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ISOLATION AND CHARACTERIZATION OF TWO KINDS OF HETEROGALACTAN FROM THE FRUIT BODIES OF *Ganoderma applanatum* BY EMPLOYING A COLUMN OF CONCANAVALIN A–SEPHAROSE 4B

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ABSTRACT

A fucogalactan and a mannofucogalactan were isolated from the fruit bodies of *Ganoderma applanatum*, by employing a column of concanavalin A–Sepharose 4B. The former consists predominantly of a main chain of (1→6)-linked α-D-galactopyranose residues, ~30% of which are substituted at O-2 by single-unit α-L-fucopyranosyl side-chains. The latter consists mainly of a backbone of (1→6)-linked α-D-galactopyranose residues, ~50% of which are substituted either with a 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl or α-L-fucopyranosyl side-chains. Both are partially substituted by *O*-acetyl groups. These structural attributions were supported by results of methylation analysis, g.l.c. analysis of polyhydric compounds, and by proton- and ^{13}C-n.m.r. spectroscopy.

INTRODUCTION

Several reports have dealt with the isolation and characterization of mannofucogalactans of such *Basidomycetes*[1–9] as *Polyporaceae*. Most of the chemical structures thus far proposed for the polysaccharides are essentially similar. A fucogalactan has also been isolated from aqueous extracts of the fruit bodies of *Fomes annosus*[10].

Affinity chromatography on a column of concanavalin A (Con A)–Sepharose 4B was applied for purification of a heterogalactan containing mannose from the fruit bodies of a *Polyporaceae species, Ganoderma applanatum*. Con A, a lectin (phytohemagglutinin) obtained from jack bean, is known to interact specifically with polysaccharides having terminal D-mannopyranosyl residues and related structures[11]. The present paper reports the isolation of a fucogalactan and a mannofucogalactan from the fruit bodies of *G. applanatum* and structural correlations between the two heterogalactans.

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RESULTS AND DISCUSSION

Fruit bodies of *G. applanatum* were ground in a blender, immediately washed with 90% ethanol, and extracted with hot water. The hot-water extracts were applied to a column of DEAE-cellulose (Cl^- form) that was eluted with water. The effluent was concentrated and fractionated into fractions designated F-I, F-II, and F-III by fractional precipitation with ethanol. Hydrolyzates of F-I and F-III contained D-glucose as the predominant sugar, whereas, F-II, precipitated at two volumes of ethanol, and gave D-galactose, D-mannose, L-fucose, and small proportion of D-glucose. Fractional precipitation of F-II with Cetavlon in borate buffer at different pH values yielded three subfractions[7]. The first fraction (F-IIa), recovered from the Cetavlon complex precipitated at pH 8.5, was shown to be a glucose-free heterogalactan. On acid hydrolysis, it gave rise to D-galactose, L-fucose, and D-mannose in the molar ratio of 9.0:3.4:1.0. The heterogalactan gave a single, symmetrical peak on ultracentrifugation, on zone electrophoresis, and on gel filtration through Sephadex G-100. The molecular weight was estimated by sedimentation analysis to be 4.65×10^4, and the polysaccharide readily formed a precipitate with Con A. Further double-diffusion reactions on agar gel[12] between F-IIa and Con A produced a single precipitation band, as shown in Fig. 1. It was, however, proved that F-IIa was heterogeneous by employing a column of Con A–Sepharose 4B as mentioned next.

The homogeneity of F-IIa was further investigated on a column of Con A–Sepharose 4B by affinity chromatography. As shown in Fig. 2, a non-adsorbed fraction (F-IIaP) was eluted with 0.1M sodium phosphate buffer at pH 7.0. When the eluant was changed to 0.1M D-glucose–M sodium chloride at pH 7.0, the adsorbed

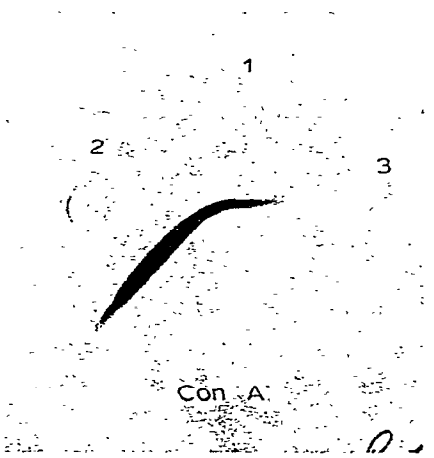


Fig. 1. Double-diffusion pattern of concanavalin A with heterogalactans: 1, F-IIa; 2, F-IIaG; and 3, F-IIaP. Portions (0.1 mL) of the polysaccharide solutions (0.5 mg/mL) were added to the peripheral wells. Concanavalin A extract (0.1 mL, 6 mg/mL) was added to the center well and precipitation bands were allowed to develop overnight at room temperature.

