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

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Water-soluble polysaccharides, designated as GIa and GIb, were isolated from the bark of *Melia azadirachta* (Meliaceae) and their inhibiting effect on subcutaneously inoculated Sarcoma-180 was tested. GIa and GIb each gave a single peak on ultracentrifugation and gel filtration. Methylation, periodate oxidation and ^{13}C-NMR spectroscopic studies suggested that GIa is composed of the following repeating unit; α-D-Glc 1→4α-D-Glc 1→4α-D-Glc 1→4α-D-Glc 1→4α-D-Glc 6←1α-L-Araf. GIb 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-arabinofuranosyl units. 3-*O*-substituted fucopyranose is attached to the α-(1→4) glucose units at the 4 position in the main chain.

Keywords——*Melia azadirachta*; heteropolysaccharide; antitumor activity; Sarcoma-180; acetolysis; methanolysis; ^{13}C-NMR

Melia azadirachta, commonly known as Indian Lilac, is an evergreen tree belonging to the Meliaceae family. The bark, leaves and fruits have been used in the Ayurvedic system of medicine from very ancient times and are mentioned in most of the ancient Sanskrit medicinal writings, such as the Susrutasanhita. The bark is regarded as a bitter, tonic, astringent and useful in fever, thirst, nausea, vomiting and skin diseases.[1)]

As a part of our structural studies on the polysaccharides, a water-soluble polysaccharide component of the neem gum, a typical plant gum exudate from the tree, was investigated.[2)]

We examined the structure of polysaccharides extracted from the bark of *Melia azadirachta* and their inhibiting effect was tested on subcutaneously implanted Sarcoma-180. The crude polysaccharide fraction, which was obtained by adding ethanol to a non-dialyzable fraction of the hot water extract, was chromatographed on a column of Sephadex G-100 to give three fractions (GI, GII, GIII) as shown in Fig. 1. The fractions GI, GII, and GIII were collected separately and lyophilized (GI 40%, GII 20%, GIII 40% yield).

