A Glucan with Immunological Activities from the Tuber of *Alisma orientale*

Noriko SHIMIZU, Sadanori OHTSU, Masashi TOMODA,* Ryōko GONDA, and Naoko ŌHARA

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

Received June 27, 1994; accepted August 3, 1994

A glucan, called alisman SI, was isolated from the tuber of *Alisma orientale* JUZEPČZ. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was $1.1 \times 10^6$. It is composed solely of d-glucose. Methylolation analysis, nuclear magnetic resonance and enzymic degradation studies indicated that it has a high-branched glucan type structure mainly composed of z-1,4-linked d-glucopyranosyl residues with partially z-1,6-linked units and both 3,4- and 4,6-branching points. The polysaccharide exhibited significant reticuloendothelial system-potentiating activity in a carbon clearance test, as well as a pronounced anti-complementary activity.

**Keywords** glucan; polysaccharide structure; immunological activity; *Alisma orientale; alisman SI; tuber

We recently isolated and elucidated the structural features of two acidic polysaccharides from the tuber of *Alisma orientale* JUZEPČZ, which had reticuloendothelial system (RES)-potentiating and anti-complementary activities, and called them alisman PII and alisman PIIF. The tuber of this plant is a characteristic constituent of a traditional crude drug. These two substances were obtained as major acidic polysaccharides having immunological activities. The present paper describes the isolation, structural analysis, and RES-potentiating and anti-complementary activities of a novel glucan from the water extract of the tuber of *Alisma orientale*.

**MATERIALS AND METHODS**

Isolation of Polysaccharide The material was imported from China. The sliced dry tubers (100 g) were extracted with hot water (1 l) under stirring for 30 min in a boiling water bath. After suction filtration and centrifugation, the residue was similarly extracted with hot water (500 ml). The supernatants were combined and 1% sodium sulfate (12 ml) was added; 5% cetyltrimethylammonium bromide (CTAB, 70 ml) was then added to the solution. After centrifugation, the supernatant obtained was poured into two volumes of ethanol. The precipitate was dissolved in water, then dialyzed and lyophilized. Yield, 1.43 g. This fraction (fr. CTAB-Sup, 10 g) was dissolved in water and applied to a column (5 x 43 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (acetate form). The column was successively eluted with water (600 ml) and 0.1 M ammonium acetate (900 ml). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method.33 The eluates obtained from tubes 49 to 69 were combined, dialyzed, concentrated and applied to a column (5 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 32 to 40 were combined, concentrated and lyophilized. Yield, 323 mg. This fraction (fr. P, 40 mg) was dissolved in 1/15 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 1 mM MgCl2 and 1 mM CaCl2, and applied to a column (1.5 x 37.5 cm) of concanavalin A (Con A)-Sephrose (Pharmacia Co.). The column was equilibrated and successively eluted with the same buffer (180 ml) and buffer containing 10 mM methyl z-D-mannoside (240 ml) at 4 °C. Fractions of 10 ml were collected, and the eluates obtained from tubes 31 to 35 were combined, dialyzed, concentrated and applied to a column (2.6 x 88 cm) of Sephadex G-25. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 22 to 25 were combined, concentrated and lyophilized. Alisman SI was obtained as a white powder. Yield, 23.3 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described previously40 on Whatman GF/83 glass-fiber paper at 570 V for 1 h with 0.025 M Na2HPO4, 10 H2O, 0.1 N NaOH (10:1; pH 9.3). Alisman SI gave a single spot at a distance of 125 mm from the origin toward the cathode.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0) and applied to a column (2.6 x 94 cm) of Toyopearl HW-55F, 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 same 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 hydrolyzate into alditol acetate, was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame-atomization detector as described previously.46

Nuclear Magnetic Resonance (NMR) The NMR spectrum was recorded on a JEOI JNM-A 500 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30 °C.

Methylation Analysis Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously. The yield was 6.7 mg from 8.0 mg of alisman SI. The product was 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. x 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C per min from 160 to 220 °C at helium flow of 1 ml...
per min. GC-MS was carried out 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.

**Enzymic Degradation with x-Amylase** The glucan (11.0 mg) was dissolved in 0.05 M acetate buffer (pH 5.0, 1 ml), and an x-amylase preparation (2.8 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 (2.6 x 88 cm) of Sephadex G-25. The column was eluted with water, and fractions of 5 ml were collected and analyzed using the phenol-sulfuric acid method. The eluates obtained from the column were divided into five groups: Fraction 1, tubes 37 to 42; Fraction 2, tubes 43 to 55; Fraction 3, tubes 56 to 59; Fraction 4, tubes 60 to 68; Fraction 5, tubes 69 to 78. Fractions 3 to 5 were desalted by treatment with Dowex 50W-X8 (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 the 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 the eluent as described previously.⁵⁻⁶

**Phagocytic Activity** This was measured by an in vivo carbon clearance test as described previously.⁶ 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 for 5d.

**Anti-complementary Activity** This was measured as described in a previous report.¹⁰ 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 (TCH₉₀) was determined using immunoglobulin M (Ig M)-hemolysis-sensitized sheep erythrocytes. NHS was incubated with water and GVB²⁺ to provide a control, and the activities of the samples were expressed as percentage inhibition of the TCH₉₀ of the control. Plantago-mucilage A from the seed of Plantago asiatica L.¹¹ was used as a positive control.

