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Further Studies on the Structure of Polysaccharides from the Bark of *Melia azadirachta*

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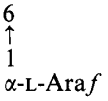
Water-soluble polysaccharides, designated as GIIa and GIIIa, were isolated together with GIa and GIb from the bark of *Melia azadirachta* (Meliaceae), and their anti-inflammatory effect on carrageenan-induced edema was tested. GIIa and GIIIa each gave a single peak on high-performance liquid chromatography and gel filtration. Methylation, periodate oxidation and carbon-13 nuclear magnetic resonance spectroscopic studies suggested that GIIa is composed of the following repeating unit: α-D-Glcl→4 α-D-Glcl→3 α-D-Glcl→3 α-D-Glc6←1 α-L-Ara*f* GIIIa is



a branched arabinofucoglucan containing a main chain of 1→4-linked α-D-glucopyranosyl units substituted in the 6 position with side chains of α-L-arabinofuranose and β-L-fucopyranose.

Keywords——*Melia azadirachta*; heteropolysaccharide; anti-inflammatory; carrageenan-induced edema; methylation analysis; ^{13}C-NMR

We have previously reported[1] a structural investigation on the water-soluble polysaccharides, designated as GIa and GIb, isolated from the bark of *Melia azadirachta* (Meliaceae). GIa is a α-1→4-D-glucan with one α-1→6-L-arabinofuranosyl group for every five glucose residues, and GIb is a branched arabinofucoglucan. GIa and GIb showed a strong antitumor effect against Sarcoma-180.

In the present work, we examined the structures of the polysaccharides designated as GIIa and GIIIa (isolated together with GIa and GIb) and their anti-inflammatory activities. These polysaccharides exhibited significant anti-inflammatory effect on carrageenan-induced edema. There have been several publications[2] on the anti-inflammatory activity of polysaccharide preparations.

The crude polysaccharide fraction, which was obtained by adding ethanol to the non-dialyzable fraction of the hot water extract, was chromatographed on a column of Sephadex G-100 to give three fractions. The fractions GI, GII, and GIII were collected separately and lyophilized (GI 40%, GII 20%, GIII 40% yield). The GII fraction was further purified by Sephadex G-50 column chromatography to give an elution pattern consisting of three fractions (GIIa, GIIb, GIIc), as shown in Fig. 1. The GIII fraction was also purified by Sephadex LH-20 column chromatography to give an elution pattern consisting of three fractions (GIIIa, GIIIb, GIIIc) as shown in Fig. 2. GIIa and GIIIa each yielded a single peak on high-performance liquid chromatography (HPLC), gel filtration and sedimentation analysis. Furthermore, they each gave a single spot on glass-fiber electrophoresis.

Polysaccharide preparations were subjected to bioassay to investigate their inhibitory effects on carrageenan-induced hind foot edema in mice according to the method reported in our previous paper.[3] The results are shown in Table I; the inner part of the dialysate, GII and

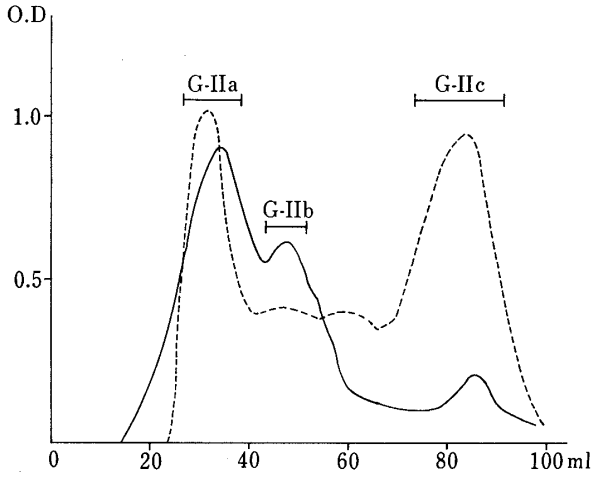


Fig. 1. Gel Filtration on a Sephadex G-50 Column

——, 620 nm; – – – –, 280 nm.

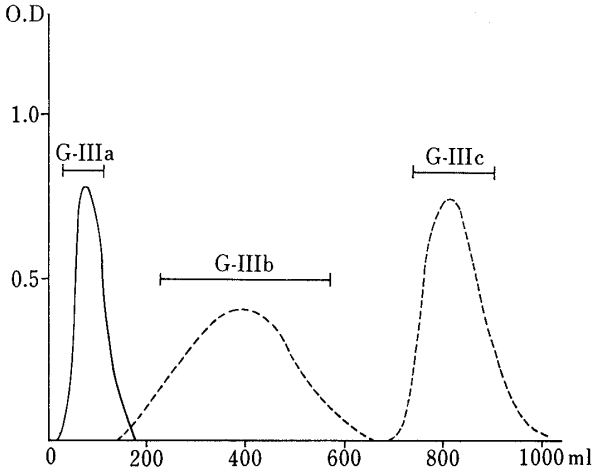


Fig. 2. Sephadex LH-20 Column Chromatography of G-III

——, 620 nm; – – – –, 280 nm.

