Characterization of an Acidic Polysaccharide Having Immunological Activities from the Tuber of *Alisma orientale*

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

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

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An acidic polysaccharide, called alisman PII, was isolated from the tuber of *Alisma orientale* JUZEPCZ. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 5.25×10^4 . It is composed of L-arabinose:D-galactose:D-glucuronic acid in the molar ratio of 4:9:2, in addition to some of O-acetyl groups. Reduction of carboxyl groups, methylation analysis, nuclear magnetic resonance and controlled Smith degradation indicated 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 -D-galactosyl side chains at position 6. Both α -1,5-linked L-arabinosyl side chains and terminal β -D-glucuronic acid residues are linked to the core galactan units. Alisman PII showed significant potentiation of the reticuloendothelial system using a carbon clearance test and also potent anti-complementary activity.

Keywords polysaccharide structure; immunological activity; *Alisma orientale*; tuber; acidic arabinogalactan; alisman PII

The tuber of *Alisma orientale* JUZEPCZ. (Alismataceae) is a traditional Chinese crude drug used as a diuretic and an antidyspeptic under the name of zexie in China (Japanese name, Takusha). It has been used as an important component of many pharmaceutical preparations in Japanese herbal medicine. In addition to a large quantity of starch and protein, glucose, fructose, sucrose, choline, lecithin,¹⁾ valine and its derivative,²⁾ alisols A, B, C and their acetates,^{3–6)} alismol, alismoxide,⁷⁾ derivatives of alisols B, C and D,^{8,9)} and orientols A, B and C¹⁰⁾ have been isolated from this crude drug. Recently, we proposed new methods for identification of the decoction of alisma rhizome, and identified sodium salts of myo-inositol hexaphosphate as useful indicators.¹¹⁾ We also demonstrated the presence of acidic polysaccharides in the crude drug during this study. The present paper describes the isolation, structural analysis and immunological activities of a novel acidic polysaccharide from the water extract of the tuber of *Alisma orientale*.

MATERIALS AND METHODS

Isolation of Polysaccharide The material was imported from China. The sliced tubers (200 g) were extracted with hot water (2 l) under stirring for 30 min in a boiling water bath. After suction filtration and centrifugation, the residue was similarly extracted with hot water (1 l). The supernatants were combined (2400 ml) and 1% sodium sulfate (24 ml) was added; 5% cetyltrimethylammonium bromide (CTAB, 140 ml) was then added to the solution. After centrifugation, the precipitate was extracted with 0.2 M sodium chloride (300 ml). After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was dissolved in water, then dialyzed and lyophilized. Yield, 400 mg. This fraction (CTAB-Ppt, 4.3 g) was dissolved in 0.01 M phosphate buffer (pH 7.2) and applied to a column (5 × 47 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia Co.). The column was equilibrated and eluted with the same phosphate buffer (1120 ml), then successively eluted with phosphate buffers

containing 0.1 M NaCl (940 ml) and 0.2 M NaCl (600 ml). Fractions of 20 ml were collected and analyzed using the phenol-sulfuric acid method.¹²⁾ Fraction A was obtained from tubes 17 to 43, and fr. B from tubes 118 to 127. After dialysis and gel chromatography using a column (5 × 86 cm) of Sephadex G-25 with water, frs. PI and PII were obtained from frs. A and B, respectively in yields of 464.3 and 180.9 mg. Fraction PII 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 (2.6 × 38 cm) of concanavalin A (Con A)-Sephacel (Pharmacia Co.). The column was equilibrated and eluted with the same buffer at 4 °C and fractions of 20 ml were collected. The eluates obtained from tubes 8 to 15 were combined, dialyzed, concentrated and applied to a column (5 × 87 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 39 were combined, concentrated and lyophilized. A polysaccharide, called alisman PII, was obtained as a white powder. Yield, 161.8 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described previously¹³⁾ on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M Na₂B₄O₇ · 10H₂O–0.1 N NaOH (10:1, pH 9.3). Alisman PII gave a single spot at a distance of 103 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 × 95 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.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described previously.¹⁴⁾ The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α -methylbenzylamino-alditol derivatives.¹⁵⁾

