A Glucan Having Reticuloendothelial System-Potentiating and Anti-complementary Activities from the Tuber of *Pinellia ternata*

Masashi Tomoda,* Ryoko Gonda, Naoko Ohara, Noriko Shimizu, Chiho Shishido, and Yuka Fujiki

Kyoritsu College of Pharmacy, Minato-ku, Tokyo 105, Japan.

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A glucan, called pinellin G, 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 $1.5 \times 10^6$. It is composed solely of $\alpha$-glucose, in addition to a few O-acetyl groups. Methylation analysis, nuclear magnetic resonance and enzymic degradation studies indicated that it is a branched glucan mainly composed of $\alpha$-1,4-linked $\alpha$-glucopyranose residues with partially $\alpha$-1,3-linked units and 4,6-branching points. The glucan showed significant reticuloendothelial system-potentiating activity in a carbon clearance test, as well as pronounced anti-complementary activity.

**Keywords** glucan; polysaccharide structure; immunological activity; *Pinellia ternata*; pinellin G

The tuber of *Pinellia ternata* Breit. (Araceae) is a well-known traditional Chinese crude drug used as an anti-emet, anti-inflammatory, sedative, antitussive and expectorant under the name of Ban-Xia in China (Japanese name, Hange). Reported constituents of this crude drug are $\beta$-sitosterol and its $\alpha$-glucopyranose, stigmastanol, fourteen amino acids, 3,4-dihydroxybenzaldehyde glycoside, ephedrine, a lectin and guanosine. More recently, a heteropolysaccharide having an arabian backbone was isolated as an anti-emetic principle, and an amylase with anti-inflammatory activity were reported. We have now isolated a glucan from the water extract of the tuber of *P. ternata*. The present paper describes the structural features, and reticuloendothelial system (RES)-potentiating and anti-complementary activities of this polysaccharide.

**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, 480 ml) was then added to the solution. After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was treated with ethanol and, after centrifugation, was dissolved in water, then dialyzed and lyophilized. Yield, 2.3 g. This fraction (fr. CTAB-Sup; 2.1 g) was dissolved in 0.01 M phosphate buffer (pH 7.2) and applied to a column (5 x 45 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia Co.). The column was equilibrated and eluted with the same phosphate buffer (980 ml). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method. The eluates obtained from tubes 15 to 40 were combined, dialyzed, concentrated and lyophilized. The yield of this fraction (fr. A) was 1.02 g. Fraction A (204 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (5 x 88 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 20 ml were collected, and the eluates obtained from tubes 44 to 58 were combined, dialyzed, concentrated and applied to a column (5 x 88 cm) of Sephacryl G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 34 to 41 were concentrated and lyophilized. Pinellin G was obtained as a white powder. Yield, 121.6 mg.

**Glass-Fiber Paper Electrophoresis** This was carried out as described previously on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M Na$_2$B$_4$O$_7$·10H$_2$O–0.1 M NaOH (10:1, pH 9.3). Pinellin G gave a single spot at a distance of 107 mm from the origin toward the cathode.

**Gel Chromatography** The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer and applied to a column (2.6 x 93 cm) of Toyopearl HW-55F as described above. Fractions of 5 ml were collected and analyzed using the phenol-sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) with known molecular masses were run on the column to obtain a calibration curve.

**Component Sugar Analysis** Hydrolysis and cellulose thin-layer chromatography (TLC) of a component sugar were performed as described previously. Analysis by gas chromatography (GC), after conversion of the hydrolysate into alditol acetate, was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector as described previously.

**Nuclear Magnetic Resonance (NMR)** The NMR spectrum was recorded on a JEOL JMN-GX270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30°C.

**Determination of O-Acetyl Groups** The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described previously.

**Methylation Analysis** Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously. The yield was 5.1 mg from 6.0 mg of pinellin G. The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced
and acetylated as described previously.\textsuperscript{15} 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 carried out with 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.