TABLE I. Inhibitory Effects of Polysaccharides on the Swelling of Mouse Hind Paw Induced by Carrageenan

	Dose (mg/kg)	Inhibition (%)
Prednisolone	25.0	45.4[d]
Inner	50.0	40.2[d]
Outer	50.0	12.5[a]
G-I	50.0	0.00
G-II	50.0	41.5[d]
G-III	50.0	54.5[d]
Prednisolone	25.0	67.9[d]
G-IIa	50.0	47.8[d]
	25.0	37.8[c]
Prednisolone	25.0	47.7[d]
G-IIIa	50.0	31.1[b]
G-IIIb	50.0	12.9[a]
G-IIIc	50.0	11.4
Prednisolone	25.0	52.9[d]
G-IIIa	10.0	31.1[b]
	2.00	41.3[d]
	0.40	38.4[c]

Significantly different from the control: *a*) $p<0.05$, *b*) $p<0.025$, *c*) $p<0.005$, *d*) $p<0.001$.
λ-Carrageenan (1.0%, 0.05 ml), average of 6 male mice.

GIII at doses of 50 mg/kg *p.o.* produced 40.2, 41.5 and 54.5% decreases respectively, in the foot edema ($p<0.001$) at 5 h after the induction of the edema. On the other hand, the outer part of the dialysate and GI were ineffective. GIIIa at the dose of 2 mg/kg *p.o.* produced a 41.3% decrease ($p<0.001$).

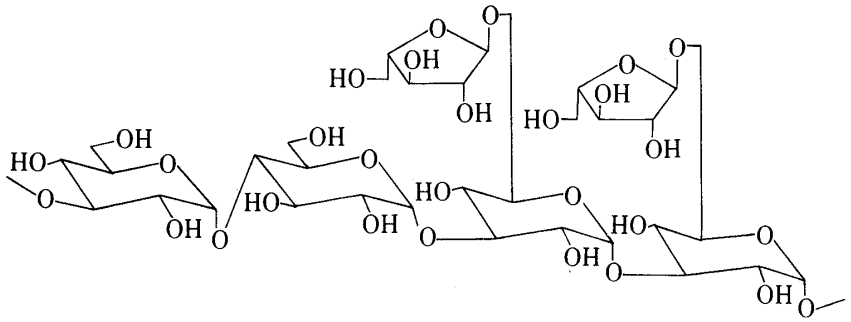
The component sugars of GIIa were identified as glucose and arabinose by thin layer and gas liquid chromatographies (TLC and GLC) of the acid hydrolysate, and their molar ratio was estimated to be approximately 2 : 1 by GLC, while GIIIa was found to consist of glucose, arabinose and fucose in the ratio of 6 : 1 : 1 by GLC. In the ^{13}C nuclear magnetic resonance

TABLE II. ^{13}C-Chemical Shifts of G-IIa Examined in D_2O

Carbon assignment	Chemical shifts (δ)	
	G-IIa	References
Arabinose		Methyl α-L-arabinofuranoside
C-1	109.2	109.3
C-2	82.9	81.9
C-3	78.3	77.5
C-4	85.7	84.9
C-5	62.8	62.4
Glucose (1→3)		α-(1→3)Glucan
C-1	101.2	101.3
C-2	72.3	72.7
C-3	83.9	83.2
C-4	71.8	71.7
C-5	73.3	73.7
C-6	62.3	62.2
Glucose (1→4)		Amylose
C-1	100.6	100.9
C-2	73.0	72.7
C-3	74.4	74.5
C-4	78.5	78.4
C-5	72.3	72.4
C-6	62.2	61.8
Glucose (1→3, 1→6)		
C-1	99.4	
C-6	68.6	

