Characterization of a Novel Acidic Polysaccharide with Immunological Activities from the Rhizome of *Cnidium officinale*

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An acidic polysaccharide, called cnidirhan SIIB, was isolated from the rhizome of *Cnidium officinale* MAKINO. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 7.9×10^4. It was composed of L-arabinose, D-galactose and D-galacturonic acid in the molar ratio of 9:11:3, in addition to a few *O*-acetyl groups. Reduction of carboxyl groups, methylation analysis, nuclear magnetic resonance and controlled Smith degradation studies indicated that its structural features are composed primarily of α-L-arabino-β-3,6- and 3,4-branched D-galactan type units. The polysaccharide showed very pronounced reticuloendothelial system-potentiating activity in a carbon clearance test and marked anti-complementary activity.

Keywords polysaccharide structure; immunological activity; acidic arabinogalactan; *Cnidium officinale*; cnidirhan SIIB

We recently isolated an acidic polysaccharide, a glucan and a heteroglucan from the rhizome of *Cnidium officinale* MAKINO, and determined the structural features of these polysaccharides, which had reticuloendothelial system (RES)-potentiating and anti-complementary activities, and called them cnidirhan AG, cnidirhan SI and cnidirhan SIIA, respectively.[1-3] The rhizome of this plant is a representative Japanese material of a traditional crude drug. Cnidirhan AG was obtained as a major acidic polysaccharide, while cnidirhan SI was isolated as a main neutral polysaccharide from this crude drug. Cnidirhan SIIA was found to be a heteroglucan having much greater immunological activities than those of the other polysaccharides obtained by us from various crude drugs.

The present paper describes the isolation, structural features, RES-potentiating and anti-complementary activities of a novel acidic polysaccharide from the water extract of the rhizome of *Cnidium officinale*.

Materials and Methods

Isolation of the Polysaccharide The material plant was cultivated in Hokkaido. Sliced dry rhizomes (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 added with 1% sodium sulfate (2 ml); 5% cetyltrimethylammonium bromide (CTAB; 210 ml) was then added to the solution. The precipitate produced by the addition of CTAB afforded cnidirhan AG. 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, 2.6 g. This fraction (fr. CTAB-Sup; 2.0 g) was dissolved in 0.01 M phosphate buffer (pH 7.2) and applied to a column (5×32 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia Co.). The column was equilibrated and eluted with the same phosphate buffer (800 ml). The eluate afforded cnidirhan SI. After elution with the phosphate buffer, the column was eluted with phosphate buffer containing 0.1 M NaCl (900 ml). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[4] The eluates obtained from tubes 23 to 43 were combined, dialyzed, concentrated and lyophilized. The yield of this fraction (fr. B) was 108 mg. Fraction B (100 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (5×88 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 30 to 34 afforded cnidirhan SIIA. The eluates obtained from tubes 39 to 45 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. The eluates obtained from tubes 30 to 32 were combined, concentrated and lyophilized. Cnidirhan SIIB was obtained as a white powder. Yield, 18.6 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described previously[5] on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M $Na_2B_4O_7\cdot10H_2O$–0.1 N NaOH (10:1, pH 9.3). Cnidirhan SIIB gave a single spot at a distance of 103 mm from the origin toward the cathode.

Gel Chromatography The sample (2 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6×98 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Component Sugar Analysis Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described previously.[6] The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylamino-alditol derivatives.[7] Neutral sugars were determined by GC after conversion of the hydrolyzate into alditol acetates as described previously.[8] GC was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector. Galacturonic acid was measured by the *m*-hydroxydiphenyl method.[9]

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.[10]

Nuclear Magnetic Resonance (NMR) The NMR spectrum was recorded on a JEOL JNM-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 previously.[11] The reduction was repeated three times under the same conditions. Yield was 12.0 mg from 22.9 mg of cnidirhan SIIB.

Methylation This was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.[12] The yields were 1.4 mg from 2.0 mg of cnidirhan SIIB, and 1.2 mg from 2.0 mg of its carboxyl-reduced derivative.

Analysis of the Methylated Products The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.[13] 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. $\times$ 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-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.

