Characterization of a Novel Heteroglucan from the Rhizome of *Cnidium officinale* Exhibiting High Reticuloendothelial System-Potentiating and Anti-complementary Activities

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Received December 21, 1993; accepted February 22, 1994

A novel β -heteroglucan, called cnidirhan SIIA, was isolated from the rhizome of *Cnidium officinale* MAKINO. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was more than 1×10^7 . It was composed of D-glucose, D-galactose and L-arabinose in the molar ratio of 85:5:8, in addition to small numbers of O-acetyl groups. Methylation analysis and nuclear magnetic resonance studies indicated its main structural features composed of β -1,6-linked D-glucopyranosyl residues with both 3,6- and 4,6-branching points. In addition, it has both β -1,4-linked L-arabinopyranosyl and β -1,6-linked D-galactopyranosyl residues. The polysaccharide showed very pronounced reticuloendothelial system-potentiating activity in a carbon clearance test, as well as a marked anti-complementary activity.

Keywords heteroglucan; polysaccharide; structure; immunological activity; *Cnidium officinale*; cnidirhan SIIA

We recently isolated an acidic polysaccharide and a glucan from the rhizome of *Cnidium officinale* MAKINO, and elucidated the structural features of these polysaccharides, which had reticuloendothelial system (RES)-potentiating and anti-complementary activities, and called them cnidirhan AG and cnidirhan SI, respectively.^{1,2)} 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, while cnidirhan SI was isolated as a major neutral polysaccharide from this crude drug. The present paper describes the isolation, structural features, RES-potentiating and anti-complementary activities of a novel heteroglucan 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, 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 $\times$ 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 phosphate buffer, the column was eluted with the phosphate buffer containing 0.1 M NaCl (900 ml). Fractions of 20 ml were collected and analyzed by the

phenol-sulfuric acid method.³⁾ 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 $\times$ 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 were combined, dialyzed, concentrated and applied to a column (5 $\times$ 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 SIIA was obtained as a white powder. Yield, 14.5 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described previously⁴⁾ on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M Na₂B₄O₇ · 10 H₂O–0.1 N NaOH (10:1, pH 9.3). Cnidirhan SIIA gave a single spot at a distance of 128 mm from the origin in the direction of 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 $\times$ 97 cm) of Toyopearl HW-75F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. Fraction numbers of the peaks of both cnidirhan SIIA and native dextran (Tokyo Kasei Co.) were 44.

Component Sugar Analysis The sample was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 20 h in a sealed tube and analyzed by cellulose thin-layer chromatography (TLC),⁵⁾ then the product was reduced, acetylated and analyzed by gas chromatography (GC).⁶⁾ GC was carried out using a fused silica capillary column (0.53 mm i.d. $\times$ 15 m) of SP-2380 (Supelco Co.) on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of O-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC.⁷⁾ GC was performed using a column (3.2 mm i.d. $\times$ 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.

Methylation Analysis Methylation was carried out using powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.⁸⁾ The yield was 2.0 mg from 2.2 mg of cnidirhan SIIA. The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described previously.⁹⁾ 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. Relative retention times of the corresponding alditol acetates derived from 2,3,4,6-tetra-*O*-methyl-, 2,3,4-tri-*O*-methyl-, 2,4-di-*O*-methyl- and 2,3-di-*O*-methyl-D-glucoses, 2,3,4-tri-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-L-arabinose were 1.00, 1.43, 1.84, 1.92, 1.62 and 1.14, respectively. GC-MS was carried out with a JEOL JMS-DX 303 mass spectrometer.

Phagocytic Activity This was measured by an *in vivo* carbon clearance test as described previously.⁶⁾ The samples 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.¹⁰⁾ Gelatin-veronal-buffered saline (pH 7.4) containing 500 μ M Mg^{2+} and 150 μ M Ca^{2+} (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 (TCH₅₀) 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 TCH₅₀ 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 rhizome of *Cnidium officinale* was treated with CTAB and the supernatant was poured into ethanol. The precipitate was dialyzed and applied to a column chromatography of DEAE-Sepacel. 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 SIIA, was obtained from the high molecular weight fraction, followed by dialysis and gel chromatography with Sephadex G-25.

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

a) Number of residues. Glcp, glucopyranose; Arap, arabinopyranose; Galp, galactopyranose.

