polysaccharide The material was imported from China. The sliced dry tubers (200 g) were extracted with hot water (2 l) under stirring for 30 min in a boiling water bath. After centrifugation, the residue was similarly extracted with hot water (1 l). The supernatants were combined (2300 ml) and 1% sodium sulfate (23 ml) was added; 5% cetyltrimethylammonium bromide (CTAB, 280 ml) was then added to the solution. After centrifugation, the precipitate was extracted with 0.2 M sodium chloride (500 ml). After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was dissolved in water, then dialyzed and lyophilized. Yield, 201 mg. This fraction (CTAB-Ppt, 2 g) was dissolved in 0.01 M phosphate buffer (pH 7.2) and applied to a column (3 × 38 cm) of diethylaminoethyl (DEAE)-Sephadex (Pharmacia Co.). The column was equilibrated and eluted with the same phosphate buffer (420 ml), then successively eluted with phosphate buffers containing 0.1 M NaCl (600 ml) and 0.2 M NaCl (640 ml). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method.⁴ The eluates obtained from tubes 64 to 75 were combined, dialyzed and concentrated. One sixth of this solution was applied to a column (5 × 83 cm) of Toyopearl HW-55F, pre-equilibrated and developed with 0.1 M Tris–HCl buffer (pH 7.0). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method. The eluates obtained from tubes 35 to 46 were combined, dialyzed, concentrated and applied to a column (5 × 88 cm) of Sephacryl G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 35 to 46 were combined, concentrated and lyophilized. A polysaccharide, called pinellian PA, was obtained as a white powder. Yield, 68.5 mg.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out at 5 mA/tube for 1 h as described previously.⁴ Pinellian PA gave a single clear band at a distance of 70 mm from the origin.

Gel Chromatography This was performed as described previously.⁵

Qualitative Analysis and Determination of Component Sugars and O-Acetyl Groups These were carried out as described previously.⁵ The molar ratio of galacturonic acid and glucuronic acid was estimated from the result of the analysis of the carboxyl-reduced derivative.

Nuclear Magnetic Resonance (NMR) The NMR spectrum was recorded on a JEOL JMN-GX 270 FT NMR spectrometer in heavy water at 30 °C.

Reduction of Carboxyl Groups The polysaccharide was reduced as described previously.⁶ The reduction was repeated three times under the same conditions. Yield was 41.9 mg from 60.7 mg of pinellian PA.

Methylation Analysis Methylation was carried out with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.⁷ The yields were 3.3 mg from 6.6 mg of pinellian PA, 3.6 mg from 5.0 mg of the carboxyl-reduced product, 1.3 mg from

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2.6 mg of the controlled Smith degradation product (SDP) and 3.8 mg from 2.5 mg of the secondary Smith degradation product (2nd SDP). The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously. The partially methylated alditol acetates obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) using a fused-silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed using a JEOL JMS-DX303 mass spectrometer. The relative retention times of the products with respect to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol in GC are listed in Table I.

**Periodate Oxidation** Pinellin PA (55.8 mg) was dissolved in 0.1 N sodium hydroxide (2.8 ml) and allowed to stand at room temperature for 10 min, then the solution was neutralized with 10 N acetic acid. The solution was adjusted to 13 ml with water, then 0.1 M sodium metaperiodate (13 ml) was added. The reaction mixture was kept at 4 °C in the dark, and the periodate consumption was measured by a spectrophotometric method. Oxidation was complete after 2 d. The reaction mixture was successively treated with ethylene glycol (0.5 ml) at 5 °C for 1 h and sodium borohydride (0.15 g) at 5 °C for 18 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (2.6 × 41 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 10 to 13 were combined, concentrated and lyophilized. The yield of the product was 49.9 mg.

