Reticuloendothelial System-Potentiating and Alkaline Phosphatase-Inducing Activities of Plantago-Mucilage A, the Main Mucilage from the Seed of Plantago asiatica, and Its Five Modification Products

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Five kinds of chemically modified products were prepared from Plantago-mucilage A, the representative mucous polysaccharide isolated from the seed of Plantago asiatica L., and their reticuloendothelial system-potentiating and alkaline phosphatase-inducing activities have been investigated. Both activities were markedly enhanced when the mucilage was de-0-acetylated. The products obtained by periodate oxidation, controlled Smith degradation, and partial acid hydrolysis under the two conditions were not effective. Structural features of the partial hydrolyzates were elucidated, and it was shown that these products lost all O-acetyl groups, all xylose branches and many hexuronic acid arabinose side chains.

Keywords: Plantago-mucilage A; Plantago asiatica; seed; polysaccharide; chemical modification; immunological activity; reticuloendothelial system; alkaline phosphatase; deacetylation effect

Plantago-mucilage A is a representative mucous polysaccharide obtained from the seed of Plantago asiatica L. The seed of this plant is a well-known crude drug used as an antiphlogistic, a diuretic, an antidiarrheic and a cough medicine. Structural studies on Plantago-mucilage A were performed by controlled Smith degradation and methylation analysis, and the results indicated that the mucilage possesses a backbone chain composed of β-1,4-linked D-xylopyranose residues having three kinds of branches composed of a β-D-xylopyranose, α-D-glucopyranosyluronic acid-(1→3)-α-L-arabinofuranose and α-D-galactopyranosyluronic acid-(1→3)-α-L-arabinofuranose at position 3. Thus, the all D-xylose residues in the backbone carry side chains, and the ratio of these three types of branches is 15:10:2. In addition, the mucilage contains 4.8% O-acetyl groups, and these groups were located at position 2 of a L-arabinofuranosyl and D-xylopyranosyl residues. The results of structural studies showed that the molar ratios of D-xylopyranose and 2-O-acetyl-D-xylopyranose are approximately 3:2 in the terminal units and approximately 8:1 in the intermediate units, and that the molar ratio of L-arabinose and 2-O-acetyl-L-arabinose is approximately 3:1.

Recently, Tomoda et al. reported on the anti-complementary activity and the hypoglycemic activity of Plantago-mucilage A and its modified products. Because of the importance of macrophages and lymphocytes for the body’s defense system against microbial infections and tumors, the present paper describes two immunological activities of this mucous polysaccharide and the five products obtained by chemical modification of it on both reticuloendothelial system (RES)-potentiating and alkaline phosphatase-inducing activities. The modification products were examined for the purpose of studies on the relationship between structures and biological activities.

Materials and Methods
Isolation of Polysaccharide This was performed as described in a previous report.¹
Deacetylation The mucilage (50 mg) was dissolved in water (5 ml), then 0.02 N sodium hydroxide (5 ml) was mixed. After standing at room temperature for 10 min, the solution was neutralized with 1 M acetic acid. The resulting mixture was applied to a column (5 x 82 cm) of Sephadex G-25, and the column was eluted with water. Fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 32 to 44 were combined, concentrated and lyophilized. Yield, 42 mg. The disappearance of a signal at δ 2.16 ppm, corresponding to acetyl groups was confirmed by proton nuclear magnetic resonance (1H-NMR) spectrum. The 1H-NMR spectrum was recorded on a JEOL JNM-GX270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 300 °C.
Periodate Oxidation The deacetylated product (200 mg) was oxidized with 0.05 M sodium metaperiodate (100 ml) at 4 °C. The periodate consumption was measured by a spectrophotometric method. Oxidation was completed after 7 h. The reaction mixture was successively treated with ethylene glycol (1 ml) at 4 °C for 1 h and sodium borohydride (1 g) at 4 °C for 16 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 x 85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates made from tubes 33 to 41 were combined, concentrated and lyophilized. Yield, 147 mg.
Controlled Smith Degradation The product (200 mg) obtained by periodate oxidation was dissolved in 0.5 N sulfuric acid (10 ml). After standing at room temperature for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and passed through a column (0.7 x 4 cm) of Dowex 50W-X8 (H⁺). The eluate with water was concentrated and dialyzed against water, then the non-dialyzable fraction was applied to a column (5 x 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 44 were combined, concentrated and lyophilized. Yield, 80 mg.
Partial Acid Hydrolysis The mucilage (300 mg) was dissolved in 0.1 M trifluoroacetic acid (45 ml) and heated under reflux at 60 °C for 1 h. After removal of acid by evaporation, the residue was dissolved in water and applied to a column (5 x 85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 37 were combined, concentrated and lyophilized. Yield, 161 mg. On the other hand, the partial hydrolysis was carried out at 80 °C for 1 h under conditions similar to those described above. In this case, the yield was 157 mg from 300 mg of the mucilage.
Determination of Component Sugars Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into dialdul acetates as described in a previous report.⁷ Hexuronic acid was determined by the m-hydroxybiphenyl method.⁸
Reduction of Carboxyl Groups This was performed with 1-cyclohexyl-3-(2-morpholinoethy1)carbodiimide metho-p-toluenesulfonate and sodium borohydride as described in a previous report.⁴
Methylation Analysis Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described in a previous report. The products obtained were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.¹ The partially methylated alditol acetates obtained were analyzed.
TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methyalted Alditol Acetates

