An Acidic Polysaccharide with Immunological Activities from the Root of *Paeonia lactiflora*

Masashi TOMODA,* Keiko MATSUMOTO, Noriko SHIMIZU, Ryōko GONDA, Naoko ŌHARA, and Keiko HIRABAYASHI

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan.
Received March 15, 1994; accepted May 13, 1994

An acidic polysaccharide, called peonan PA, was isolated from the root of *Paeonia lactiflora* PALLAS. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 6.0 × 10⁵. Peonan PA is composed of L-arabinose: D-galactose: D-galacturonic acid in the molar ratio of 2:1:10, in addition to small amounts of O-acetyl groups and peptide moieties. About forty percent of the hexuronic acid residues in peonan PA exist as methyl esters. Reduction of carboxyl groups, methylation analysis, lithium degradation and nuclear magnetic resonance studies indicated that its main structural features involve both α-1,5-linked L-arabinino-β-3,6-branched D-galactan type and α-1,4-linked D-galacturanic type structural units. The polysaccharide exhibited remarkable reticuloendothelial system-potentiating activity in a carbon clearance test and considerable anti-complementary activity.

**Keywords** polysaccharide structure; immunological activity; *Paeonia lactiflora*; acidic arabinogalactan; peonan PA

We recently isolated and elucidated the structural features of a neutral and an acidic polysaccharide from the root of *Paeonia lactiflora* PALLAS (Paeoniaceae) which exhibited reticuloendothelial system (RES)-potentiating and anti-complementary activities, and called them peonans SA and SB. The root of this plant is a very important crude drug in China and Japan. Peonans SA and SB were obtained as major polysaccharides from the principally neutral sugar fraction. The present paper describes the isolation, structural features and immunological activities of a novel acidic polysaccharide from the water extract of this crude drug.

**MATERIALS AND METHODS**

**Isolation of Polysaccharide** The material plant was cultivated in Nara prefecture. The sliced dry roots (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 and the solution (2.4 l) was mixed with 1% sodium sulfate (24 ml); 5% cetyltrimethylammonium bromide (CTAB, 300 ml) was then added to the solution. The supernatant afforded peonan SA and peonan SB after purification by ion-exchange and gel chromatographies. After centrifugation, the precipitate obtained was extracted with 0.2M sodium chloride (960 ml). After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was treated with 80% ethanol and, after centrifugation, the precipitate was dissolved in water, then dialyzed and lyophilized. Yield, 168 mg. This fraction (CTAB-Ppt, 429 mg) was dissolved in 0.01M phosphate buffer (pH 7.2) and applied to a column (2 × 40 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia Co.). The column was equilibrated and eluted with the same phosphate buffer (220 ml), then successively eluted with the phosphate buffers containing 0.1M NaCl (300 ml) and 0.2M NaCl (480 ml). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method. The eluates obtained from tubes 30 to 40 were combined, dialyzed, concentrated and applied to a column (5 × 85 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 tubes 30 to 35 were combined, concentrated and lyophilized. Peonan PA was obtained as a white powder. Yield, 269 mg.

**Polyacrylamide Gel Electrophoresis (PAGE)** This was carried out in an apparatus equipped with gel tubes (4 × 140 mm) and 5 mm Tris-glycine buffer (pH 8.3) at 5 mA/tube for 1 h. Gels were stained by the periodate-Schiff (PAS) procedure and with Coomassie blue reagent. Peonan PA gave a single clear band at a distance of 85 mm from the origin.

**Gel Chromatography** The sample (2 mg) was dissolved in 0.1M Tris–HCl buffer (pH 7.2) and applied to a column (2.6 × 96 cm) of Toyopearl HW-65F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed using the phenol-sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

**Qualitative Analysis of Component Sugars** Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described previously. The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylamine-alditol derivatives. GC was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector.

**Determination of Components** Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described previously. Galacturonic acid was determined by the m-hydroxydiphenyl method. Peptide determination was performed by the method of Lowry et al. using bovine serum albumin as a standard.

**Determination of O-Acetyl Groups** The sample was hydrolyzed with 0.2N hydrochloric acid in a sealed tube
at 100 °C for 2 h. The hydrolyzate was directly applied to GC with propionic acid as an internal standard. GC was performed using a column (3.2 mm i.d. x 2.1 m long spiral glass) packed with 5% Thermion-3000 on Shincarbon A (60 to 80 mesh) at 120 °C with a helium flow of 30 ml per min; tR (min), acetic acid 5.2; propionic acid (internal standard) 7.9.

**Determination of O-Methyl Groups in Methyl Esters**

The sample was dissolved in 0.5 N sodium hydroxide containing ethanol as an internal standard, and the solution was left at 20 °C for 30 min. The product was directly subjected to GC using a column (3.2 mm i.d. x 2.1 m long spiral glass) packed with 15% PEG 6000 on Shimalite F (40 to 80 mesh) at 80 °C with a helium flow of 40 ml per min; tR (min), methanol 4.5; ethanol (internal standard) 5.7.