**Controlled Smith Degradation** The periodate oxidation-reduction product (47.0 mg) was dissolved in 0.5 N sulfuric acid (5 ml). After standing at 20 °C for 18 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and passed through a column (1 × 15 cm) of Dowex 50W-X8 (H⁺). The eluate with water was concentrated and applied to a column (2.6 × 40 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 10 to 12 were combined, concentrated and lyophilized. The yield of the SDP was 14.6 mg.

**Secondary Smith Degradation** SDP (8.4 mg) was oxidized with 0.05 M sodium metaperiodate (4 ml) at 5 °C for 3 d in the dark. The reaction mixture was successively treated with ethylene glycol (0.1 ml) and sodium borohydride (30 mg) as described above. After the addition of acetic acid up to pH 5.0, the solution was applied to a column (2.6 × 40 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 10 and 11 were combined and lyophilized. This product was treated with 0.5 N sulfuric acid as described above and, after neutralization with Dowex 2 (OH⁻), the solution was applied to a column (2.6 × 40 cm) of Sephadex G-25. The 2nd SDP was obtained from the eluates in tubes 10 and 11 of fractions of 10 ml each. Yield, 2.5 mg.

**Phagocytic Activity** This was measured by in vivo carbon clearance test as described previously. The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and administered i.p. (20 mg/kg body weight) to male mice (ICR-SPF) once a day for 5 d.

**Anti-complementary Activity** This was measured as described in a previous report. Gelatin-veronal-buffered saline (pH 7.4) containing 500 μM Mg²⁺ and 150 μM Ca²⁺ (GVB²⁻) was prepared, and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the samples in water were incubated and the residual total hemolytic complement (TCH₀) was determined using immunoglobulin M (Ig M)-hemolysin-sensitized sheep erythrocytes. NHS was incubated with water and GVB²⁻ to provide a control, and the activities of the samples were expressed as the percentage inhibition of the TCH₀ of the control. Plantago mucilagin A from the seed of *Plantago asiatica* L.²¹ was used as a positive control.

**RESULTS**

The hot water extract obtained from the tuber of *Pinellia ternata* was treated with CTAB in the presence of small amounts of sodium sulfate. After centrifugation, the supernatant afforded pinellin G.²² The precipitate was extracted with 0.2 M sodium chloride, and the extract was poured into ethanol. After dialysis, the solution of the precipitate was applied to a column chromatography on DEAE-Sephacel, then stepwise elution with sodium chloride in a dilute phosphate buffer was carried out. The eluate obtained with 0.2 M sodium chloride in a phosphate buffer was dialyzed and subjected to gel chromatography on Toyopearl HW-55F. A pure polysaccharide, designated as pinellin PA, was obtained from the first polysaccharide fraction, followed by dialysis and gel chromatography on Sephadex G-25.

The polysaccharide gave a single band on PAGE, and gave a single peak on gel chromatography. It has [ξ]D²⁰ +15.⁹ (H₂O, c=0.1). Gel chromatography gave a value of 11.8 × 10⁴ for the molecular mass.

Pinellin PA is composed of α-l-arabinose, D-galactose, L-rhamnose, D-galacturonic acid and D-glucuronic acid. Quantitative analysis showed that it contained 14.0% arabinose, 55.2% galactose, 3.3% rhamnose, 11.7% galacturonic acid, 11.7% glucuronic acid and 4.2% peptide moiety. The molar ratio of these component sugars was 5:15:1:3:3.

The carbon-13 NMR (¹³C-NMR) spectrum of pinellin PA showed six signals due to anomic carbons at δ 102.12, 102.72, 105.47, 106.17, 107.57 and 111.73 ppm. These were assigned to the anomic carbons of α-D-galactopyranosyluronic acid, α-L-rhamnopyranosyluronic acid, β-D-glucopyranosyluronic acid, β-D-galactopyranosyluronic acid, α-L-arabinopyranosyluronic acid and α-L-arabinofuranosyluronic acid, respectively.¹³,¹⁴ Further, the ¹³C-NMR spectrum showed signals at δ 51.67 and 173.26 ppm, suggesting the presence of O-acetyl groups. This was confirmed by GC of the hydrolyzate, and the content of acetyl groups was 0.7%.