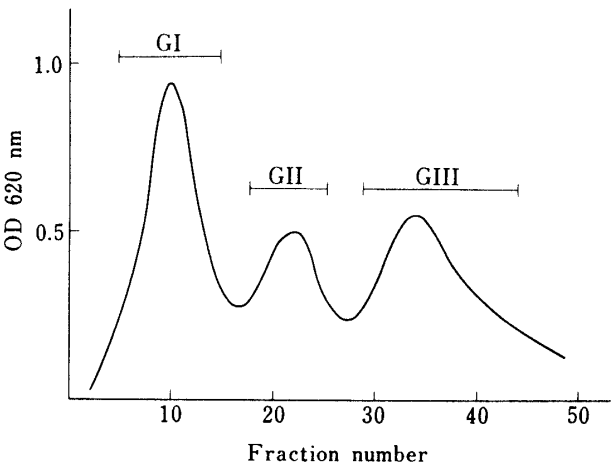

Fig. 1. Gel Filtration on a Sephadex G-100 Column

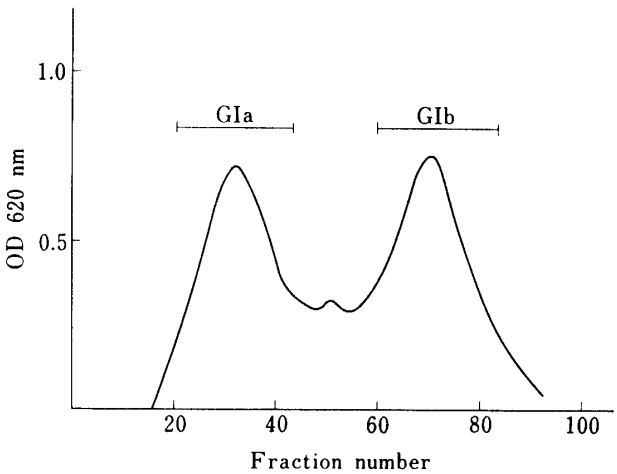

Fig. 2. Sephadex G-200 Column Chromatography of GI

The GI fraction was further purified by Sephadex G-200 column chromatography to give an elution pattern consisting of two fractions (GIa, GIb) as shown in Fig. 2. Each fraction yielded a single peak on ultracentrifugation and gave a single spot on glass-fiber paper electrophoresis. The component sugars of GIa 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 5: 1 by GLC, while GIb was found to consist of glucose, arabinose and fucose in the ratio of 5: 2: 1 by GLC. Both heteropolysaccharides showed a greenish-blue color reaction in the iodine test. In the ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectrum of GIa, all of the carbon lines were resolved, and their chemical shifts are recorded in Table I. By comparison of the GIa spectra with those of amylose (α-1→4 glucan),[3] maltotriose[3] and methyl α-L-arabinofuranoside,[4] a complete assignment of GIa resonances could be made. As the chemical shifts for C-1 in maltose and maltotriose are δ 101.1 and 101.0 ppm, respectively,[3] the signal at 101.1 ppm might be regarded as due to C-1 linked α-1→4. The resonance at 99.2 ppm is clearly due to C-1 of an α-1→6 link by comparison with the data for isomaltose,[3] the α-1→6 glucan[3] and glycogen.[3] Accordingly, the peaks at 78.7 and 67.3 ppm are attributed to 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 108.9, 82.4, 78.1, 85.5 and 62.7 ppm are attributable to an arabinofuranose by comparison with the values for methyl α-L-arabinofuranoside. The remaining resonances in the intermediate field range can be assigned by comparison with the data for amylose and the α-1→6 glucan.

Table I. ^{13}C-NMR Resonances in the 100-MHz Spectra of Polysaccharide Preparations in D_2O[a]

Carbon assignment	Chemical shift (δ)[b]			References
	GIa	GIb		
Arabinose				Methyl α-L-arabinofuranoside[4]
C-1	108.9	109.1	108.7	109.3
2	82.4	82.9	82.5	81.9
3	78.1	78.3	78.3	77.5
4	85.5	85.6	85.6	84.9
5	62.7	62.3	62.3	62.4
Glucose (1→4)				Amylose [3]
C-1	101.1	101.2		100.9
2	72.9	73.2		72.7
3	74.8	74.9		74.5
4	78.7	78.9		78.4
5	72.9	73.2		72.4
6	62.1	62.7		61.8
Glucose (1→4) (1→6)				Glycogen[3]
C-1	99.2	99.4		99.3
6	67.3	67.4		67.8
Fucose				Methyl β-L-fucopyranoside[4,c]
C-1		105.0		105.8
2		72.0		72.0
3		83.9		75.3
4		73.2		72.6
5		71.1		71.3
6		18.2		17.1

a) Measured at 70°C.
b) In ppm relative to a tetramethylsilane external standard.
c) For signal assignments in pyridine-d_5.

Gas liquid chromatography of the methanolysis products of GIa methyl ether prepared by the Hakomori method[5] and the Kuhn procedure[6] revealed the liberation of methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside and methyl 2,3-di-*O*-methyl-glucopyranoside in a 1 : 4 : 1 ratio; these products were identified by comparison with authentic samples.

The acetolysis of GIa afforded arabinose tetraacetate, glucose pentaacetate, maltose octaacetate and maltotriose undecaacetate, which were identified by comparison with authentic samples (TLC and ^{13}C-NMR spectra). Enzymic treatment of GIa with α-amylase gave glucose, maltose, maltotriose and *O*-α-D-glucopyranosyl-(1→4)-*O*-[α-L-arabinofuranosyl-(1→6)]-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose, which were separated by droplet counter-current chromatography and identified by ^{13}C-NMR spectroscopy. When GIa was subjected to periodate oxidation, 1.04 mol of periodate per anhydro sugar unit was consumed. Periodate oxidation followed by reduction and acid hydrolysis gave 0.19 mol of glycerol, 0.86 mol of erythritol and 1.2 mol of glycolaldehyde, in good agreement with the methylation data.

Based on the above evidence, it is proposed that GIa is composed of repeating units of a hexasaccharide having the following structure (Chart 1). The molecular weight of GIa, based on estimation of reducing end groups by the Park–Johnson method[7] and by gel filtration (Fig. 3) was determined to be about 94000.