(^{13}C-NMR) spectrum of GIIa, all of the carbon lines were resolved, and their chemical shifts are recorded in Table II. By comparison of the GIIa spectrum with those of amylose (α-1→4 glucan),[4] α-(1→3)glucan[4] and methyl α-L-arabinofuranoside,[5] complete assignment of the GIIa resonances was possible. As the chemical shift for C-1 in α-(1→3)glucan is δ 101.3 ppm, the signal at 101.2 ppm might be due to C-1 linked α-1→3. The resonance at 100.6 ppm is clearly due to C-1 linked α-1→4. The C-1 resonance of 3,6-di-*O*-substituted residues at 99.4 ppm shows a β-shift of -1.8 ppm compared with that of 3-*O*-substituted residues. Accordingly, the peaks at 83.9, 78.5 and 68.6 ppm are attributed to C-3 in an α-1→3 link, C-4 in an α-1→4 link and C-6 in an α-1→6 link, respectively, located in glycosidic bonds. The resonances at 109.2, 82.9, 78.3, 85.7 and 62.8 ppm are attributable to arabinofuranose by comparison with the values for methyl α-L-arabinofuranoside. GLC of the methanolysis products of GIIa methyl ether prepared by the modified Hakomori method[6] revealed the liberation of methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,4,6-tri-*O*-methyl-glucopyranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside and methyl 2,4-di-*O*-methyl-glucopyranoside in a 2:1:1:2 ratio (these products were identified by comparison with authentic samples). When GIIa was subjected to periodate oxidation, 0.48 mol of periodate per anhydro sugar unit was consumed. Periodate oxidation followed by reduction and acid hydrolysis[7] gave 0.34 mol of glycerol, 0.34 mol of glycolaldehyde and 0.16 mol of nigerotriosyl-erythritol, in good agreement with the methylation data. Based on the above

GIIa



GIIIa

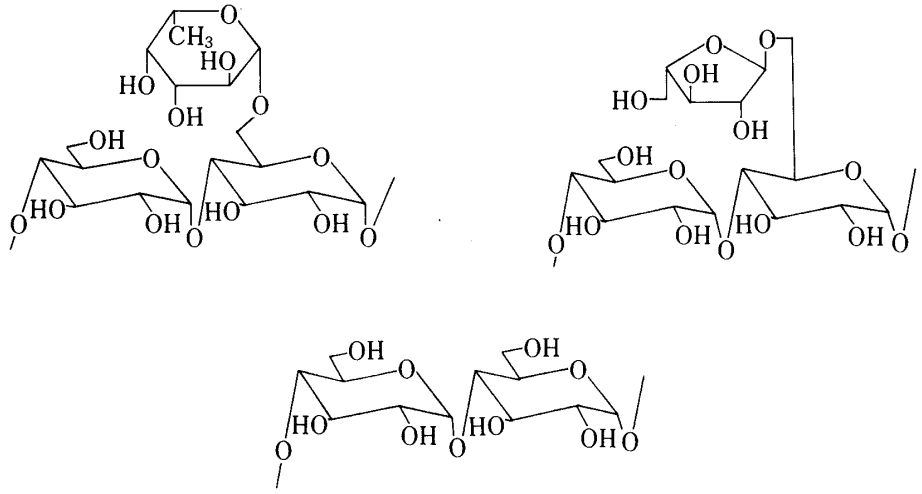


Chart 1. Structures of GIIa and GIIIa

evidence, it is proposed that GIIa is composed of repeating units of a hexasaccharide having the following structure (Chart 1). The molecular weight of GIIa, based on estimation of reducing end groups by the Park–Johnson method[8] was determined to be about 8400. On the other hand, in the ^{13}C-NMR spectrum of GIIIa, as shown in Table III, many of the carbon lines are very similar to those of GIa except for signals based on fucose.[1] The signal at 101.5 ppm might be due to C-1 linked α-1→4. The resonance at 99.6 ppm is clearly due to C-1 of an α-1→6 link by comparison with the data for α-1→6-glucan and glycogen. The resonances at 105.1, 72.0, 74.5, 73.2, 71.3 and 18.3 ppm are attributable to a fucopyranose by comparison with the values for methyl β-L-fucopyranoside.[5] GLC of the methanolysis products of GIIIa methyl ether prepared by the modified Hakomori method revealed the liberation of methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,3,4-tri-*O*-methyl-fucopyranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside and methyl 2,3-di-*O*-methyl-glucopyranoside in a 1 : 1 : 4 : 2 ratio. These products were identified by comparison with authentic samples. On periodate oxidation of GIIIa, the consumption of periodate was 1.13 mol per anhydro sugar unit. Smith degradation of GIIIa gave 0.79 mol of erythritol, 0.98 mol of glycolaldehyde and 0.18 mol of 1,2-propanediol. The analytical data mentioned earlier indicate that GIIIa has a structure that contains a main chain consisting principally of (1→4)-linked α-D-glucopyranosyl units substituted in some of the 6-positions by side chains of α-L-arabinofuranose and β-L-fucopyranose. The molecular weight of GIIIa was determined to be about 8000 by the same method as used in the case of GIIa.