**Nuclear Magnetic Resonance (NMR)**

NMR spectra were recorded on a JEOL JMN-GX 270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70 °C.

**Reduction of Carboxyl Groups**

This was carried out with 1-cyclohexyl-1-(2-morpholinooethyl)carbodiimide metho-p-toluenesulfonate and sodium borohydride as described previously.8) The reduction was repeated five times under the same conditions. The yield was 6.5 mg from 25.0 mg of peonan PA.

**Methylation Analysis**

Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.9) The yield was 3.4 mg from 5.0 mg of the carboxyl-reduced peonan PA. The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.10) 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. x 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 with a JEOL JMS-DF 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 1.

**Selective Degradation by Lithium Treatment**

The polysaccharide (100 mg) was suspended in ethylenediamine (20 ml) and sonicated for 1 h. Then small pieces of lithium wire (200 mg) were added to the mixture under stirring. After the solution turned deep blue, additional small pieces of lithium wire were added as required to maintain the deep blue color. After treatment for 1 h, the reaction mixture was cooled in an ice bath and then mixed with water (20 ml) to quench the reaction. The resulting solution was evaporated following the addition of toluene and the residue was dissolved in water. After addition of acetic acid up to pH 4.5, the solution was applied to a column (1 x 20 cm) of Dowex AG-50W-X12 (H+). The column was eluted with water (200 ml), and the eluate was concentrated and applied to a column (1 x 15 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted with water (200 ml), and the eluate was concentrated and applied to a column (2.6 x 90 cm) of Sephadex G-25.

The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 9 to 11 were combined, concentrated and lyophilized. Yield, 4.1 mg.

**Phagocytic Activity**

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

**Anti-complementary Activity**

This was measured as described previously.11) 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 (TCH50) was determined using immunoglobulin M (IgM)-hemolysis-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 TCH50 of the control. Plantago-mucilage A from the seed of Plantago asiatica L.12) was used as a positive control.13)

**RESULTS**

The hot water extract obtained from the root of Paeonia lactiflora was treated with CTAB in the presence of small amounts of sodium sulfate. The supernatant obtained was poured into ethanol. The precipitate (fr. CTAB-Sup) afforded two polysaccharides designated as peonan SA and peonan SB.11) The precipitate obtained by treatment with CTAB was extracted with 0.2 M sodium chloride, and the resulting precipitate (fr. CTAB-Ppt) from the extract by addition with ethanol was applied to a column chromatography of DEAE-Sephalac. The stepwise elution with sodium chloride in a phosphate buffer was carried out. The eluate with 0.2 M sodium chloride in a phosphate buffer was dialyzed and purified by gel chromatography with Sephadex G-25, and a pure polysaccharide designated as peonan PA was obtained.

Peonan PA produced a single band on PAGE, and a single peak on gel chromatography with Toyopearl HW-65F. Gel chromatography gave a value of 6.0 x 10⁴ for the molecular mass. It had [α]D²⁰ +172.7° (c=0.1, H₂O).

Quantitative analyses showed that peonan PA contained 11.2% l-arabinose, 7.4% d-galactose, 80.2% d-galacturonic acid and 1.2% peptide moieties. The molar ratio of component sugars was 2:1:10.

The carbon-13 NMR (13C-NMR) spectrum of peonan PA showed signals at δ 21.67 and 173.19 ppm suggesting the presence of O-acetyl groups. It also showed a signal at δ 55.50 ppm suggesting the presence of O-methyl groups as carboxylic acid methyl esters. The presence of these groups was confirmed by GC of the hydrolyzate, and the contents of acetyl and methoxyl groups were 2.7% and 5.6%, respectively. Thus, about 43% of the hexuronic acid residues in peonan PA are as methyl esters.

Furthermore, the 13C-NMR spectrum of peonan PA showed three signals due to anomeric carbons at δ 102.72,
Table I. Methylation Analysis of the Carboxyl-Reduced Derivative and the Lithium Degradation Product of Peonan PA

<table>
<thead>
<tr>
<th>Relative retention time</th>
<th>Molar ratios</th>
<th>Carboxy-</th>
<th>Lithium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4-Ac₂-2,3,5-Me₃-α-L-arabinitol</td>
<td>0.68</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>1.4,5-Ac₂-2,3,6-Me₂-α-L-arabinitol</td>
<td>1.14</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>1.5-Ac₂-2,3,4,6-Me₄-δ-galactitol</td>
<td>1.10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1.3,5-Ac₂-2,4,6-Me₃-δ-galactitol</td>
<td>1.38</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1.4,5-Ac₂-2,3,6-Me₃-δ-galactitol</td>
<td>1.50</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>1.5,6-Ac₂-2,4,5-Me₃-δ-galactitol</td>
<td>1.62</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1.3,4,5-Ac₂-2,6-Me₂-δ-galactitol</td>
<td>1.67</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1.2,4,5-Ac₂-3,6-Me₂-δ-galactitol</td>
<td>1.79</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>1.3,5,6-Ac₂-2,4-Me₂-δ-galactitol</td>
<td>2.02</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

a) Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (e.g., 1,4-Ac₂-2,3,5-Me₃-γ = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-).