Periodate Oxidation The polysaccharide (31 mg) was dissolved in 0.1 N sodium hydroxide (1.6 ml) and allowed to stand at room

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temperature for 10 min, then the solution was neutralized with 10 M acetic acid. The solution was adjusted to 8 ml with water, then 0.1 M sodium metaperiodate (8 ml) was added. The reaction mixture was kept at 4 °C in the dark, and the periodate consumption was measured by a spectrophotometric method.[14] Oxidation was completed after 3 d. The reaction mixture was successively treated with ethylene glycol (0.3 ml) at 4 °C for 1 h and sodium borohydride (150 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 × 93 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 21 to 24 were combined, concentrated and lyophilized. Yield, 30 mg.

Controlled Smith Degradation The periodate oxidation-reduction product (27.5 mg) was dissolved in 0.5 N sulfuric acid (2.8 ml). After standing at 23 °C for 16 h, the solution was neutralized with barium carbonate. The filtrate was applied to a column (0.5 × 4 cm) of Dowex 50W-X8 (H^+). The column was eluted with water, and the eluate was concentrated and applied to a column (2.6 × 91 cm) of Sephadex G-25. This column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 20 to 22 were combined, concentrated and lyophilized. Yield of the controlled Smith degradation product (SDP) was 5.2 mg.

Phagocytic Activity This was measured by *in vivo* carbon clearance test as described previously.[8] The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved or 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.[15] Gelatin-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 (Ig M)-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 percentage inhibition of the TCH_{50} of the control. Plantago-mucilage A from the seed of *Plantago asiatica* L.[16] was used as a positive control.

Results

The hot water extract obtained from the rhizome of *Cnidium officinale* was treated with CTAB in the presence of small amounts of sodium sulfate. The supernatant was poured into ethanol, and the precipitate was dialyzed and applied to column chromatography of DEAE-Sephacel. After elution with dilute phosphate buffer, the eluate with 0.1 M sodium chloride in a dilute phosphate buffer was dialyzed and subjected to gel chromatography with Toyopearl HW-55F. A pure polysaccharide, designated as cnidirhan SIIB, was obtained from the second polysaccharide fraction, followed by dialysis and gel chromatography with Sephadex G-25.

The polysaccharide gave a single spot on electrophoresis, and it gave a single peak on gel chromatography with Toyopearl HW-55F. Gel chromatography gave a value of 7.9×10^4 for the molecular mass of cnidirhan SIIB. It had $[\alpha]_D^{24}$ $-36.5°$ (H_2O, $c=0.1$).

Cnidirhan SIIB is composed of L-arabinose, D-galactose and D-galacturonic acid. Quantitative analysis showed that cnidirhan SIIB contained 34.6% arabinose, 50.8% galactose and 14.3% galacturonic acid. The molar ratio of these component sugars was 9 : 11 : 3. It contained no nitrogen.

The carbon-13 NMR (^{13}C-NMR) spectrum of cnidirhan SIIB showed three signals due to anomeric carbons at δ 102.8, 106.1 and 110.2 ppm. These were assigned to the anomeric carbons of α-D-galacturonopyranosyl, β-D-galactopyranosyl and α-L-arabinofuranosyl units, respectively.[17] Further, the ^{13}C-NMR spectrum showed signals at δ 21.8 and 178.6 ppm, suggesting the presence of *O*-acetyl groups. This was confirmed by GC of the hydrolyzate, and the content of acetyl groups was 0.1%.

The carboxyl groups of hexuronic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[18] Both cnidirhan SIIB and its carboxyl-reduced derivative were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[19] The two methylated products thus obtained were hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I, which indicate that D-galacturonic acid residues in the polysaccharide produced 2,3,4,6-tetra-*O*-methyl D-galactose, 2,3,6-tri-*O*-methyl D-galactose and 2,6-di-*O*-methyl D-galactose in the molar ratio of 1 : 3 : 1 in the methylation products from its carboxyl-reduced derivative.

Thus these results indicated that the minimal unit of cnidirhan SIIB is composed of twelve kinds of component sugar units, as shown in Chart 1.