<table>
<thead>
<tr>
<th>Relative retention time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Main fragments (m/z)</th>
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<tbody>
<tr>
<td>1.4-Ac&lt;sub&gt;2&lt;/sub&gt;-2,3,5-Me&lt;sub&gt;2&lt;/sub&gt;-l-arabinitol</td>
<td>0.69</td>
</tr>
<tr>
<td>1.3-Ac&lt;sub&gt;2&lt;/sub&gt;-2.5-Me&lt;sub&gt;2&lt;/sub&gt;-l-arabinitol</td>
<td>1.04</td>
</tr>
<tr>
<td>1.4-Ac&lt;sub&gt;2&lt;/sub&gt;-2,3-Me&lt;sub&gt;2&lt;/sub&gt;-d-xylitol</td>
<td>1.21</td>
</tr>
<tr>
<td>1.3,4,5-Ac&lt;sub&gt;3&lt;/sub&gt;-2-Me&lt;sub&gt;2&lt;/sub&gt;-d-xyitol</td>
<td>1.54</td>
</tr>
<tr>
<td>1.5-Ac&lt;sub&gt;2&lt;/sub&gt;-2,3,4,6-Me&lt;sub&gt;4&lt;/sub&gt;-d-glucitol</td>
<td>1.00</td>
</tr>
<tr>
<td>1.5-Ac&lt;sub&gt;2&lt;/sub&gt;-2,3,4,6-Me&lt;sub&gt;4&lt;/sub&gt;-d-galactitol</td>
<td>1.09</td>
</tr>
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</table>

<sup>a</sup> 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-Ac<sub>2</sub>-2,3,5-Me<sub>2</sub>-l-arabinitol).

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 min from 160 to 220 °C at a helium flow of 1 m l min. GC-MS was performed with a JEOL JMS-GX 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 and the main fragments in MS are listed in Table I.

Phagocytic Activity
This was measured as described in a previous report.<sup>3</sup> The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (50 and 20 mg/kg body weight) once a day. The phagocytic index, K, was calculated by means of the following equation:

\[ K = (O_D - L - O_D^0) / (t_1 - t_0) \]

where \( O_D \) and \( O_D^0 \) are the optical densities at times \( t_1 \) and \( t_0 \), respectively. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF). The comparison of results was performed by means of Student’s t-test.