**Enzymic Degradation with α-Amylase** Pinellin G (20 mg) was dissolved in 0.05 M acetate buffer (pH 5.0, 1 ml), and an α-amylase preparation (5 μl; Sigma Co.) was added. The solution was incubated with a drop of toluene at 37°C for 7 h. After being heated in a boiling water bath for 5 min, the solution was applied to a column (5 × 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method. The eluates obtained from the column were divided into six groups: Fraction 1, tubes 28 to 33; fr. 2, tubes 34 to 44; fr. 3, tubes 45 to 53; fr. 4, tubes 54 to 56; fr. 5, tubes 57 to 59; fr. 6, tubes 60 to 62. The yields were 3.8 mg for fr. 1, 6.2 mg for fr. 2, 4.0 mg for fr. 3, 3.6 mg for fr. 4, 0.5 mg for fr. 5, and 0.8 mg for fr. 6. Fractions 3 to 6 were desalted by treatment with Dowex 50WX8 (H+) followed by evaporation.

**Analysis of Degradation Products** This was carried out by TLC on Merck pre-coated Kieselgel 60 plates with n-butanol-acetic acid–water (2:1:1, v/v) as a developing solvent, and by the high-performance liquid chromatography (HPLC) system using a column of Asahipak NH 2P-50 with acetonitrile–water (2:1, v/v) as an eluent as described previously.\textsuperscript{16}

**Phagocytic Activity** This was measured by an in vivo carbon clearance test as described previously.\textsuperscript{12} The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. to male mice (ICR-SPF) once a day for 5 d.

**Anti-complementary Activity** This was measured as described in a previous report.\textsuperscript{17} Gelatin-veronal-buffered saline (pH 7.4) containing 500 μM Mg\textsuperscript{2+} and 150 μM Ca\textsuperscript{2+} (GVB\textsuperscript{2+}) 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 (TCH\textsubscript{50}) was determined using immunoglobulin M (IgM)-hemolysis-sensitized sheep erythrocytes. NHS was incubated with water and GVB\textsuperscript{2+} to provide a control, and the activities of the samples were expressed as percentage inhibition of the TCH\textsubscript{50} of the control. Plantago-mucilage A from the seed of *Plantago asiatica* L.\textsuperscript{18} was used as a positive control.

**RESULTS**

The hot water extract obtained from the tuber of *Pinellia ternata* was treated with CTAB, and the supernatant obtained was poured into ethanol. The precipitate was dialyzed and applied to ion-exchange chromatography with DEAE-Sephacel. The eluate with a phosphate buffer was dialyzed and subjected to gel chromatography with Toyopearl HW-55F. A pure polysaccharide, designated as pinellin G, was obtained from the main fraction, followed by dialysis and gel chromatography with Sephadex G-25.

The polysaccharide gave a single spot on electrophoresis, and gave a single peak on gel chromatography. Gel chromatography gave a value of 1.5 × 10\textsuperscript{4} for the molecular mass of pinellin G. It had [a]\textsubscript{D}25 +156.0° (H\textsubscript{2}O, c = 0.1).

Pinellin G is composed solely of d-glucose, and it contains no nitrogen. The carbon-13 NMR (\textsuperscript{13}C-NMR) spectrum showed signals at δ 21.64 and 175.35 ppm, suggesting the presence of O-acetyl groups. This was confirmed by GC of the hydrolysate, and the content of acetyl groups was 0.4%. Further, the \textsuperscript{13}C-NMR spectrum showed a signal due to an anomeric carbon of d-glucopyranose at δ 102.31 ppm.\textsuperscript{19}

This glucan was degraded by treatment with α-amylase, followed by gel chromatography with Sephadex G-25. Six fractions (i.e. frs. 1 to 6) were obtained, and the major fraction (frs. 2 to 4) was identified as a mixture of maltotetraose and maltotriose (fr. 2), and maltotriose and maltose (frs. 3 and 4) by TLC and HPLC analysis.