To elucidate the galactan core structure of cnidirhan SIIB, the polysaccharide was de-acetylated and subjected to periodate oxidation followed by reduction. The product was treated with dilute sulfuric acid under a mild condition, and the controlled Smith degradation product (SDP) was obtained.

SDP gave a single peak on gel chromatography, and

TABLE I. Methylation Analysis of Cnidirhan SIIB, Its Carboxyl-Reduced Derivative and the Controlled SDP

Methylated sugars (as alditol acetates)	Relative retention times[a]	Molar ratio		
		Original	Carboxyl-reduced	SDP
2,3,5-Me_3-L-arabinose	0.69	10	10	—
2,3-Me_2-L-arabinose	1.14	2	2	—
2-Me-L-arabinose	1.44	3	3	—
2,3,4,6-Me_4-D-galactose	1.10	1	2	4
2,4,6-Me_3-D-galactose	1.39	4	4	4
2,3,6-Me_3-D-galactose	1.47	3	6	—
2,3,4-Me_3-D-galactose	1.62	3	3	3
2,6-Me_2-D-galactose	1.66	3	4	1
2,4-Me_2-D-galactose	2.02	5	5	3

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

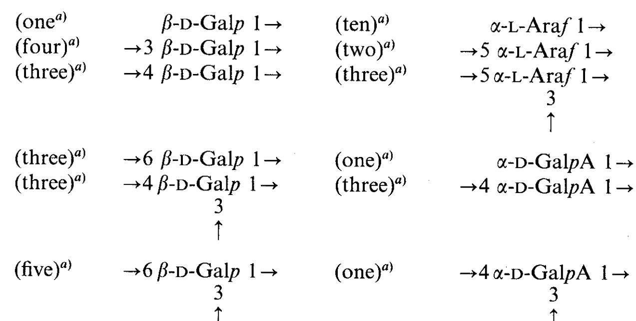

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Cnidirhan SIIB

a) Number of residues. Gal*p*, galactopyranosyl unit; Ara*f*, arabinofuranosyl unit; Gal*p*A, galacturonopyranosyl unit.

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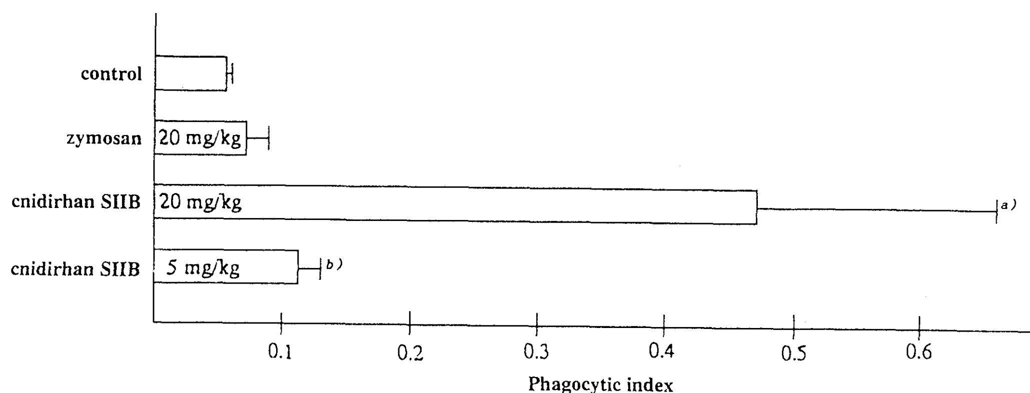

Fig. 1. Effects of Cnidirhan SIIB on Phagocytosis
Significantly different from the control, *a)* $p<0.01$; *b)* $p<0.001$.

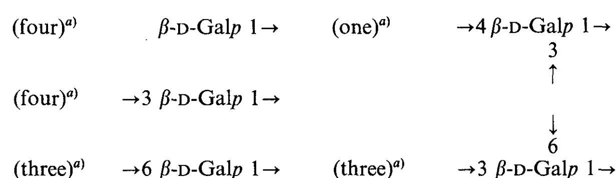

Chart 2. Component Sugar Residues in the Minimal Unit in the Structure of the Controlled Smith Degradation Product
a) Number of residues.

showed a value of 3.98×10^4 for the molecular mass. SDP was composed of D-galactose alone, and it was methylated, hydrolyzed, then converted into partially methylated alditol acetates. The result of analysis by GC-MS is also shown in Table I. This result indicated that the minimal unit of SDP is composed of five kinds of component sugar units, as shown in Chart 2.