The carboxyl groups of hexuronic acid residues in the
polysaccharide were reduced to give the corresponding neutral sugar residues.\textsuperscript{15} Both pinellian PA and its carboxyl-reduced derivative were methylated with solid sodium hydride and methyl iodide in dimethyl sulfoxide.\textsuperscript{16} The two methylated products thus obtained were hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I. These results indicate that D-galacturonic acid residues in the original polysaccharide produced most of the 2,3,6-tri-O-methyl D-galactose in the products from its carboxyl-reduced derivative, and that D-glucuronic acid residues in the original produced 2,3,4,6-tetra-O-methyl and 2,3,6-tri-O-methyl D-glucose units in the molar ratio of 2:1 in the methylation products from the carboxyl-reduced derivative.

Pinellian PA was subjected to periodate oxidation followed by reduction. The product was treated with dilute sulfuric acid at room temperature overnight.\textsuperscript{17} The SDP obtained was composed of D-galactose alone. SDP was methylated, hydrolyzed, then converted into the partially methylated alditol acetates. The results of the GC-MS analysis are shown in Table I. In order to elucidate the structural features of SDP, secondary Smith degradation was performed by periodate oxidation followed by successive reduction and mild hydrolysis under the same conditions used for the preparation of SDP. The result of methylation analysis of the product (2nd SDP) is also shown in Table I.

Based on the accumulated evidence described above, it can be concluded that pinellian PA has the structural features shown in Chart 1. It can be presumed that SDP, the core of pinellian PA, has a backbone chain composed of β-1,3-linked D-galactose residues, and that three-tenths of the galactose units in the backbone chain must carry β-1,6-linked galactosyl side-chains at position 6. Some of the β-1,6-linked galactosyl residues in the side-chains carry terminal galactose units at position 3. Possible structural features of SDP are given in Chart 2.

The effect of pinellian PA on the RES was demonstrated by a modification\textsuperscript{18} of the in vivo carbon clearance test\textsuperscript{18} using ymosan as a positive control. As shown in Fig. 1, the phagocytic index was significantly increased, suggesting activation of the RES following i.p. injection of pinellian PA.

The anti-complementary activity of pinellian PA is shown in Fig. 2. The polysaccharide showed remarkable activity, markedly superior to that of the positive control, Planto-mucilage A.

### Table I. Methylation Analysis of Pinellian PA, Its Carboxyl-Reduced Derivative and the Controlled Smith Degradation Products (SDP)