Pinellin G was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.\textsuperscript{20} The methylated products obtained were hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I. The polysaccharide appeared to have slightly bluish red coloration with an iodine test.

The effect of pinellin G on the RES was demonstrated by a modification\textsuperscript{21} of the in vivo carbon clearance test.\textsuperscript{21} As shown in Fig. 1, the phagocytic index was significantly increased, suggesting activation of the RES by i.p. injection of this substance.

**Table I. Methylation Analysis of Pinellin G**

<table>
<thead>
<tr>
<th>Methylated sugars (as alditol acetates)</th>
<th>Relative retention times (a)</th>
<th>Molar ratio</th>
<th>Structural features</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Me\textsubscript{4}-d-glucose</td>
<td>1.00</td>
<td>4</td>
<td>Glc 1 →</td>
</tr>
<tr>
<td>2,4,6-Me\textsubscript{3},d-glucose</td>
<td>1.42</td>
<td>1</td>
<td>→-3 Glc 1 →</td>
</tr>
<tr>
<td>2,3,6-Me\textsubscript{3},d-glucose</td>
<td>1.51</td>
<td>52</td>
<td>→-4 Glc 1 →</td>
</tr>
<tr>
<td>2,3-Me\textsubscript{2},d-glucose</td>
<td>1.92</td>
<td>4</td>
<td>→-6 Glc 1 →</td>
</tr>
</tbody>
</table>

(a) Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-d-glucitol. Abbreviations: Me = methyl, Glc, D-glucopyranose.

![Graph showing phagocytic index](image)

**Fig. 1. Effect of Pinellin G on Phagocytosis**

Significantly different from the control, a) \( p < 0.001, (n = 5) \).
The anti-complementary activity of pinellian G is shown in Fig. 2. It showed potent activity, nearly equal to that of the positive control, Plantago-mucilage A.

**DISCUSSION**

To date, our series of studies on the immunologically active polysaccharides in crude drugs obtained from various plant sources have identified thirty-three substances as RES-activating polysaccharides, and elucidated their structural features. Among them, the acidic arabinono-3,6-galactan group is very common. On the other hand, seven substances are neutral polysaccharides. These are cinnamann AX from the bark of *Cinnamomum cassia*, glycyrrhizin UC from the root of *Glycyrrhiza uralensis*, MVS-I from the seed of *Malva verticillata*, ukonan D from the rhizome of *Cucuma longa*, peonan SA from the root of *Paonia lactiflora*, and cnidirhan SI and cnidirhan SIIA from the rhizome of *Cnidium officinale*. Cinnamann AX is an arabinoxylan, while glycyrrhizin UC, MVS-I, ukonan D, peonan SA and cnidirhan SIIA are glucose-rich heteropolysaccharides. Glycyrrhizin UC, ukonan D and peonan SA possess both arabinono-3,6-galactan and 3-4,6-glucan moieties as their main parts in common. The major parts of MVS-I and cnidirhan SIIA, in contrast, are occupied by β-1,3- or β-1,6-linked d-glucose units.

Among these seven neutral polysaccharides, cnidirhan SI is the only d-glucan with immunological activity. Thus, pinellian G is the second example of a branched d-glucan with phagocytosis-stimulating and anti-complementary activities. Both amylopectin and glycogen, as the ordinary 4,6-branching d-glucans, are completely inactive in the RES. In addition to α-1,4-linear linkage and 4,6-branching points, pinellian G possesses exceptional α-1,3-linked units. There is an average of about one branch for every fifteen glucose units in this glucan. Pinellian G showed slightly bluish red coloration (∆λmax = 490 nm) with an iodine test. The degree of branching in this glucan is lower than cnidirhan SI, and characteristic 3,4-branching points in the latter polysaccharide are not found in pinellian G. The presence of α-1,3-linked units may contribute to the activity of pinellian G.

The yield of pinellian G from the material tuber is relatively high compared with the other constituents, so it seems reasonable to assume that pinellian G is a representative polysaccharide of the tuber of *Pinellia ternata.*

**REFERENCES**