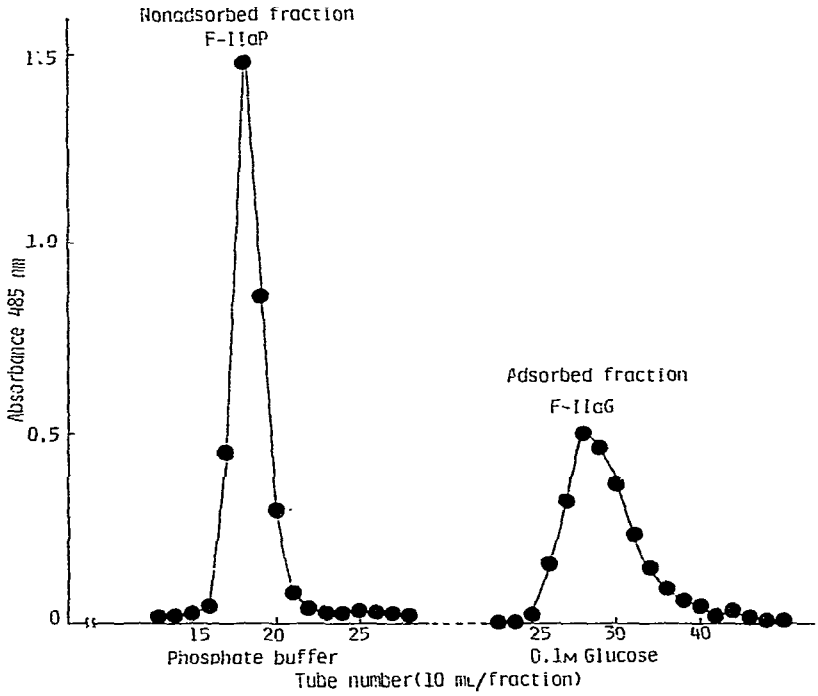


Fig. 2. Fractionation of F-IIa (50 mg) on a column of Con A–Sepharose 4B with 0.1M sodium phosphate buffer (pH 7.0) followed by 0.1M glucose–M sodium chloride (pH 7.0).

fraction (F-IIaG) was eluted. Thus, the elution of F-IIa from insoluble Con A by the two buffers allowed identification of at least two fractions in what appeared, from some chemical and physical criteria, to be a homogeneous sample.

Fraction F-IIaP had $[\alpha]_D$ +108° and on acid hydrolysis it yielded D-galactose and L-fucose in the molar ratio of 3.0:1.0. F-IIaG had $[\alpha]_D$ +87° and contained D-galactose, L-fucose, and D-mannose in the molar ratio of 2.4:1.3:1.0. It should be noted that this fractionation depends on the presence of mannopyranose residues in the sample. The specific rotation of F-IIaP and F-IIaG indicated mainly α-D-galactose residues. Each heterogalactan showed i.r. absorption of 8.0 and 5.8 μm, suggesting the presence of ester linkages. Fractions F-IIaP and F-IIaG gave a single symmetrical peak in zone electrophoresis and in gel filtration on Sephadex G-100. Furthermore, F-IIa and F-IIaG each gave one precipitation line in agar-gel, double-diffusion reactions against Con A. On the other hand, F-IIaP as expected, did not produce any precipitation line. The polysaccharides were methylated by the Hakomori method[13], and the methylated polysaccharides were hydrolyzed and the resulting sugars reduced with sodium borohydride to alditols, which were subsequently acetylated. The mixtures of partially methylated alditol acetates were then analyzed by g.l.c.–m.s. spectroscopy with use of characteristic retention-times (*T*-values)[14] and response-factors previously determined for the individual constituents[15]. The results

