Characterization of a Novel Glucan, Which Exhibits Reticuloendothelial System-Potentiating and Anti-complementary Activities, from the Rhizome of *Cnidium officinale*

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A novel glucan, called cnidirhan SI, 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 1.3×10^4. It is composed of D-glucose alone, in addition to small numbers of *O*-acetyl groups. Methylation analysis, nuclear magnetic resonance and enzymic degradation studies indicated it has a high-branched glucan type structure composed of α-1,4-linked D-glucopyranose residues with both 3,4- and 4,6-branching points. The glucan showed significant reticuloendothelial system-potentiating activity in a carbon clearance test, as well as pronounced anti-complementary activity. This substance is the first example of a branched α-glucan with phagocytosis-stimulating and anti-complementary activities.

Keywords glucan; polysaccharide structure; immunological activity; *Cnidium officinale*; cnidirhan SI

We recently isolated and elucidated the structural features of an acidic polysaccharide from the rhizome of *Cnidium officinale* MAKINO which had reticuloendothelial system (RES)-potentiating and anti-complementary activities, and called it cnidirhan AG.[1] The rhizome of this plant is a representative Japanese material of a traditional crude drug. Cnidirhan AG was obtained as a major acidic polysaccharide having immunological activities. The present paper describes the isolation, structural features, RES-potentiating and anti-complementary activities of a novel glucan 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 to 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, the precipitate 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). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[2] The eluates obtained from tubes 11 to 33 were combined, dialyzed, concentrated and lyophilized. The yield of this fraction (fr. A) was 1.04 g. Fraction A (50 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (5 × 84 cm) of Toyopearl HW-55F, pre-eqilibrated and developed with the same buffer. Fractions of 20 ml were collected, and the eluates obtained from tubes 44 to 53 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 33 were combined, concentrated and lyophilized. Cnidirhan SI was obtained as a white powder. Yield, 30.5 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described previously[3] on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M $Na_2B_4O_7 \cdot 10H_2O$–0.1 N NaOH (10 : 1, pH 9.3). Cnidirhan SI gave a single spot at a distance of 140 mm from the origin toward the cathode.

Gel Chromagotraphy The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer and applied to a column (2.6 × 98 cm) of Toyopearl HW-55F as described above. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) with known molecular masses were run on the column to obtain a calibration curve.

Component Sugar Analyses Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described previously.[4] 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 ionization detector as described previously.[5]

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.

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.[6]

Methylation Analysis Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.[7] The yields were 2.1 mg from 3.0 mg of cnidirhan SI, and 2.1 mg from 2.4 mg of β-limit-dextrin SI (LDSI). The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.[8] 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 carried out 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.

Enzymic Degradation with α-Amylase Cnidirhan SI (100 mg) was dissolved in 0.05 M acetate buffer (pH 5.0, 5 ml), and an α-amylase preparation (25 μl; Sigma Co.) was added. The solution was incubated with a few drops 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 (5 × 88 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method. The eluates obtained from the column were divided into seven groups: Fraction 1, tubes 32 to 38; fr. 2, tubes 39 to 45; fr. 3, tubes 46 to 50; fr. 4, tubes 51 to 57; fr. 5, tubes 58 to 61; fr. 6, tubes 62 to 64; fr. 7, tubes 65 to 68. The yields were 24.0 mg for fr. 1, 3.7 mg for fr. 2, 6.1 mg for fr. 3, 21.6 mg for fr. 4, 14.2 mg for fr. 5, 22.2 mg for fr. 6, and 8.2 mg for fr. 7. Fractions 5 to 7 were desalted by treatment with Dowex 50W-X8 (H^+) followed by evaporation.