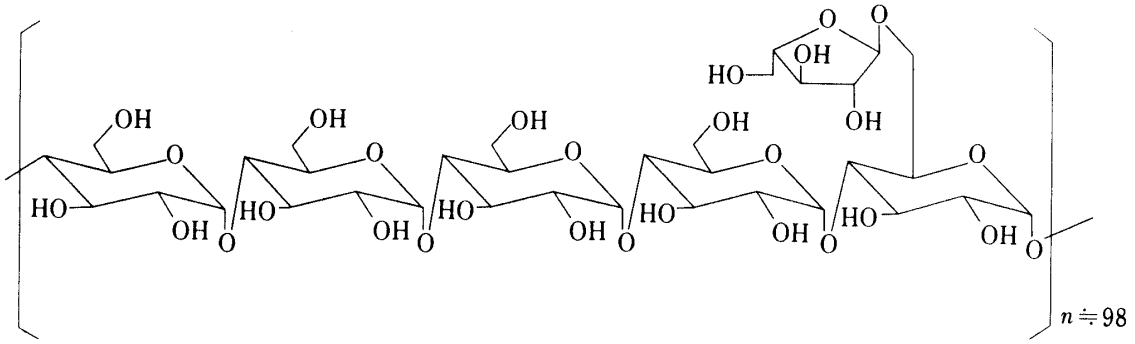

Chart 1. Structure of GIa

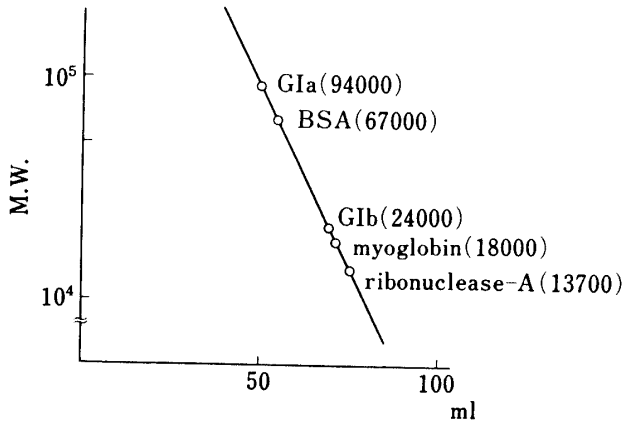

Fig. 3. Determination of the Molecular Weights of GIa and GIb by Gel Filtration (Sephadex G-200)

On the other hand, in the ^{13}C-NMR spectrum of GIb, as shown in Table I, many of the carbon lines are very similar to those of GIa. The signals based on the 3 position linked fucose are new. Gas liquid chromatography of the methanolysis products of GIb methyl ether prepared by the Hakomori method as well as the Kuhn method revealed the liberation of

methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside, methyl 2,4-di-*O*-methyl-fucopyranoside and methyl 2,3-di-*O*-methyl-glucopyranoside in a 2:3:1:2 ratio; these products were identified by comparison with authentic samples. On periodate oxidation of GIb, the consumption of periodate was 1.08 mol per anhydro sugar unit. Smith degradation[8] of GIb gave 0.24 mol of glycerol, 0.88 mol of erythritol, 0.85 mol of glycolaldehyde and 0.13 mol of fucosylerythritol.

The analytical data mentioned earlier indicate that GIb 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. Clearly 3-*O*-substituted fucopyranose moieties are attached to the α-(1→4) glucose units at the 4 positions in the main chain. The molecular weight of GIb was determined to be about 24000 by the same methods as used in the case of GIa.

The antitumor activities of fractions GIa and GIb are shown in Tables II and III. Hitherto, certain polysaccharides derived from other natural sources,[9] such as higher plants, fungi, yeasts, and lichens, have been shown to inhibit the growth of transplanted tumors.

Table II. Antitumor Activities of Polysaccharide Preparations given for 4 d from the Day after Subcutaneous Inoculation of Sarcoma-180 Cells

Sample	Dose (mg/kg × d)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression[a]
GI	50 × 4	1.00	74	1/6
	25 × 4	1.32	66	1/6
Control	—	3.90	—	0/6
GIa	50 × 4	0.21	94	3/6
	25 × 4	1.57	54	1/6
	5 × 4	1.10	68	1/6
GIb	50 × 4	0.28	92	3/6
	25 × 4	1.32	61	0/6
	5 × 4	3.04	11	0/6
Control	—	3.42	—	0/6
GII	50 × 4	3.57	10	0/6
GIII	50 × 4	2.38	40	0/6
Control	—	3.96	—	0/6

a) No. of tumor free mice/No. of mice treated.