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.¹⁶⁾ Glucuronic acid was determined by the *m*-hydroxybiphenyl method.¹⁷⁾

Determination of *O*-Acetyl Groups The sample was hydrolyzed with 0.2*N* 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. × 2.1 m long spiral glass) packed with 5% Thermon-3000 on Shincarbon A (60 to 80 mesh) at 120 °C with a helium flow of 30 ml per min; *t_R* (min), acetic acid 5.2; propionic acid (internal standard) 7.9.

Nuclear Magnetic Resonance (NMR) The NMR spectrum was 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 30 °C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.¹⁸⁾ The reduction was repeated three times under the same conditions. The yield was 37.5 from 45.2 mg of alisman PII.

Methylation This was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.¹⁹⁾ The yields were 3.3 from 4.7 mg of alisman PII, 3.4 from 4.9 mg of the carboxyl-reduced product, 1.9 from 3.0 mg of the controlled Smith degradation product (SDP) and 2.0 from 3.0 mg of the secondary Smith degradation product.

Analysis of the Methylated Products The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.²⁰⁾ 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 JEOL JMS-DX 303 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 The polysaccharide (12.0 mg) was dissolved in 0.05 *M* sodium metaperiodate (6 ml) and kept at 4 °C in the dark. The periodate consumption was measured by a spectrophotometric method.²¹⁾ Oxidation was complete after 4 d. The reaction mixture was successively treated with ethylene glycol (0.1 ml) at 4 °C for 1 h and sodium borohydride (30 mg) at 4 °C for 16 h, then adjusted to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (2.6 × 38 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 5 to 7 were combined, concentrated and lyophilized. Yield, 11.5 mg.

Controlled Smith Degradation The periodate oxidation-reduction product (8.1 mg) was dissolved in 0.5 *N* sulfuric acid (0.8 ml). After standing at 22 °C for 18 h, the solution was neutralized with Dowex 2 (OH⁻). The filtrate was concentrated and applied to a column (1.9 × 40 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 5 and 6 were combined, concentrated and lyophilized. The yield of the product (SDP) was 4.3 mg.

Secondary Smith Degradation SDP (30 mg) was oxidized with 0.05 *M* sodium metaperiodate (15 ml) at 4 °C for 3 d in the dark. After successive treatment with ethylene glycol (0.15 ml) and sodium borohydride (100 mg) as described above, the solution was neutralized with acetic acid 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 9 to 12 were combined, concentrated and lyophilized. Yield, 17.4 mg. This product was treated with 0.5 *N* sulfuric acid as described above, and after neutralization, the solution was applied to a column (2.6 × 40 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 9 to 11. Yield, 5.8 mg.

Phagocytic Activity This was measured by *in vivo* carbon clearance test as described previously.¹⁶⁾ The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 mg/kg body weight) to male mice (ICR-SPF) once a day for 5 d.

TABLE I. Methylation Analysis of Alisman PII, Its Carboxyl-Reduced Derivative and the Controlled Smith Degradation Products

	Relative retention time ^{a)}	Molar ratios			
		Original	Carboxyl-reduced	Primary SDP	Secondary SDP
1,4-Ac ₂ -2,3,5-Me ₃ -L-arabinitol	0.69	6	6	—	—
1,4,5-Ac ₃ -2,3-Me ₂ -L-arabinitol	1.14	6	6	—	—
1,5-Ac ₂ -2,3,4,6-Me ₄ -D-glucitol	1.00	—	6	—	—
1,5-Ac ₂ -2,3,4,6-Me ₄ -D-galactitol	1.10	1	1	2	1
1,3,5-Ac ₃ -2,4,6-Me ₃ -D-galactitol	1.39	8	8	5	24
1,4,5-Ac ₃ -2,3,6-Me ₃ -D-galactitol	1.47	1	1	—	—
1,5,6-Ac ₃ -2,3,4-Me ₃ -D-galactitol	1.62	4	4	2	2
1,3,4,5-Ac ₄ -2,6-Me ₂ -D-galactitol	1.68	1	1	—	—
1,3,5,6-Ac ₄ -2,4-Me ₂ -D-galactitol	2.02	12	12	2	2