106.20 and 110.20 ppm. These were assigned to the amonic carbons of α-D-galactopyranosyluronic acid, β-D-galactopyranose and α-L-arabinofuranose, respectively. It also showed a carbonyl signal of galacturonic acid at 176.56 ppm.

Methylation analysis of peonan PA was unsuccessful owing to its poor solubility in dimethyl sulfoxide, and so the carboxyl groups of galacturonic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues. The carboxyl-reduced derivative was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide. The methylated product was hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I.

The carboxyl-reduced derivative was composed of L-arabinose and D-galactose in the molar ratio of 1:4. This result showed that partial degradation and the loss of galacturonic acid residues had taken place during the process of reduction. The result of methylation analysis of the derivative indicated that most of the galacturonic acid units were α-1,4-linked in the polysaccharide. To determine the other kind of galacturonic acid linkage, isolation of the neutral sugar moieties in the polysaccharide was carried out by treating it with lithium in ethylenediamine. Enzymic degradation of peonan PA with pectinase (from Aspergillus niger) was unsatisfactory.

The selective degradation product obtained by lithium treatment gave a single peak on gel chromatography with Toyopearl HW-55F. It was composed of L-arabinose and D-galactose in the molar ratio of 1:0.6. Methylation analysis of this product was performed under the same conditions as for the carboxyl-reduced derivative. The results are also shown in Table I.

Based on the accumulated evidence described above, it can be concluded that peonan PA has the structural features shown in Chart 1.

The effect of peonan PA on the RES was demonstrated by a modification of the in vivo carbon clearance test using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was markedly increased by peonan PA, suggesting activation of the RES, by i.p. injection of the polysaccharide.

The anti-complementary activity of peonan PA shows in Fig. 2. And it can be seen that the polysaccharide had potent activity compared with the positive control, Plantago-mucilagin A.

**DISCUSSION**

We earlier isolated two immunologically-active polysaccharides, called peonan SA and peonan SB, from the CTAB-Sup fraction of the hot water extract of the root of Paeonia lactiflora. Peonan SA is a unique arabino-
galacto-glucan having a degree of 2,4-branching similar to the 3,6-branching in its β-d-galactose moieties and both 3,4- and 4,6-branching points to the same extent in its α-d-glucose moieties. On the other hand, peonan SB is a typical acidic α-1,5-linked L-arabinino-β-3,6-branched D-galactan. This polysaccharide possesses additional 2,5- and 3,5-branched L-arabinose units and 3,4-branched D-galactose units.13

To date, in our studies of the immunologically-active polysaccharides in crude drugs obtained from various plant sources, we have isolated thirty-three substances shown to be RES-activating polysaccharides, and elucidated their structural features. Among them, acidic arabinogalactan is the major group. In this group, only three substances are commonly composed of α-L-arabinose, β-d-galactose and α-d-galacturonic acid. Those are saposnikovian A from the root and rhizome of Saponosikovia divaricata,20 ginsen S-IA from the root of Panax ginseng,20 and peonan SB. Their structural features include mainly α-1,5-linked L-arabinino-β-3,6-branched D-galactan type structural units.

Peonan PA is also composed of the same component sugars as these three acidic arabinogalactans. In contrast to these polysaccharides, however, peonan PA is essentially an arabinogalacto-galacturonan. The molar ratios of arabinose, galactose and galacturonic acid were 6:15:10 in saposnikovian A, 8:8:1 in ginsen S-IA, 4:3:2 in peonan SB, and 2:1:10 in peonan PA. The major part of peonan PA consists of α-1,4-linked D-galacturonic acid residues with partial 2,4-branching points. In addition, this polysaccharide has the usual α-1,5-linked L-arabinino-β-3,6-branched D-galactan type units as its neutral sugar moieties.

Both the RES-potentiating and anti-complementary activities of peonan PA are similar to those exhibited by peonan SB.They have a common minor 3,4-branching in addition to the major 3,6-branched D-galactose residues. Peonan PA possesses additional β-1,4-linked D-galactose units. Peonan SB has no branching D-galacturonic acid unit, although it has α-1,3-linked and both α-2,5- and α-3,5-branched L-arabinose units. This complicated branching that they possess may contribute to their activities.

Further investigations of the relationship between the structural features and biological activities of these polysaccharides are in progress.

REFERENCES