TABLE I

METHYL ETHERS FROM THE HYDROLYZATES OF METHYLATED HETEROGALACTANS

Peak	*Methylated sugar*[a]	T[b]	T[c]	*Molar ratio*[d]	
				F-IIaP	*F-IIaG*
A	2,3,4-Me_3-Fuc	0.62	0.61	1.0	0.1
B	2,3,4,6-Me_4-Man	1.00	1.00		1.0
C	2,4-Me_2-Fuc	1.15	1.04		1.1
D	3,4,6-Me_3-Gal	2.44	2.18	0.1	0.1
E	2,3,4-Me_3-Gal	3.38	2.83	2.2	1.3
F	4,6-Me_2-Gal	3.57	3.10	0.1	0.1
G	Unknown	4.50	3.82	±[f]	±
H	3,4-Me_2-Gal	6.80	5.41	0.9	1.1

[a]2,3,4,6-Tetra-*O*-methyl-D-mannitol, etc. [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-*O*-methyl-D-mannitol on a column of ECNSS-M at 180°. [c]As in *b*, but an OV-225 column at 190°. [d]Molar response-factors for partially methylated alditol acetates according to effective carbon response. [f]Trace.

are summarized in Table I. All sugar residues are present in the pyranose form. Methylation analysis of F-IIaP revealed the presence of three major components (peaks A, E, and H). An L-fucose residue is exclusively located at nonreducing, terminal positions. D-Galactose residues are present, both as chain residues linked at O-6, and as branch points at O-2. In F-IIaG, there were two additional peaks (B and C), corresponding to the nonreducing mannopyranosyl and 3-*O*-substituted fucopyranosyl residues. It is noteworthy that these additional components have almost equal intensities. Minor components (peaks D, F, and G) were found in both cases. Peak D was identified as 3,4,6-tri-*O*-methyl-D-galactose. Peaks F and G were not identified from their mass spectra, but the former was presumed to be 4,6-di-*O*-methyl-D-galactose from the *T*-value of its alditol acetate and from the g.l.c. analysis of polyhydric compounds, which provided a small amount of unoxidized galactitol, as shown later.

Based on the results of i.r. spectra, F-IIaP and F-IIaG were treated with dilute alkali in order to remove their *O*-acetyl groups. The resulting *O*-deacetylated products obtained were oxidized with periodate and then reduced by borohydride. The periodate-oxidized products were completely acid hydrolyzed, acetylated, and the products analyzed by g.l.c.[16]. G.l.c. analysis of the product from *O*-deacetylated F-IIaP gave glycerol, together with a trace of galactitol, and from *O*-deacetylated F-IIaG glycerol and fucitol were obtained in 4.5:1.0 molar ratio, together with a trace of galactitol. The occurrence of fucitol and galactitol, suggesting the presence of 3-*O*-linked sugar residues, supported the methylation data.

The proton-n.m.r. spectra of three heterogalactans in the anomeric region were compared as shown in Fig. 3. Taking into account the sugar components of each polysaccharide, it is possible that the most prominent peak at δ 5.02 arises from the

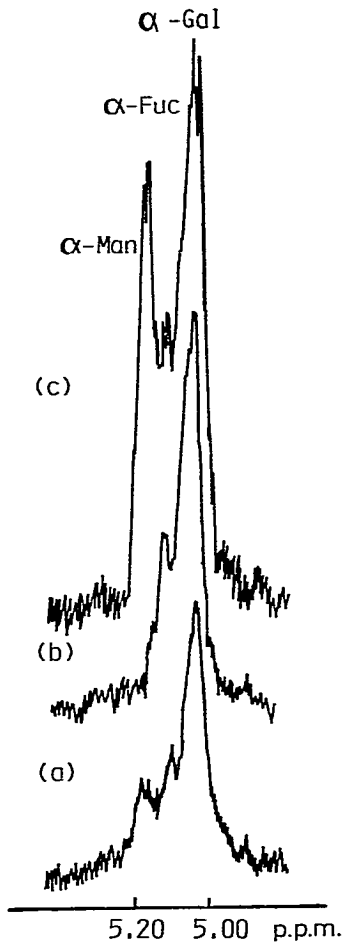


Fig. 3. ^{1}H-N.m.r. spectra at 100 MHz of solutions in D_2O at 80°; only the anomeric-proton region is shown. (a), F-IIa; (b), F-IIaP; (c), F-IIaG.

anomeric proton of a D-galactose residue, and the peak at δ 5.17, which did not appear in the spectrum of F-IIaP, to that of a D-mannose residue. As a result, the remaining peak at δ 5.10 may reasonably be assigned to the glycosidic proton of an L-fucose residue. It should be noted that the coupling constants of signals at δ 5.10 and 5.02 showed $J_{1,2}$ values near 2.0 Hz, suggesting the *cis* disposition, if each L-fucose and D-galactose residue in the polysaccharides adopts a chair conformation[16–18]. Thus, both residues appear to be α-linked. Further, from the reactivity of the polysaccharide with Con A, it may be inferred that the mannopyranose residues have the α-D configuration[11].