The effect of cnidirhan SIIB on the RES was demonstrated by a modification[8] of the *in vivo* carbon clearance test[20] using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was extraordinarily increased, suggesting very powerful activation of RES by i.p. injection of this polysaccharide. Further, the anti-complementary activity of cnidirhan SIIB is shown in Fig. 2. The polysaccharide exhibited a marked activity compared with the positive control, Plantago-mucilage A.

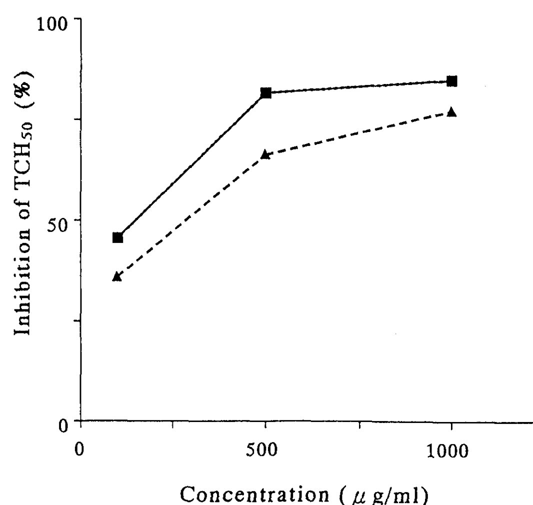

Fig. 2. Anti-complementary Activity of Cnidirhan SIIB
Cnidirhan SIIB, —■—; plantago-mucilage A, ---▲---. Each point represents the mean ($n=3$).

Discussion

We have already obtained three immunologically active polysaccharides, called cnidirhan AG, cnidirhan SI and cnidirhan SIIA, from the hot water extract of Cnidium rhizome. Structural studies indicated that cnidirhan AG is a typical α-1,5-linked L-arabino-β-3,6-branched D-galactan with terminal β-D-glucuronic acid units.[1] Cnidirhan SI is a high-branched α-1,4-linked D-glucan with both 3,4- and 4,6-branching points,[2] and the main structural features of enidirhan SIIA are β-1,6-linked D-glucan with both 3,6- and 4,6-branching points.[3]

During our studies to date on the immunologically active polysaccharides in crude drugs obtained from various plant sources, we have isolated thirty-four substances which are RES-activating polysaccharides, and determined their structural features. Acidic arabinogalactans form the major group. Among them, only three polysaccharides are composed of α-L-arabinose, β-D-galactose and α-D-galacturonic acid. Those are saposhnikovan A from the root and rhizome of *Saposhnikovia divaricata*,[8] ginsenan S-IA from the root of *Panax ginseng*,[17] and peonan SB from the root of *Paeonia lactiflora*.[21] These are acidic α-1,5-linked L-arabino-β-3,6-branched D-galactans. D-Galacturonic acid residues are α-1,4-linked minor components in ginsenan S-IA and peonan SB, while saposhnikovan A possesses a backbone chain composed of α-1,4-linked D-galacturonic acid residues with a few 2,4- and 3,4-branching units.

The main parts of cnidirhan SIIB are also occupied by α-1,5-linked L-arabino-β-3,6-branched D-galactan moieties. In contrast to the typical arabino-3,6-galactan, this polysaccharide has relatively high content of α-3,5-branched L-arabinose, β-1,4-linked and 3,4-branched D-galactose units. Further, this substance possesses relatively many terminal and α-3,4-branched D-galacturonic acid residues in addition to the usual α-1,4-linked units.

The RES-potentiating activity of cnidirhan SIIB is greater than those of cnidirhan AG and cnidirhan SI. The complicated branching manner in the polysaccharide may

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contribute to the elevation of this activity. The remarkable anti-complementary activity of cnidirhan SIIB is at almost the same level as cnidirhan AG. Further investigation of the relationship between the biological activities and structural features is in progress.

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