Alkaline Phosphatase Assay
ICR-SPF male mice were killed by cervical dislocation and the cell suspension from the spleen was prepared by teasing the organ in ice cold RPMI-1640 medium (Nissui Seiyaku Co.) supplemented with 5% HEPES (Sigma Co.), 100 U/ml penicillin and 100 μg/ml streptomycin (Meiji Seika Co.). After centrifugation (1200 rpm for 5 min at 4 °C), the packed cells were treated with ACK buffer (8.29 g ammonium chloride, 1 g potassium bicarbonate, 37.2 mg disodium ethylenediaminetetraacetate in 1 l. pH 7.2) to lyse red cells. The treated cells were washed with fresh medium three times, resuspended with the above medium containing 10% FCS (Flow Lab.) and viability was assessed by the trypan blue dye exclusion test. The cell suspension was adjusted to 5 x 10<sup>6</sup> viable cells per ml. Each test sample solution (100 μl) was placed in a bottomed 96-well tissue culture plate and 100 μl of the cell suspension prepared above were added to each well. The cell cultures were incubated at 37 °C for 4 h in a humidified atmosphere of 5% CO<sub>2</sub>. Each of the resultant cell suspensions (150 μl) was placed in an appropriate test tube. To these tubes, 600 μl of 10% diethanolamine-HCl buffer (pH 9.8) containing 0.1% p-nitrophenylphosphate was added. The reaction mixture was incubated at 37 °C for 4 h and was terminated by the addition of 600 μl of 0.25% sodium hydroxide. The absorbance at 405 nm was measured and the results were expressed as the arithmetic mean ± S.D. of triplicate cultures.

Results
The deacetylated product (DAP) of Plantago-mucilage A was obtained by treatment with alkali under a very mild condition. DAP was subjected to periodate oxidation followed by reduction. The product (POP) was treated with dilute sulfuric acid at room temperature overnight, then the controlled Smith degradation product (SDP) was isolated. As reported previously,<sup>2</sup> DAP had the same composition and almost the same molecular mass as Plantago-mucilage A. Both POP and SDP were composed of d-xylene and l-arabinose in a molar ratio of 9:4,<sup>3</sup> and their molecular masses were 15.4 x 10<sup>4</sup> and 11.1 x 10<sup>4</sup>, respectively.

In addition, the mucilage was partially hydrolyzed under the two conditions with dilute trifluoroacetic acid at 60°C or 80°C for 1 h. Both the products (HP60 and HP80) gave a single peak on gel chromatography, respectively. Gel chromatography gave values of 8.6 x 10<sup>4</sup> and 2.26 x 10<sup>4</sup> for the molecular masses of HP60 and HP80, respectively. Quantitative analyses of component sugars showed that HP60 was composed of d-xylene, l-arabinose, d-gluconic acid and d-galacturonic acid in the molar ratio of 52:14:11:1, and that HP80 was composed of the same components as HP60 in the molar ratio of 80:15:13:1. 1H-NMR spectra showed no acetyl signals in HP60 and HP80.

The carbohydrate groups of hexuronic acid residues in both HP60 and HP80 were reduced to give the corresponding neutral sugar residues.<sup>12</sup> The ratio of two hexuronic acids was confirmed by analysis of the carboxyl-reduced derivatives. Both the original products (HP60 and HP80) and their carboxyl-reduced derivatives were methylated with solid sodium hydride and methyl iodide in dimethyl sulfoxide.<sup>13</sup> The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Hexuronic acid methyl ethers from the original samples were removed from the hydrolysates by treatment with an union-exchange resin. Analysis by GC-MS<sup>14</sup> gave the results shown in Tables II and III.

The structural feature of Plantago-mucilage A<sup>2</sup> and the results of methylation analysis described above indicated that HP60 and HP80 contain the units shown in Chart 1, respectively.

The effects of Plantago-mucilage A and five kinds of chemical modification products, DAP, POP, SDP, HP60 and HP80, on the RES were demonstrated by a modification<sup>7</sup> of the in vivo carbon clearance test<sup>13</sup> using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was significantly increased, suggesting the activation of RES by i.p. injection of Plantago-mucilage A. The activity was markedly enhanced when the mucilage was deacetylated. HP60 showed weak activity, but the other three products, POP, SDP and HP80, had no effect.

The alkaline phosphatase activity of murine spleen cell was induced by stimulating directly with B cell mitogen or indirectly via lymphokines with T cell mitogen.<sup>16</sup> So the measurements of alkaline phosphatase with Plantago-
Chart 1. Component Units in the Structures of HP60 and HP80
Abbreviations: Xylp, xylopyranose; AraF, arabinofuranose; GlcpA, glucopyranosyluronic acid; GalpA, galactopyranosyluronic acid.

![Chart Image]

Fig. 1. Effects of Plantago-Mucilage A and Its Chemical Modification Products on Phagocytosis.
Significantly different from the control, a) p<0.05, b) p<0.01, c) p<0.001.