Analysis of Degradation Products TLC was performed on Merck pre-coated Kieselgel 60 plates using *n*-butanol–acetic acid–water (2 : 1 : 1,

v/v) as a developing solvent. Detection was done by spraying 0.2% orcinol in 20% sulfuric acid followed by heating at 110 °C for 5 min. *Rf* values of maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, maltose and glucose were 0.22, 0.26, 0.30, 0.35, 0.43, 0.50 and 0.59, respectively. The high-performance liquid chromatography (HPLC) system used consisted of a Hitachi L-6200 intelligent pump, a Shodex SE-51 RI monitor and a Hitachi D-2000 chromatointegrator. The analysis was carried out using a column (0.46 × 25 cm) of Asahipak NH 2P-50 with acetonitrile–water (2 : 1, v/v) as an eluent at a flow rate of 1 ml per min at room temperature. Retention times of glucose, maltose, maltotriose and maltotetraose were 5.47, 6.83, 8.75 and 10.77 min.

Enzymic Degradation with β-Amylase and Isolation of the Main Product Cnidirhan SI (200 mg) was dissolved in water (4.51 ml), then β-amylase preparation (0.49 ml; Seikagaku-Kōgyō Co., 844 unit/mg) and 0.1 M acetate buffer (pH 4.8, 5 ml) were added. The solution was incubated with a few drops of tolene under dialysis against 0.05 M acetate buffer (pH 4.8, 800 ml) at 37 °C. The external buffer solution was replaced twice after 4 h and 21 h. After incubation for 24 h, the internal solution was dialyzed against water, then the solution was applied to a column (0.7 × 3.5 cm) of Dowex 50W-X8 (H^+). The column was eluted and washed with water. The eluate and washings were combined, concentrated, and half of the solution was applied to a column (5 × 87 cm) of Toyopearl HW-55F, pre-equilibrated and developed with 0.1 M Tris–HCl buffer (pH 7.0). Fractions of 20 ml were collected, and the eluates obtained from tubes 48 to 58 were combined, dialyzed and purified by column chromatography with Sephadex G-25 as described above. β-Limit-dextrin SI (LDSI) was obtained by lyophilization; the yield was 42.8 mg.

Phagocytic Activity This was measured by an *in vivo* carbon clearance test as described previously.[5] The samples 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.

Anti-complementary Activity This was measured as described in a previous report.[9] 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[10] was used as a positive control.

Results

The hot water extract obtained from the rhizome of *Cnidium officinale* was treated with CTAB, and the supernatant obtained was poured into ethanol. The precipitate was dialyzed and applied to a column chromatography of DEAE–Sephacel. The eluate with a dilute phosphate buffer was dialyzed and subjected to gel chromatography with Toyopearl HW-55F. A pure polysaccharide, designated as cnidirhan SI, was obtained from the main fraction, followed by dialysis and gel chromatography with Sephadex G-25.

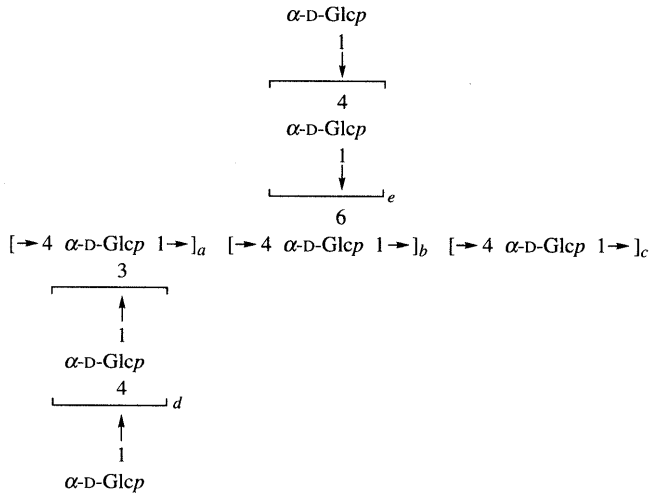
The polysaccharide gave a single spot on electrophoresis, and gave a single peak on gel chromatography. Gel chromatography gave a value of 1.3×10^4 for the molecular mass of cnidirhan SI. It had $[\alpha]_D^{21}$ +138° ($c = 0.1$, H_2O).