Table III. Antitumor Activities of Polysaccharide Preparations given for 5 d from 10 d after Subcutaneous Inoculation of Sarcoma-180 Cells

Sample	Dose (mg/kg × d)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression[a]
GI	50 × 5	0.83	86	3/6
	25 × 5	1.98	67	2/6
	10 × 5	2.34	61	1/6
GIa	50 × 5	0.80	86	1/7
	25 × 5	0.66	89	3/7
	10 × 5	0.96	84	1/5
GIb	50 × 5	0.93	84	1/7
	25 × 5	0.99	83	3/7
	10 × 5	3.02	49	0/7
Control	—	5.94	—	0/7

a) No. of tumor free mice/No. of mice treated.

During observation for 3 weeks, GIa and GIb showed a strong antitumor effect with many complete regressions of the tumors when given to mice at daily dose of 50 mg/kg for 4 d from 24 h after subcutaneous inoculation of Sarcoma-180 cells (Table I). GII and GIII were inactive. Table III shows the curative effects of the polysaccharide preparations. According to the method described by Hamuro *et al.*,[10] the test samples were intraperitoneally injected once daily for 5 d, starting 10 d after tumor implantation. The antitumor activity of GIa and GIb is obvious, and seems to be higher than when the samples were administered starting 24 h after tumor implantation. Even at lower doses, the antitumor activity of GIa seems to be higher than that of GIb. The mechanism of action of these polysaccharides is not clear yet, but the results show that GIa and/or GIb have no direct cytocidal effect on Sarcoma-180 cells *in vitro*.

Experimental

Optical rotations were recorded with a Union Giken PM-201 automatic digital polarimeter. The infrared (IR) spectra were measured with a Japan Spectroscopic Co. Model IRA-2 spectrometer. Gas liquid chromatographic analyses were carried out with a Shimadzu Model GC-6A gas chromatograph attached with a hydrogen flame 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 electrophoreses were performed on Whatman glass fiber GF 83 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 at 110°C.

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) as shown in Fig. 1. GI fraction (570 mg) was further purified by Sephadex G-200 column chromatography to give two fractions (GIa 280 mg, GIb 280 mg) as shown in Fig. 2. GIa; $[\alpha]_D^{22}$ −35.0° (c=0.4, H_2O), GIb; $[\alpha]_D^{22}$ −46.0° (c=0.28, H_2O).

Sugar Components of GIa and GIb——GIa and GIb 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 the ratio 1: 5. In the case of GIb, arabinose, glucose and fucose were identified in the ratio 2: 5: 1.

Permethylation of GIa and GIb——A solution (2 ml) of methylsulfinyl carbanion was added to a stirred solution of GIa (50 mg) in dimethyl sulfoxide (5 ml) under argon gas, and the mixture was stirred at room temperature for 5 h. The mixture was poured into ice-water and extracted with chloroform. The organic layer was washed with water and concentrated. The residue was methylated by Kuhn's method using dimethylformamide (5 ml), Ag_2O (0.5 g) and methyl iodide (2 ml) at 40°C in the dark. The product showed no hydroxyl absorption in the infrared spectrum. GIb (50 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 GIa, methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside and methyl 2,3-di-*O*-methyl-glucopyranoside were identified. In the sample from GIb, methyl 2,3,5-tri-*O*-methyl-arabinofuranoside, methyl 2,3,6-tri-*O*-methyl-glucopyranoside, methyl 2,3-di-*O*-methyl-glucopyranoside and methyl 2,4-di-*O*-methyl-fucopyranoside were detected.

Periodate Oxidation, Mild Smith Degradation and Analysis of Products——GIa (56 mg) was added to a solution of 0.02 M sodium periodate (25 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.[11] When the oxidation was complete (after 18 h), the oxidized GIa was reduced with sodium borohydride (20 mg), and hydrolyzed with 0.1 N sulfuric acid (5 ml) at room temperature for 16 h. Glycerol, erythritol and glycolaldehyde were identified as their trimethylsilylated derivatives by GLC [3% ECNSS-M on Gas chrom Q, column temperature 190°C; N_2 flow rate 40 ml min^{-1}]. In the sample from GIb (47 mg), glycerol, glycolaldehyde, erythritol and fucosyleryhthritol were identified.