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- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

Anti-complementary Activity This was measured as described in a previous report.²²⁾ Geratin-veronal-buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{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_{50}) was determined using immunoglobulin M (IgM)-hemolysis-sensitized sheep erythrocytes. NHS was incubated with water and GVB^{2+} to provide a control, and the activities of the samples were expressed as the percentage inhibition of the TCH_{50} of the control. Plantago-mucilage A from the seed of *Plantago asiatica* L.²³⁾ was used as a positive control.

RESULTS

The hot water extract obtained from the tuber of *Alisma orientable* was treated with CTAB in the presence of small amounts of sodium sulfate. The precipitate obtained was extracted with 0.2 M sodium chloride, and the extract was poured into ethanol. After dialysis, the precipitate was applied to a column chromatography on DEAE-Sephacel, then the stepwise elution with sodium chloride in a dilute phosphate buffer was carried out. The eluate obtained with dilute phosphate buffer afforded a glucan having no biological activity (Fig. 1). The eluate obtained with 0.1 M sodium chloride in phosphate buffer was dialyzed and purified by gel chromatography on Sephadex G-25. This fraction was further subjected to affinity chromatography on Con A-Sepharose. A pure polysaccharide, called alisman PII, was obtained from the passed-through fraction, followed by dialysis and gel chromatography on Sephadex G-25.

The polysaccharide gave a single spot on electrophoresis, and gave a single peak on gel chromatography. It had $[\alpha]_D^{21} -21.8^\circ$ (H_2O , $c=0.1$). Gel chromatography gave a value of 5.25×10^4 for the molecular mass.

Alisman PII is composed of L-arabinose, D-galactose and D-glucuronic acid. Quantitative analysis showed that it contained 21.4% arabinose, 63.2% galactose and 15.4% glucuronic acid. The molar ratio of these component sugars was 4:9:2.

The carbon-13 NMR (^{13}C -NMR) spectrum of alisman PII showed signals at δ 21.77 and 178.32 ppm, suggesting the presence of O-acetyl groups. The presence of acetyl groups was confirmed by GC of the hydrolyzate, and the content was 3.4%. The ^{13}C -NMR spectrum gave no signal

suggesting the presence of O-methyl groups as carboxylic acid methyl esters.

Further, the ^{13}C -NMR spectrum showed four signals due to anomeric carbons at δ 105.33, 106.55, 110.14 and 111.92 ppm. The first and second were assigned to the anomeric carbons of β -D-glucopyranosyluronic acid and β -D-galactopyranose, respectively,²⁴⁾ while signals at δ 110.14 and 111.92 ppm were assigned to the anomeric carbons of α -L-arabinofuranose.²⁴⁾

The carboxyl groups of hexuronic acid residues in alisman PII were reduced to give the corresponding neutral sugar residues.²⁵⁾ Both alisman PII and its carboxyl-reduced derivative were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.²⁶⁾ The methylated products were hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I. These indicate that D-glucuronic acid residues in the original polysaccharide produced 2,3,4,6-tetra-O-methyl-D-glucose in the products from its carboxyl-reduced derivative.

Alisman PII was subjected to periodate oxidation followed by reduction. The product was treated with dilute sulfuric acid at room temperature overnight.²⁷⁾ The controlled Smith degradation product (SDP) obtained was composed of D-galactose alone. SDP was methylated, hydrolyzed, then converted into the partially methylated alditol acetates. The result of the GC-MS analysis is shown in Table I. In order to elucidate the structural features of SDP, the 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 its methylation analysis is also shown in Table I.