Cnidirhan SI is composed of D-glucose alone. The carbon-13 NMR (^{13}C-NMR) spectrum showed signals at δ 21.61 and 178.28 ppm, suggesting the presence of *O*-acetyl groups. This was confirmed by GC of the hydrolyzate, and the content of acetyl group was 0.2%. Further, the ^{13}C-NMR spectrum showed a signal due to an anomeric carbon of α-D-glucopyranose at δ 102.28 ppm.[11]

Table I. Methylation Analysis of Cnidirhan SI and LDSI

Methylated sugars (as alditol acetates)	Relative retention times[a]	Molar ratios		Structural features
		Cnidirhan SI	LDSI	
2,3,4,6-Me_4-D-glucose	1.00	7	5	Glc 1→
2,3,6-Me_3-D-glucose	1.49	21	28	→4 Glc 1→
2,6-Me_2-D-glucose	1.68	4	1	→4 Glc 1→ 3 ↑
2,3-Me_2-D-glucose	1.92	3	4	→6 Glc 1→ 4 ↑

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



cnidirhan SI, $a : b : (c + d + e) = 4 : 3 : 21$

LDSI, $a : b : (c + d + e) = 1 : 4 : 28$

Chart 1. Possible Structural Units of Cnidirhan SI and LDSI

The polysaccharide was degraded by treatment with α-amylase, followed by gel chromatography with Sephadex G-25. Seven fractions (*i.e.* frs. 1 to 7) were obtained, and the major part (frs. 4 to 7) was identified as a mixture of maltoheptaose, maltohexaose, maltopentaose and maltotetraose (fr. 4), and of maltotriose, maltose and glucose (frs. 5 to 7, respectively) by TLC and HPLC analysis.

On the other hand, treatment with β-amylase under dialysis, followed by gel chromatography with Toyopearl HW-55F, afforded a product designated as LDSI. It had $[\alpha]_D^{21}$ +116° ($c = 0.1$, H_2O). The gel chromatography gave a value of 1.07×10^4 for the molecular mass of LDSI.

Both cnidirhan SI and LDSI were separately methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[12] The methylated products obtained were hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS gave the results shown in Table I.

Based on the accumulated evidence described above, it can be concluded that cnidirhan SI and LDSI have the structural features shown in Chart 1. Cnidirhan SI appeared reddish violet with an iodine test.

The effects of cnidirhan SI and LDSI on the RES were demonstrated by a modification[5] of the *in vivo* carbon

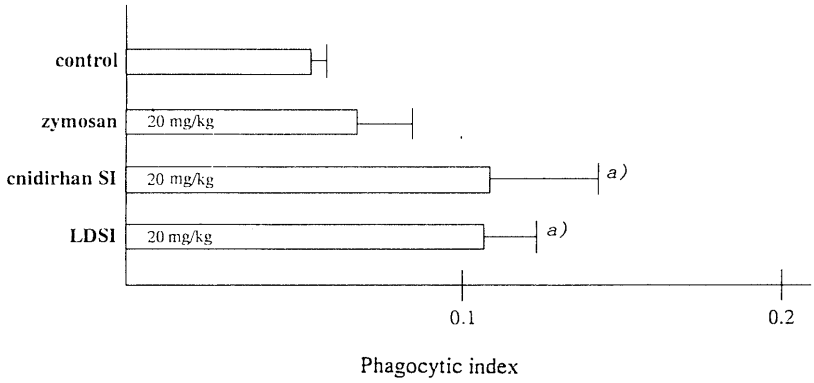


Fig. 1. Effects of Cnidirhan SI and LDSI on Phagocytosis

Significantly different from the control, *a*) $p<0.001$.

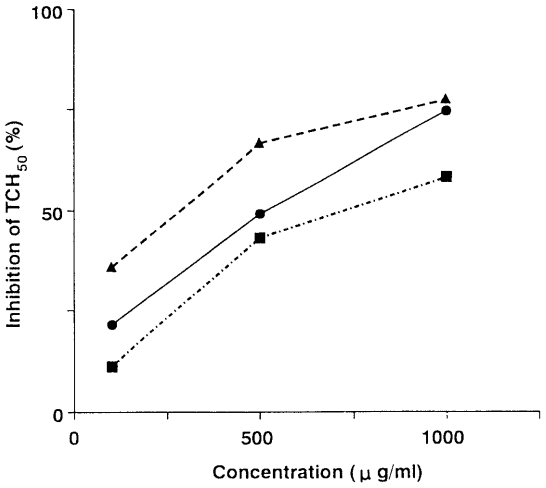


Fig. 2. Anti-complementary Activity of Cnidirhan SI and LDSI

Cnidirhan SI, —●—; LDSI, -·-■-·-; Plantago-mucilage A, ----▲---. Each point represents the mean ($n=3$).