Correlation of the spectra of F-IIaP and F-IIaG in the region of δ 2.23–1.15 is shown in Table II. For F-IIaP, it is possible that the additional peaks at δ 1.15 and 2.23 arise from the proton signals of the deoxy methyl group and *O*-acetyl group,

TABLE II

COMPARISON OF PROTON CHEMICAL SHIFTS AND RELATIVE PEAK HEIGHTS OF *O*-ACETYL TO DEOXY METHYL IN THE HETEROGALACTANS

Compound	*Chemical shifts in δ (p.p.m.)*			
	O-acetyl methyl		*deoxy methyl*	
F-IIaP	1.26	1.15	2.23	2.18
	(0.4)[a]	(0.2)	(1.0)	(0.4)
F-IIaG	—	1.15	2.23	—
		(0.2)	(1.0)	—
O-Deacetylated F-IIaP	—	—	2.22	—

[a]Relative peak height relative to deoxy methyl signal at δ 2.23.

TABLE III

SUMMARY OF THE ASSIGNMENTS OF SIGNALS IN ^{13}C-N.M.R. SPECTRA OF F-IIaP AND F-IIaG FROM *G. applanatum*

Signal, δc (p.p.m.)		*Assignments*
F-IIaP	F-IIaG	
	103.0[a]	C-1 of α-D-mannopyranosyl nonreducing end-groups
102.2[a]	102.3[a]	C-1 of α-L-fucopyranose residues
99.0[b]	99.0[b]	C-1 of 6-*O*-substituted α-D-galactopyranose residues
78.9[a]	78.8[a]	C-2 of 2-*O*-substituted α-D-galactopyranose residues of -α-D-Gal*p*-(1→2)-L-Fuc*p*-
	78.4[a]	C-3 of 3-*O*-substituted α-L-fucopyranose residues of α-D-Man*p*-(1→3)-L-Fuc*p*-
	74.1[a]	C-5 of α-D-mannopyranosyl nonreducing end-groups[c]
72.9[a]	72.8[a]	C-2 of α-L-fucopyranose residues
70.6[b]	70.6[b]	C-2 and C-4 of 6-*O*-substituted α-D-galactopyranose residues
70.1[b]	70.1[b]	C-5 of 6-*O*-substituted α-D-galactopyranose residues
69.4[b]	69.4[b]	C-3 of 6-*O*-substituted α-D-galactopyranose residues
68.0[b]	68.0[b]	C-6 of 6-*O*-substituted α-D-galactopyranose residues
67.5[a]	67.5[a]	C-5 of α-L-fucopyranose residues
	62.0[a]	C-6 of α-D-mannopyranosyl nonreducing end-groups
16.5[a]	16.6[a]	C of methyl group of α-L-fucopyranose residues
16.0[a]		C of methyl group of α-L-fucopyranose residues

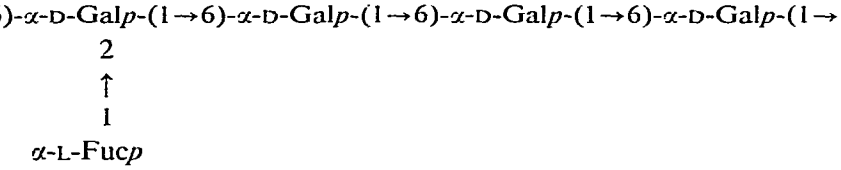
[a]Minor peak. [b]Major peak. [c]Uncertainty concerning the relative assignments.

respectively, of a 4-*O*-acetylated L-fucose residue. Shielding of the deoxy methyl group might be anticipated through steric interaction generated by the *cis*-relationship of substituents at C-4 and C-5, which would not occur in the non-substituted residues. Taking into account their relative intensities, most of the *O*-acetyl groups in F-IIaP appear to be located at position 4 of L-fucose residues. Further experiments are in progress to confirm the location of *O*-acetyl groups.