Acetolysis of GIa——GIa (100 mg) was suspended in acetolysis reagent (25 ml of acetic anhydride, 25 ml of acetic acid and 2 ml of sulfuric acid), and acetolysis was carried out at room temperature for 12 h. The reaction solution was extracted with chloroform and the organic layer was washed with water, then dried over sodium sulfate. After removal of the solvent, the resulting syrup was chromatographed on silica gel using benzene: acetone (20: 1) as a developing solvent. Glucose pentaacetate, arabinose tetraacetate, maltose octaacetate and maltotriose undecaacetate were identified by ^{13}C-NMR. Glucose pentaacetate (C-1; 89.8, C-2; 67.4, C-3; 69.9, C-4; 68.0, C-5; 69.9, C-6; 62.6), Arabinose tetraacetate (C-1; 99.4, C-2; 82.4, C-3; 76.9, C-4; 85.8, C-5; 63.1), Maltose octaacetate (C-1; 88.9, C-2; 67.5, C-3; 68.0, C-4; 77.2, C-5; 68.7, C-6; 61.4, C-1′; 95.9, C-2′; 69.3, C-3′; 69.8, C-4′; 68.0, C-5′; 70.1, C-6′; 62.5), maltotriose undecaacetate (C-1; 88.8, C-2; 67.4, C-3; 68.5, C-4; 77.2, C-5; 69.1, C-6; 61.4, C-1′; 95.7, C-2′, 69.1, C-3′; 70.5, C-4′; 77.2, C-5′; 71.0, C-6′; 62.3, C-1″; 95.9, C-2″; 69.4, C-3″; 69.7, C-4″; 68.0, C-5″; 70.1, C-6″; 62.3).

Hydrolysis of GIa with α-Amylase——GIa (100 mg) in standard buffer solution (pH 6.86) (10 ml) was incubated with α-amylase (100 mg) from *B. subtilis* (purchased from Sigma Chemical Company) at 37°C for 30 h. After inactivation of the enzyme at 100°C for 5 min, the hydrolysate was subjected to droplet counter-current chromatography (moving phase, upper layer; stationary phase, lower layer of $CHCl_3$–MeOH–H_2O (35: 65: 40)) to give glucose, maltose, maltotriose and tetrasaccharide (*O*-α-D-glucopyranosyl-(1→4)-*O*-[α-L-arabinofuranosyl-(1→6)]-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose), which were identified by ^{13}C-NMR spectroscopy and thin-layer chromatography. Tetrasaccharide (C-1α; 93.1, C-2α; 73.0, C-3α; 74.5, C-4α; 78.6, C-5α; 71.6, C-6α; 62.4, C-1β; 97.2, C-2β; 75.7, C-3β; 77.8, C-4β; 78.5, C-5β; 76.2, C-6β; 62.4, C-1′; 99.2, C-2′; 74.3, C-3′; 74.6, C-4′; 78.5, C-5′; 73.4, C-6′; 67.3, C-1″; 101.0, C-2″; 74.3, C-3″; 74.6, C-4″; 71.0, C-5″; 73.4, C-6″; 62.5, Arabinose C-1; 108.8, C-2; 82.5, C-3; 78.0, C-4; 85.6, C-5; 62.7).

Assay of Antitumor Activity——ICR mice weighing about 25 g (5 week) were used for the antitumor assay. The ascites tumor cells (10^7) of Sarcoma-180 were transplanted subcutaneously into the right groin of mice. The test samples were dissolved in phosphate-buffered saline (pH 7.4) and the solutions were intraperitoneally injected daily for 4 d, starting 24 h after tumor implantation. At the end of the 3rd week, the mice were sacrificed, and the tumors were extirpated and weighed. The inhibition ratios were calculated by the use of the following formula: Inhibition ratio $(\%) = [C - T/C] \times 100$, where C is the average tumor weight of the control group, and T is that of the treated group. To investigate the curative effect of the samples according to the method of Hamuro *et al.*, the test samples were intraperitoneally injected once daily for 5 d, starting 10 d after tumor implantation.

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