### RESULTS

The hot water extract obtained from the tuber of *Alisma orientale* was treated with CTAB, and the supernatant obtained was poured into ethanol. The precipitate was dialyzed and subjected to ion-exchange chromatography with DEAE-Sephadex A-25. The eluate with a dilute ammonium acetate was dialyzed and subjected to affinity chromatography with Con A-Sepharose. The fraction adsorbed was eluted with methyl α-D-mannoside in a phosphate buffer, and the resulting eluant was dialyzed and applied to gel chromatography with Sephadex G-25. A pure polysaccharide, designated as alisman SI, was obtained.

The polysaccharide gave a single spot on electrophoresis and a single peak on gel chromatography. Gel chromatography gave a value of 1.1 x 10⁴ for the molecular mass of alisman SI. It had [α]D2⁴ = 156.3° (H₂O, c = 0.1). Alisman SI is composed solely of D-glucose, and it contained no nitrogen. The carbon-13 NMR (¹³C-NMR) spectrum showed a signal due to an anomeric carbon of α-D-glucopyranosyl units at δ 102.33 ppm.¹²

The glucan was degraded by treatment with x-amylase, followed by gel chromatography with Sephadex G-25. Five fractions (i.e., frs. 1 to 5) were obtained, and the major components (frs. 2 to 4) were identified as a mixture of maltopentaose and maltotetraose (fr. 2), maltotetraose and maltotriose (fr. 3), and maltotriose and maltose (fr. 4) by TLC and HPLC.

Alisman SI was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹³ The methylated product obtained was hydrolyzed, then converted into partially methylated aldito acetates. Analysis by GC-MS gave the results shown in Table I. The glucan produced a crimson lake coloration when the iodine test was applied.

**DISCUSSION**

We earlier isolated two immunologically active polysaccharides, called alisman P11 and alisman P11IF, from the CTAB-precipitate fraction of the hot water extract of the tuber of *Alisma orientale*.¹,² Structural studies indicated that alisman PI1 is a typical arabino-3,6-galactan with terminal glucuronic acid units, and that alisman P11IF possesses mainly both arabino-3,6-galactan and rhamnogalacturonic type structural units. We have now obtained a glucan, designated as alisman SI, as the major component of the CTAB-supernatant fraction from this crude drug.

During our studies to date on the immunologically active polysaccharides in crude drugs obtained from various plant sources, thirty-eight substances have been isolated.

<table>
<thead>
<tr>
<th>Table 1. Methylated Analysis of Alisman SI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylated sugars</strong></td>
</tr>
<tr>
<td>(as aldito acetates)</td>
</tr>
<tr>
<td>2,3,4,6-Me₂α-D-glucose</td>
</tr>
<tr>
<td>2,3,4-Me₂α-D-glucose</td>
</tr>
<tr>
<td>2,3,6-Me₂α-D-glucose</td>
</tr>
<tr>
<td>2,6-Me₂α-D-glucose</td>
</tr>
<tr>
<td>2,3-Me₂α-D-glucose</td>
</tr>
</tbody>
</table>

a Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. Abbreviations: Me, methyl; G1, α-D-glucopyranose.
and characterized as RES-potentiating polysaccharides. Among them, acidic arabinio-3,6-galactan is a major group. In addition, eight substances are neutral polysaccharides. These are cinnaminal AX from the bark of *Cinnamomum cassia*, glycyrrhizian UC from the root of *Glycyrrhiza uralensis*, MVS-I from the seed of *Malva verticillata*, ukonan D from the rhizome of *Curcuma longa*, peonan SA from the root of *Paeonia lactiflora*, cnidirhan SI and cnidirhan SIIA from the rhizome of *Cnidium officinale*, and pinellian G from the tuber of *Pinellia ternata*.

Among these eight neutral polysaccharides, cnidirhan SI and pinellian G are α-glucans with immunological activity. Thus, alisman SI is the third example of a branched α-glucan with phagocytosis-stimulating and anti-complementary activities. Both amylopectin and glycogen, ordinary 4,6-branching α-glucans, are completely inactive in the RES.

Alisman SI is mainly composed of α-1,4-linked D-glucopyranosyl units, and it has 3,4-branching points in addition to 4,6-branching. These two branching modes were also observed in cnidirhan SI and peonan SA, although pinellian G has no 3,4-branching. Pinellian G possesses exceptional α-1,3-linked units in addition to the usual α-1,4-linear linkage. On the other hand, the presence of α-1,6-linked units in alisman SI is characteristic. There is an average of almost one branch for every thirteen glucose units in this glucan. The degree of branching in this substance is between that for cnidirhan SI and pinellian G. The presence of the α-1,6-linkage and 3,4-branching may contribute to the activity of alisman SI.

The yield of alisman SI from the material tuber is relatively high compared with the other biologically active polysaccharides, so it seems reasonable to assume that alisman SI is a representative polysaccharide of the tuber of *Alisma orientale*.

REFERENCES