The ^{13}C-n.m.r. data for F-IIaP and F-IIaG are shown in Table III, and show close similarity in the chemical shifts. Resonances were assigned by comparing both

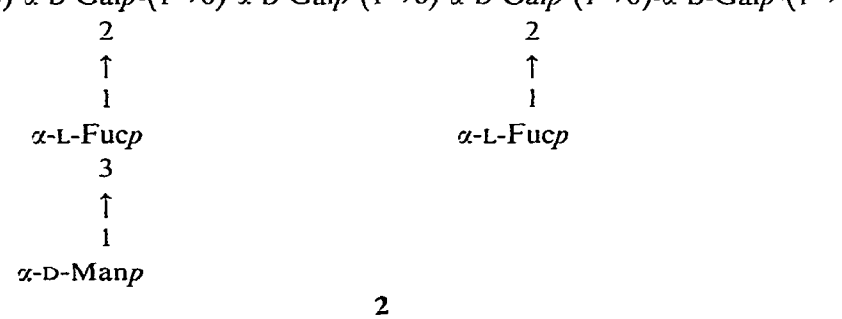
spectra with earlier data[26–28]. These assignments are compatible with the results deduced by the chemical procedures already mentioned. The signals of anomeric carbon atoms were the most clearly differentiated. The resonance at δ 78.8 corresponds to C-2 of an α-D-galactopyranose residue 2-*O*-substituted by an α-L-fucopyranosyl group[27]. Five of the major signals in the ^{13}C-n.m.r. spectra gave chemical shifts very close to those of α-D-galactopyranoside derivatives[20,22–25]. On the other hand, the minor peaks at δ_c 103.0, 78.4, 74.1, and 62.0 were observed in the spectrum of F-IIaG, but were absent in that of F-IIaP. Such signals may be attributed to resonances from the α-D-mannopyranosyl residues[20–26,28]. However, it was laborious to determine the peaks attributed to *O*-acetyl group, because of their low content.

By combining the chemical data with the ^{13}C- and proton-n.m.r. spectra, the preponderant structure of F-IIaP may be represented as **1**. Every third (1→6)-linked α-D-galactopyranose residue is substituted at O-2 by single L-fucopyranosyl side-chains, together with a significant proportion of partial *O*-acetylation.



1

If there had been a possible alternative structure of a branched D-galactan in which the side-chains were terminated with L-fucose, the anomeric region of the ^{13}C-n.m.r. spectrum would be expected to have shown a different spectral pattern from that of F-IIaP, because 2-*O*-substitution of an α-D-galactopyranosyl group by an α-D-galactopyranosyl group causes the C-1 signals to move upfield by 1.0–2.0 p.p.m.[23,27,29–31]. This is also the case for the proton-n.m.r. spectrum[31]. Based on the chemical structure of F-IIaP, it is possible that F-IIaG has predominantly the structure **2** in



2

Thus every two (1→6)-linked α-D-galactopyranose residues are substituted with either a 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl or an L-fucopyranosyl group as a side-chain. The disaccharide unit and L-fucopyranose residues are present in the ratio of 10:1. The principal structure of F-IIaG is similar to those thus far proposed

for the mannofucogalactans of other fungi of the *Basidomycetes* group[2,4,5,9]. From chemical data, it should also be noted that both heterogalactans might contain a very small proportion of internal, (1→2)-linked α-D-galactopyranose residues, a proportion of which are substituted at O-3 by galactose residues.

The present investigation suggests that fungi at first synthesize a fucogalactan having single-unit fucopyranosyl side-chains, and, on the basis of this framework, they produce a mannofucogalactan having the greater proportion of α-D-Man-(1→3)-L-Fuc-(1→ side-chains in the polysaccharide.

EXPERIMENTAL

Polyporaceae fungi. — The dried fruit-bodies of wild *Gannoderma applanatum* used were collected in Senzu, Shizuoka Prefecture of Japan. The materials were identified by Dr. K. Aoshima.