<table>
<thead>
<tr>
<th>Relative retention time</th>
<th>Original</th>
<th>Carboxyl-reduced</th>
<th>SDP</th>
<th>2nd SDP</th>
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<tr>
<td>1.4-Ac\textsubscript{2},2,3,5-Me\textsubscript{3},\textalpha{-arabininitol}</td>
<td>0.69</td>
<td>20</td>
<td>20</td>
<td></td>
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<td>1.5-Ac\textsubscript{2},2,3,4-Me\textsubscript{3},\textalpha{-arabininitol}</td>
<td>0.80</td>
<td>5</td>
<td>5</td>
<td></td>
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<td>1.4,5-Ac\textsubscript{2},2,3-Me\textalpha{-arabininitol}</td>
<td>1.14</td>
<td>4</td>
<td>4</td>
<td></td>
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<tr>
<td>1.2,4,5-Ac\textsubscript{3},3-Me\textalpha{-arabininitol}</td>
<td>1.52</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1.5-Ac\textsubscript{2},2,3,4,6-Me\textsubscript{3},\textalpha{-rhamninitol}</td>
<td>0.64</td>
<td>2</td>
<td>2</td>
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<td>1.2,5-Ac\textsubscript{3},3,4,6-Me\textsubscript{3},\textalpha{-rhamninitol}</td>
<td>0.95</td>
<td>3</td>
<td>3</td>
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<tr>
<td>1.2,4,5-Ac\textsubscript{2},3,Me\textalpha{-rhamninitol}</td>
<td>1.28</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>1.5-Ac\textsubscript{2},2,3,4,6-Me\textalphat\textgammaglucitol</td>
<td>1.00</td>
<td>—</td>
<td>6</td>
<td></td>
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<tr>
<td>1.4,5-Ac\textsubscript{2},2,3,6-Me\textalphat\textgammaglucitol</td>
<td>1.48</td>
<td>—</td>
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<td>1.5-Ac\textsubscript{2},2,3,4,6-Me\textsubscript{3},\textgammaglucitol</td>
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<td>2</td>
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<td>1.3,5-Ac\textsubscript{2},2,4,6-Me\textsubscript{3},\textgammaglucitol</td>
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<td>27</td>
<td>4</td>
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<td>1.4,5-Ac\textsubscript{2},2,3,6-Me\textsubscript{3},\textgammaglucitol</td>
<td>1.44</td>
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<tr>
<td>1.5,6-Ac\textsubscript{2},2,3,4-Me\textsubscript{3},\textgammaglucitol</td>
<td>1.62</td>
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<td>1.66</td>
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<td>1.5,6,8-Ac\textsubscript{2},2,4-Me\textalpha{-galactitol}</td>
<td>2.02</td>
<td>42</td>
<td>42</td>
<td>2</td>
</tr>
</tbody>
</table>

a) Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-\textgammaglucitol. Ac = acetyl; Me = methyl (e.g., 1.4-Ac\textsubscript{2},2,3,5-Me\textsubscript{3},\textalpha{-arabininitol} = 1,4-di-O-acetyl-2,3,5-tri-O-methyl).
DISCUSSION

We have already obtained an immunologically active glucan, called pinellin G, from the hot water extract of the tuber of *Pinellia ternata*. Structural studies indicate that pinellin G is a branched α-glucan having both 1,4- and 1,3-linkages with 4,6-branching points. On the other hand, pinellin PA now obtained is an acidic arabinogalactan type polysaccharide.

During our studies to date on the immunologically active polysaccharides in crude drugs obtained from various plant sources, we have so far isolated thirty-five substances as RES-activating polysaccharides and elucidated their structural features. Acidic arabinogalactans form the major group. Most of them have d-galacturonic acid as the sole component hexuronic acid. Among them, however, glycyrrhizin GA from the stolon of *Glycyrrhiza glabra var. glandulifera* (14) AMon-S from the root of *Astragalus mongholicus* (15) and ginsenan PA and ginsenan PB from the root of *Panax ginseng* (5) possess both d-galacturonic acid and D-glucuronic acid as their components. These four polysaccharides have intermediate α-1,4-linked d-galacturonic acid and terminal β-d-glucuronic acid residues as their common hexuronic acid units.

Pinellin PA is a novel example of the known RES-activating acidic arabinogalactans having terminal glucuronic acid with intermediate galacturonic acid. In addition to the terminal units, the presence of intermediate β-1,4-linked d-glucuronic acid residues in pinellin PA is characteristic. Pinellin PA is the first example of the known RES-potentiating acidic polysaccharides to have both terminal and intermediate glucuronic acid units in addition to the usual rhamnogalacturonan type moieties.

The core structure of pinellin PA was revealed in the present study to have a backbone chain composed of β-1,3-linked d-galactose, and some of the galactose units in the backbone carry side-chains at position 6. We have identified the similar galactan backbone structure in ukonans A, B and C (4,20,21) glycyrrhizans UA and GA (22,23) enidirhan AG (24) alisman PII (25) and ginsenan PA (26) in common. The presence of a complicated branching structure may contribute the both RES-potentiating and anti-complementary activities, although the characteristic intermediate units of d-glucuronic acid in pinellin PA have no significant effect. Further investigation of the relationship between the biological activities and structural features is in progress.
REFERENCES