Fig. 2. Mitogenic Activity of Plantago-Mucilage A and Its Chemical Modification Products Assessed by Alkaline Phosphatase Activity
Plantago-mucilage A, ——— DAP, ——— POP, ——— SDP, ——— HP60, ——— HP80, ——— Control, ×: positive control, lipopolysaccharide from E. coli serotype 0111 (Sigma Co.), △.

mucilage A and its five products were performed by the \textit{in vitro} murine spleen cell assay.\textsuperscript{16} As shown in Fig. 2, Plantago-mucilage A was not so effective. However, when the cells were stimulated with DAP, the alkaline phosphatase activity was induced in a dose dependent manner. The other four modification products, POP, SDP, HP60 and HP80, on the contrary, showed no effect.

**Discussion**

We have already investigated the structure-complement activation relationship of Plantago-mucilage A.\textsuperscript{3} Plantago-mucilage A showed considerable anti-complementarity activity. Its activity was in almost the same level as that of a positive control, AR-arabinogalactan, from the root of \textit{Angelica acutiloba}.\textsuperscript{17} POP gave an effect similar to the original mucilage, and the activity of HP80 was slightly enhanced on this assay. Structural features of this partial hydrolyzate were not clear at that time. The anti-complementarity activity was markedly enhanced when the mucilage was deacylated. It was concluded that the O-acetyl groups in Plantago-mucilage A prevent the activation of the complement via the classical pathway.\textsuperscript{3}

Four years previous, Plantago-mucilage A and DAP were tested for hypoglycemic activity on administration to normal mice.\textsuperscript{20} The deacylation product of the mucilage showed a pronounced elevation in the activity. Thus, DAP belongs to the substances having the highest levels of activity by these two assays.

We have already found the RES activities of two polysaccharides obtained from the root and rhizome of \textit{Saposnikovia divaricata}.\textsuperscript{7,18} three polysaccharides from the seed of \textit{Malva verticillata},\textsuperscript{16,19,20} a polysaccharide from the bark of \textit{Cinnamomum cassia},\textsuperscript{21} three polysaccharides from the rhizome of \textit{Curcuma longa},\textsuperscript{22-24} three polysaccharides from the root of \textit{Glycyrrhiza uralesis}\textsuperscript{5,26} and two polysaccharides from the bark of \textit{Eucomnia ulmoides}.\textsuperscript{27,28} Saposnikovin A,\textsuperscript{7} MVS-III-A,\textsuperscript{19} \textit{IVA}\textsuperscript{20} and -VI,\textsuperscript{10} ukonins A\textsuperscript{23} and B,\textsuperscript{22} and glycyrrhizain UA\textsuperscript{25} possess acidic arabinono-3,6-galactan structure as their major parts. Ukonan C\textsuperscript{24} and glycyrrhizacin UC\textsuperscript{26} belong to arabinono-3,6-galactoglycan. Saposnikovin C,\textsuperscript{18} glycyrrhizauk B,\textsuperscript{25} and eucommans A\textsuperscript{27} and B\textsuperscript{28} are essentially rhamnogalacturonans having various arabinogalactan type side chains. Cinnamann A\textsuperscript{22} is not arabinogalactan but arabinoxylan having a β-1,4-linked D-xylene backbone mostly bearing L-arabinosyl side chains at position 3.

Plantago-mucilage A is a partially acetylated acidic arabinoxylan having a similar backbone to that of cinnamann AX, but its side chains are composed of terminal D-xylene and D-glucurono- or D-galacturono-L-arabinose. This substance also showed significant RES activity, although its value of the phagocytic index was lower than those of the other active polysaccharides described above. However, the RES activity was greatly increased by deacylation of this mucous polysaccharide. A similar effect of deacylation
was observed on the alkaline phosphatase inducing activity. Deacetylation of Plantago-mucilage A does not cause any reduction of molecular weight of polysaccharide itself.\textsuperscript{3} Of course DAP has the same sugar composition as that of the original mucilage. So it is conceivable that the deacetylation effect on these biological activities may be attributed to steric factors. The difference in RES activity between HP60 and HP80 indicated that both the degree of branching and molecular mass seem to be the other factors in the immunological activity.

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References