The polysaccharide gave a single spot on electrophoresis, and it gave a single peak on gel chromatography with Toyopearl HW-75F. The elution volume of the peak was the same as native dextran. Therefore, this result gave a value of more than 1×10^7 for the molecular mass of cnidirhan SIIA.¹²⁾ It had $[\alpha]_D^{24} - 180^\circ$ (H_2O , $c=0.1$).

Cnidirhan SIIA is composed of D-glucose, D-galactose and L-arabinose. Quantitative analysis showed that cnidirhan SIIA contained 88.0% glucose, 5.2% galactose and 6.8% arabinose. The molar ratio of these component sugars was 85:5:8.

The carbon-13 NMR (¹³C-NMR) spectrum of cnidirhan SIIA showed signals at δ 21.78 and 178.89 ppm, suggesting the presence of *O*-acetyl groups. This was confirmed by GC of the hydrolyzate, and the content of acetyl groups was 0.8%. Further, the ¹³C-NMR spectrum showed three signals due to anomeric carbons at δ 100.42, 106.21 and 106.88 ppm. The first and second were assigned to the anomeric carbons of β -L-arabinopyranose and β -D-galactopyranose, respectively.^{13,14)} The signal at δ 106.88 ppm was assigned to the anomeric carbon of β -D-glucopyranose.¹⁴⁾ This conclusion was supported by the corresponding signal strengths.

Cnidirhan SIIA was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹⁵⁾ The methylated product thus obtained was hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS revealed derivatives of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-glucose, 2,4-di-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-L-arabinose as the products in the molar ratio of 8:84:6:3:4:6.

These results suggested that the minimal unit of cnidirhan SIIA was composed of six kinds of component sugar unit as shown in Chart 1. The ratios of the galactose and arabinose units were corrected following the result of the quantitative analysis.

The effect of cnidirhan SIIA on the RES was demonstrated by a modification⁶⁾ of the *in vivo* carbon clearance test¹⁶⁾ 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.

The anti-complementary activity of cnidirhan SIIA is shown in Fig. 2. This polysaccharide exhibited a very marked activity compared with the positive control, Plantago-mucilage A.

Fig. 1. Effects of Cnidirhan SIIA on Phagocytosis

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

Fig. 2. Anti-complementary Activity of Cnidirhan SIIA

Cnidirhan SIIA, —□—; Plantago-mucilage A, ---▲---. Each point represents the mean ($n=3$).

DISCUSSION

During our studies to date on the immunologically active polysaccharides in crude drugs obtained from various plant sources, we have isolated thirty-two substances which are RES-activating polysaccharides and elucidated their structural features. Among them, acidic arabino-galactans form the major group. In addition, six are neutral polysaccharides: cinnaman AX from the bark of *Cinnamomum cassia*,¹⁷⁾ glycyrrhizan UC from the root of *Glycyrrhiza uralensis*,¹⁸⁾ MVS-I from the seed of *Malva verticillata*,⁸⁾ ukonan D from the rhizome of *Curcuma longa*,¹⁹⁾ peonan SA from the root of *Paeonia lactiflora*,²⁰⁾ and cnidirhan SI from the rhizome of *Cnidium officinale*.²⁾ Cinnaman AX is an arabinoxylan, and cnidirhan SI is a glucan with a highly-branched structure. Glycyrrhizan UC, ukonan D and peonan SA belong to the arabino-galacto-glucan group, although glycyrrhizan UC and ukonan D contain small amounts of L-rhamnose and D-mannose residues, respectively. These three polysaccharides have α -L-arabino- β -3,6-D-galactan and α -4,6-D-glucan moieties in common as their main components.

MVS-I also possesses both α -1,5-linked L-arabinofuranosyl and β -3,6-branched D-galactosyl units. However, the major part of this polysaccharide consists of β -1,3-linked D-glucose units with some dispersed β -1,3-

linked D-galactose units. In contrast to MVS-I, the major part of cnidirhan SIIA consists of β -1,6-linked D-glucose residues with partial 3,6- and 4,6-branching. In addition, this polysaccharide has unique β -1,4-linked L-arabinopyranose and β -1,6-linked D-galactose units. Thus, of the known RES-active polysaccharides, cnidirhan SIIA has particularly characteristic structural features.

It is very interesting that both the RES-potentiating and the anti-complementary activities of cnidirhan SIIA are much greater, even at very low doses, than those of the other immunologically active polysaccharides obtained by us.

Further investigation of the relationship between the structural features and biological activities is in progress.

Acknowledgement This work was supported in part by the Sasagawa Scientific Research Grant.

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