General methods. — Evaporations were performed under diminished pressure at 40–45°. Paper chromatography was performed on Toyo No 50 filter paper by the ascending method with 6:4:3 (v/v) pyridine–1-butanol–water. Components were detected with alkaline silver nitrate. G.l.c. of neutral sugars and their methyl ethers was usually performed, after conversion into their corresponding alditol acetates[5], with a Shimazu gas chromatograph Model GC-4CM-PF fitted with a flame-ionization detector and a glass column (0.3 × 200 cm) packed with 3% ECNSS-M on Gas Chrom Q, operated at 190 and 180° (methylated sugars), or with 3% Silicone OV-225 on Gas Chrom Q at 190° (methylated sugars). Polysaccharide samples (10 mg) were hydrolyzed with 0.5M sulfuric acid in sealed tube for 5 h at 105° and the solutions made neutral with barium carbonate. The hydrolyzate in water was reduced with sodium borohydride, the solution treated with Amberlite IR-120 (H^+), and the mixture filtered. The filtrate was evaporated and boric acid removed by repeated addition and evaporation of methanol. The sugars in the hydrolyzate were analyzed by g.l.c. or paper chromatography. Optical rotations were measured with a Yanaco OR-50D Digital Polarimeter. Infrared spectra were recorded with a KBr disk (polysaccharide) or in carbon tetrachloride (methylated polysaccharide) with a Jasco A-102 spectrometer. Zone electrophoresis of polysaccharides was conducted on Whatman GF/A glass microfiber paper (5 × 57 cm) in 0.1M sodium tetraborate (pH 9.3) at 2000–3000 V for 60 min, and the carbohydrates were detected according to M. Shida *et al.*[7]. Sedimentations were performed in a synthetic boundary-cell at 60,000 r.p.m. at 20° by using a Hitachi model UCA-1A ultracentrifuge, equipped with rotor RA60HC. The concentration of polysaccharide was 0.1% in water.

Isolation of polysaccharide. — The fungus (3 kg) was cut into small pieces immediately after harvesting and disintegrated in a blender. The resulting slurry was treated with 90% ethanol (30 L) for 2 h. The residues were extracted with hot water (20 L) for 3–4 h, and this procedure was repeated once more. The combined extracts were concentrated and dialyzed. A precipitate that formed during dialysis was removed by centrifugation and discarded, and the crude polysaccharides (12.5 g) were

precipitated by an excess of ethanol. The water-extracted fraction was dissolved in water (200 mL) and the solution applied to the top of a column of DEAE-cellulose (Cl^- form). The column was eluted with water, and the almost colorless effluent concentrated to low volume (200 mL) and poured into an equal volume of ethanol. The precipitate was centrifuged off and designated F-I (2.1 g). Ethanol (200 mL) was added to the supernatant, and the precipitate obtained was termed F-II (0.42 g). The final fraction (F-III) was recovered from the supernatant by addition of 400 mL of ethanol.

For F-II, gradual precipitation was performed by treatment with cetyltrimethylammonium chloride (Cetavlon) in borate buffer at different pH values[7]. Fraction F-II (350 mg) was dissolved in water and treated with equal volumes of 0.15M Cetavlon and borate buffer (pH 8.0). The solution was adjusted to pH 8.5 with 0.5M sodium hydroxide and the precipitate formed was washed with water, dissolved in 2M acetic acid, and the solution poured into 3 volumes of methanol. The precipitate (F-IIa) was successively washed with methanol and water, and ether, and dried; yield 150 mg. The supernatant solution at pH 8.5 was adjusted to pH 10.0 and the precipitate treated by the foregoing procedure to give 90 mg of an additional product (F-IIb). The supernatant solution at pH 10.0 was poured into three volumes of methanol to give 70 mg of precipitate (F-IIc). Further fractionation of F-IIa was performed on a column of concanavalin A–Sepharose 4B. Concanavalin A was isolated from jack-bean meal (Sigma) according to the procedure of Goldstein[12] and coupled to Sepharose 4B (Pharmacia Fine Chemicals) after activation[32] with cyanogen bromide. A solution of F-IIa (50 mg) was applied to the top of the column, which was eluted with 0.1M sodium phosphate buffer at pH 7.0 and 25°. The eluate was monitored for neutral carbohydrates by the phenol–sulfuric acid assay. When nonadsorbed carbohydrate had been eluted, the eluant was changed to 0.1M glucose–M sodium chloride at pH 7.0. The eluant containing polysaccharide, passed through a column, was continuously dialyzed by using a Zeineh Dialyzer (Funacoshi Pharmaceutical Co. Ltd), in order to remove glucose contained in the eluant. The elution volume of sample was calculated as shown in Fig. 2. The nonadsorbed fraction was concentrated, dialyzed, and freeze-dried to give 28 mg of F-IIaP. The adsorbed fraction gave 14 mg of F-IIaG.