The values ± S.D. were as follows:

	100 μg/ml	500 μg/ml	1000 μg/ml
cnidirhan SI	16.93 ± 1.46	45.31 ± 1.34	56.40 ± 0.41
LDSI	11.20 ± 2.48	35.81 ± 3.29	47.58 ± 0.66
plantago-muc. A	29.41 ± 0.47	50.29 ± 1.35	68.17 ± 0.85

clearance test.[13] As shown in Fig. 1, the phagocytic indices were significantly increased, suggesting activation of the RES by i.p. injection of these substances.

The anti-complementary activities of cnidirhan SI and LDSI are shown in Fig. 2. Cnidirhan SI showed potent activity, which is nearly equal to that of the positive control, Plantago-mucilage A, in a high concentration. The activity of LDSI was a little lower than that of cnidirhan SI.

Discussion

We earlier isolated an immunologically active polysaccharide, called cnidirhan AG, from the CTAB-precipitate fraction of the hot water extract of Cnidium rhizome.[1] Structural studies indicated that it is a typical α-1,5-linked L-arabino-β-3,6-branched D-galactan with terminal β-D-glucuronic acid units. We have now obtained a glucan, designated as cnidirhan 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, twenty-seven substances have been isolated and characterized as RES-activating polysaccharides. Among them, only five substances are neutral polysaccharides. Those are cinnaman AX from the bark of *Cinnamomum cassia*,[14] glycyrrhizan UC from the root of *Glycyrrhiza uralensis*,[15] MVS-I from the seed of *Malva verticillata*,[7] ukonan D from the rhizome of *Curcuma longa*,[11] and peonan SA from the root of *Paeonia lactiflora*.[16] Cinnaman AX is an arabinoxylan, while glycyrrhizan UC, MVS-I, ukonan D and peonan SA are glucose-rich heteropolysaccharides. MVS-I is a β-1,3-glucan with additional arabinogalactan units. In contrast, glycyrrhizan UC, ukonan D and peonan SA are rich in α-1,4-linked D-glucose residues with partially 4,6-branching. Glycyrrhizan UC is rich in arabinogalactan moieties as well. Similarly, ukonan D and peonan SA possess arabinogalactan units; however, the major parts in these polysaccharides are occupied by α-D-glucose residues.

Cnidirhan SI possesses α-D-glucose units as the sole component sugar. Thus, this substance is the first example of a branched α-glucan with phagocytosis-stimulating and anti-complementary activities. Both amylopectin and glycogen, as the ordinary 4,6-branching α-glucans, are completely inactive in the RES.[11] Cnidirhan SI has 3,4-branching points to a slightly greater extent than 4,6-branching. These two modes of branching in a glucan moiety were also observed in peonan SA to an equal extent. There is an average of about one branch for every five glucose units in cnidirhan SI. The degree of branching in this glucan is very high compared with those in amylopectin and glycogen. These factors may contribute to its activity. Cnidirhan SI showed the same reddish violet coloration ($\lambda_{max}=532.5$ nm) as that of amylopectin with an iodine test. So, it is conceivable that cnidirhan SI has relatively long α-1,4-linked side chains.

The treatment of the glucan with β-amylase afforded the product LDSI, and resulted in a decrease in both the value of molecular mass and the degree of branching. Although the immunological activities of LDSI were lowered, this product still maintained significant activity. A similar mode of branching in LDSI and cnidirhan SI may contribute to these activities.

The RES-potentiating and anti-complementary activi-

ties of cnidirhan AG[1)] are superior to those of cnidirhan SI. However, the yield of cnidirhan SI from the material rhizome is about 24 times that of cnidirhan AG. So it seems reasonable to assume that cnidirhan SI is a representative polysaccharide of the constituents of the rhizome of *Cnidium officinale*.

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