A few papers have suggested that oral administration of polysaccharides is ineffective for anti-inflammatory activity, but the GIIa and GIIIa were effective after oral administration.[2] However, the relationship between the structure and the biological activity is not well understood.

TABLE III. ^{13}C-Chemical Shifts of G-IIIa and G-Ia Examined in D_2O

Carbon assignment	Chemical shifts (δ)		
	G-IIIa	G-Ia	References
Arabinose			Methyl α-L-arabinofuranoside
C-1	109.3	108.9	109.3
C-2	82.7	82.4	81.9
C-3	78.3	78.1	77.5
C-4	85.8	85.5	84.9
C-5	63.0	62.7	62.4
Glucose (1→4)			Amylose
C-1	101.5	101.1	100.9
C-2	73.2	72.9	72.7
C-3	75.0	74.8	74.5
C-4	78.5	78.7	78.4
C-5	72.6	72.9	72.4
C-6	62.5	62.1	61.8
Glucose (1→4, 1→6)			Glycogen
C-1	99.6	99.2	99.3
C-6	68.8	67.3	67.8
Fucose			Methyl β-L-fucopyranoside
C-1	105.1		105.8
C-2	72.0		72.0
C-3	74.5		75.3
C-4	73.2		72.6
C-5	71.3		71.3
C-6	18.3		17.1

Experimental

Optical rotations were measured with a JASCO DIP-4 digital polarimeter. The infrared (IR) spectra were measured with a JASCO IRA-2 spectrometer. Gas liquid chromatographic analyses were carried out with a Shimadzu GC-6A gas chromatograph equipped with a hydrogen flame ionization detector. Sedimentation analysis was performed at 58000 rpm with a Beckman Spinco model E ultracentrifuge equipped with a schlieren optical system. The ^{13}C-NMR spectra were obtained with a JEOL FX-100 spectrometer operating at 25.0 MHz in the pulsed Fourier-transform mode. Free-induction decays were accumulated with a 45° pulse. All spectra were recorded in D_2O at 70 °C by using 8000 data points and a spectral width of 5 KHz. ^{13}C-Chemical shifts are expressed in ppm downfield from external tetramethylsilane. High-voltage paper electrophoresis was performed on Whatman GF 83 glass fiber paper at 50 V/cm, using 0.1 M borate buffer, pH 9.3. The spots were detected by spraying ammonium vanadate-H_2SO_4 reagent and heating the paper 110 °C. HPLC was carried out on a Shimadzu LC-5A instrument equipped with a RI detector. The eluent (H_2O) flow rate was 1 ml/min at 40 °C on a column of G 2000SW.

Isolation and Purification——Finely powdered bark of *Melia azadirachta* (750 g) was extracted with benzene and then with methanol in order to remove soluble components, and the residue was extracted further with distilled water on a boiling water bath. Ethanol was added to the hot filtered extracts to form precipitates, which were collected by centrifugation, washed with ethanol and ether, and dried to obtain a pale brownish water-soluble powder (47.6 g). The crude polysaccharide was dialyzed. The non-dialyzable fraction (14.3 g) was chromatographed on a column of Sephadex G-100, and elution provided three fractions (GI 5.7 g, GII 2.8 g, GIII 5.7 g). GII (2 g) was further purified by Sephadex G-50 column chromatography (4.5 × 60 cm) to give three fractions (GIIa 940 mg, GIIb 232 mg, GIIc 452 mg) as shown in Fig. 1. Fractions of 5 ml were collected and analyzed by the anthrone method.[9] GIIa; $[\alpha]_D^{27}$ +54.7° (c=0.25, H_2O). GIII (900 mg) was also further purified by Sephadex LH-20 column chromatography (2.6 × 100 cm) to give three fractions (GIIIa 26.4 mg, GIIIb 229.8 mg, GIIIc 409.2 mg). Fractions of 10 ml were

collected and analyzed by the anthrone method. GIIIa; $[\alpha]_D^{27}$ +87.4° (c=0.1, H_2O). GIa and GIb were further purified by Biogel P-200 column chromatography (2.6×100 cm), GIa; $[\alpha]_D^{25}$ +136.0° (c=0.5, H_2O), GIb; $[\alpha]_D^{25}$ +143.7° (c=0.5, H_2O). The data given in the previous paper[1] were incorrect.