Agar-gel diffusion. — Agar-gel diffusion studies were performed according to the procedure described by Goldstein[11].

Methylation of polysaccharide. — The polysaccharide (20 mg) was dissolved in dimethyl sulfoxide (2 mL) under ultrasonication (30 kHz, 3–5 min), in an atmosphere of nitrogen, and then methylated by the method of Hakomori[12], with fresh methylsulfinyl carbanion (0.5 mL) and methyl iodide (2 mL). Upon completion of reaction, the mixture was dialyzed and the methylated product extracted with chloroform. The extracts were combined and washed with water, and then dried over sodium sulfate, and the filtrate was evaporated to dryness. Methylation was repeated once more. The product showed no absorption for free hydroxyl groups in its i.r. spectrum.

The methylated product was hydrolyzed according to the method of Lindberg

et al.[4]. The mixture was treated with 90% formic acid (1 mL) for 1 h at 100° and then evaporated, and the residue was hydrolyzed with 0.25M sulfuric acid (2 mL) for 15 h at 100°. The sugars were converted into their alditol acetates as already mentioned, and analyzed by g.l.c.–m.s. A Hitachi 063 gas chromatograph coupled to a Hitachi RMU6M mass spectrometer data-system was used, with 3% ECNSS-M on Gas Chrom Q (stainless steel, 0.3 × 200 cm) at 180°.

G.l.c. analysis of polyhydric compounds, followed by sequential periodate oxidation, reduction, and complete acid hydrolysis. — *O*-Deacetylation of F-IIaP and F-IIaG was performed by dissolution of the polysaccharide (20 mg) in water with subsequent adjustment of the pH of the solution to 11.0 by using dilute ammonia solution. After 2 h, the solution was dialyzed against distilled water for 1 day, and freeze-dried. Both products showed no absorption for *O*-acetyl groups in the i.r. The *O*-deacetylated product was oxidized with 0.05M sodium metaperiodate (20 mL) at 6° in the dark. The periodate consumption was measured spectrophotometrically[33]. Oxidation was complete after 5 days, at which time 1 mL of the solution was used for the measurement of formic acid liberation by titration with 0.01M sodium hydroxide, following reduction of the excess periodate with ethylene glycol. Most of the solution remaining after periodate oxidation was made neutral with barium carbonate. The filtrate was reduced with sodium borohydride (20 mg) for 20 h, and then adjusted to pH 5.0 by addition of acetic acid. The solution was dialyzed against running water for 2 days, and freeze dried. The resulting polysaccharide polyol was hydrolyzed with 0.25M sulfuric acid for 6 h at 100°, and the hydrolyzate converted into alditol acetates, which were analyzed by g.l.c. on a column of 3% ECNSS-M, at 80–190°, programmed at 8° per min.

Gel filtration. — A solution of the polysaccharide (60–90 mg) in 2 mL of water was applied to a column (2.5 × 115 cm) of Sephadex G-100. The column was eluted with 0.05M sodium chloride, and the effluent collected in 13-mL fractions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method.

Measurement of n.m.r. spectra. — Proton-n.m.r. spectra were measured at 80° in deuterium oxide at 100 MHz, by using a JNM PS-100 100-MHz spectrometer. Chemical shifts (δ) are expressed in p.p.m. relative to sodium 4,4-dimethyl-4-silapentanesulfonate salt (TPS) as the internal standard. First-order coupling constants (J) are expressed in Hz. Spectral data were usually obtained at a concentration of about 10%. For exchange of labile hydroxyl protons with deuterons, the samples were dissolved in D_2O and the solvent was freeze-dried. This procedure was repeated three times.

^{13}C-N.m.r. spectra were obtained on samples having natural ^{13}C abundance by using a JEOL PST-60 spectrometer with Fourier transform and having an 8 k memory. Polysaccharides (70–80 mg) were dissolved in D_2O (2.5 mL) and the solution was introduced into a 10-mm outside-diameter, 18-cm n.m.r. tube. Spectra were recorded at 80° and chemical shifts (δ_c) are expressed in p.p.m. relative to external Me_4Si, whose shift relative to D_2O was determined in a separate experiment. The spectral width used was 2500 Hz, the acquisition time 1.8 s, the pulse width

25 μs, and the number of transients 20,000–50,000, according to the spectral resolution and line width.

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