Based on the accumulated evidence described above, it can be concluded that alisman PII has the structural features shown in Chart 1. It can be presumed that SDP, the core of alisman PII, has a backbone chain composed of β -1,3-linked D-galactose residues, and that one-fifth of the galactose units in the backbone chain must carry β -1,6-linked galactosyl side chains at position 6. Some of β -1,6-linked galactose 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 alisman PII on the reticuloendothelial system (RES) was demonstrated by a modification¹⁶⁾ of the *in vivo* carbon clearance test²⁸⁾ using zymosan as a positive control. As shown in Fig. 1, the phagocytic index

Fig. 1. Effect of Alisman PII on Phagocytosis
Significantly different from the control, a) $p < 0.05$.

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Alisman PII

a) Number of residues. Araf, arabinofuranose; Galp, galactopyranose; GlcpA, glucopyranosyluronic acid.

Chart 2. Possible Structural Units of the Primary and Secondary Smith Degradation Products of Alisman PII

Fig. 2. Anti-complementary Activity of Alisman PII

Alisman PII, —●—; Plantago-mucilage A, ---▲---. Each point represents the mean ($n=3$).

was significantly increased, suggesting activation of the RES by i.p. injection of alisman PII.

The anti-complementary activity of alisman PII is shown in Fig. 2. The polysaccharide 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 twenty-seven substances as RES-activating polysaccharides and their structural features have been elucidated. The acidic arabinosyl-3,6-galactan group is very common. It is present in saposhnikovan A,¹⁶⁾ MVS-IIIa, -IVa and -VI,²⁹⁻³¹⁾ ukonans A, B and C,³²⁻³⁴⁾ glycyrrhizans UA, UB and GA,^{24,35)} eucommian A,³⁶⁾ AMon-S,³⁷⁾ cnidirhan AG,³⁸⁾ ginsenosides PA, PB, S-IA and S-IIa,^{39,40)} and peonin SB.⁴¹⁾

Most of these RES-activating polysaccharides possess arabinosyl-3,6-galactan moieties as the major neutral constituents, and D-galacturonic acid as the sole component hexuronic acid. On the other hand, glycyrrhizan GA from the stolon of *Glycyrrhiza glabra* var. *glandulifera*, AMon-S from the root of *Astragalus mongholicus*, and ginsenosides PA and PB from the root of *Panax ginseng* possess both D-galacturonic acid and D-glucuronic acid as their components. In addition, cnidirhan AG from the rhizome of *Cnidium officinale* is classified as a novel type of acidic arabinosyl-3,6-galactan. Its component hexuronic acid is not D-galacturonic acid, but D-glucuronic acid. Thus, alisman PII is the second example of the RES-activating acidic polysaccharides obtained by us having D-glucuronic acid as the sole component hexuronic acid. D-Galacturonic acid is generally present in the known RES-activating acidic polysaccharides as intermediate units having α -1,4-linkage, while all of the D-glucuronic acid has been found as β -linked terminal units in the immunologically active acidic polysaccharides obtained by us.

Both cnidirhan AG and alisman PII possess basically common structural units. The main difference between them is the nature and ratio of the D-galactose structural

units. Alisman PII has β -1,4-linked and 3,4-branched D-galactosyl residues, while cnidirhan AG does not. The ratio of the other structural units, i.e., terminal α -L-arabinosyl, α -1,5-linked L-arabinosyl, terminal β -D-galactosyl, β -1,3-linked D-galactosyl, β -1,6-linked D-galactosyl, β -3,6-branched D-galactosyl and terminal β -D-glucuronic acid residues is 7:7:1:11:15:15:7 in cnidirhan AG,³⁸⁾ while the ratio of these in alisman PII is 6:6:1:8:4:12:6. The phagocytic and anti-complementary activities of cnidirhan AG is greatly superior to that of alisman PII. Values for the molecular masses of the both polysaccharides are almost identical. It can therefore be presumed that both the presence of β -1,4-linked and 3,4-branched D-galactosyl units and the ratio of β -1,3- and 1,6-linked D-galactosyl residues affect the biological activities of these polysaccharides. The latter reflects differences in their core galactan structures.

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