Anti-inflammatory Activity on Carrageenan-Induced Hind Foot Edema in Mice——Male mice of ddy strain, 5 weeks old and weighing 18—22 g, were used as experimental animals and foot edema was induced with carrageenan according to the method reported in our previous paper.[3] Groups of five mice were used. λ-Carrageenan (1.0% in physiological solution, 0.05 ml) was injected *s.c.* under the plantar surface of the right hind paw at 1 h after oral administration of a test drug. The polysaccharides were dissolved in water. Increase in the foot volume was measured as described previously and expressed as percent of the foot volume measured before the injection of carrageenan. Anti-edema effects of the test drugs were expressed in terms of percent inhibition of the foot edema in the drug-treated group compared with the foot edema in the control group treated with the vehicle.

Sugar Components of GIIa and GIIIa——GIIa and GIIIa were hydrolyzed with 1 N H_2SO_4 for 8 h at 90 °C. The hydrolysate was neutralized with ion-exchange resin (IR-45) and concentrated. Trimethylsilylation followed by GLC [2% OV-17 on Chromosorb WAN-DMCS (3 mm×2 m); column temperature 160 °C; N_2 flow rate 50 ml min^{-1}] showed the presence of arabinose and glucose in a ratio of 1 : 2. In the case of GIIIa, arabinose, fucose and glucose were identified in a ratio of 1 : 1 : 6. Relative retention times: arabinose, 0.28, 0.33, 0.38; fucose, 0.33, 0.38, 0.45; glucose, 1.00, 1.57.

Permethylation of GIIa and GIIIa——1,1,3,3-Tetramethylurea (2 ml) and methylsulfinyl carbanion solution (4 ml) were added to a solution of GIIa (50 mg) in a mixture of dimethyl sulfoxide (2 ml) and after 30 min, a large excess of methyl iodide (6 ml) was added dropwise to the mixture under vigorous stirring, with cooling to keep the reaction temperature below 50 °C. When the methylation was completed, the mixture was diluted with water, and dialyzed against running water to remove the excess of methyl iodide and tetramethylurea, as well as the sodium iodide formed. The dialysate was extracted with chloroform, and the extracts were washed with water, dried, and evaporated. The product showed no hydroxyl absorption in the IR spectrum. GIIIa (30 mg) was permethylated by the same procedures.

Methylation Analysis——The methylated product (10 mg) was methanolyzed with 5% HCl–MeOH (2 ml) in a sealed ampoule at 100 °C for 5 h. The resulting methyl glycosides were analyzed by GLC [10% DEGS on Chromosorb W (3 mm×2 m); column temperature 170 °C; N_2 flow rate 50 ml min^{-1}]. In the sample from GIIa, methyl 2,3,5-tri-*O*-methyl-arabinofuranoside (0.57), methyl 2,4,6-tri-*O*-methyl-α,β-D-glucopyranosides (3.32, 4.89), methyl 2,3,6-tri-*O*-methyl-α,β-D-glucopyranosides (3.51, 4.78) and methyl 2,4-di-*O*-methyl-α,β-D-glucopyranosides (4.92, 6.97) were identified by comparison with authentic samples, and were present in a ratio of 2 : 1 : 1 : 2. The relative retention times of the products are given with respect to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. In the sample from GIIIa, methyl 2,3,5-tri-*O*-methyl-arabinofuranoside (0.57), methyl 2,3,4-tri-*O*-methyl-L-fucopyranoside (0.73), methyl 2,3,6-tri-*O*-methyl-α,β-D-glucopyranosides (3.51, 4.78) and methyl 2,3-di-*O*-methyl-α,β-D-glucopyranosides (4.97, 7.02) were identified, and were present in a ratio of 1 : 1 : 4 : 2.

Periodate Oxidation, Mild Smith Degradation and Analysis of Products——GIIa (50 mg) was added to a solution of 0.02 M sodium periodate (20 ml). Oxidation was carried out in the dark at 7 °C. Aliquots (1 ml) were removed from the solution at intervals for estimation of their iodate.[10] When the oxidation was complete (after 48 h), the oxidized GIIa was reduced with sodium borohydride (20 mg) then hydrolyzed with 0.1 N sulfuric acid (5 ml) at room temperature for 3 h. Glycerol, glycolaldehyde and nigerotriosyl-erythritol were identified as their trimethylsilylated derivatives by GLC [3% ECNSS-M on Gaschrom Q, column temperature 190 °C; N_2 flow rate 40 ml min^{-1}]. In the sample from GIIIa (40 mg), glycolaldehyde, erythritol and 1,2-propanediol were identified.

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