Carbohydrate Research, 105 (1982) 237–245
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STRUCTURE OF AN ALKALI-SOLUBLE POLYSACCHARIDE FROM THE FRUIT BODY OF *Ganoderma japonicum* LLOYD*

SHIGEO UKAI, SHUNJI YOKOYAMA, CHIHIRO HARA, AND TADASHI KIHO
Gifu College of Pharmacy, Mitahora-higashi, Gifu 502 (Japan)
(Received December 3rd, 1981; accepted for publication, December 19th, 1981)

ABSTRACT

A water-insoluble glucan (G-A), $[\alpha]_D^{16}$ +12.8° (*c* 0.4, M sodium hydroxide), was isolated from the alkaline extract of the fruit body of *Ganoderma japonicum*. G-A was homogeneous as judged by gel filtration, electrophoresis, and ultracentrifugal analysis. The molecular weight and degree of polymerization of G-A were respectively estimated to be 82,000 and 330. The ^{1}H-n.m.r. and i.r. spectra, together with the result of chromium trioxide oxidation, indicated that the glucosidic linkages in G-A have the β-D configuration. From the results of methylation analysis, periodate oxidation, Smith degradation, and partial hydrolysis with acid, it was concluded that G-A is composed of a backbone of β-(1→3)-linked D-glucopyranosyl residues, and has side-chains of single, β-(1→6)-linked D-glucopyranosyl groups attached, on average, to every 30th residue of the backbone.

INTRODUCTION

The fruit bodies of *Ganoderma japonicum* and *G. lucidum* (Chinese name: líng zhī cǎo), which belong to the polyporaceae, have been used as a drug. In recent years, Ito *et al.*[2] reported the antitumor activities of polysaccharide preparations from the fruit bodies of *G. lucidum*, but did not elucidate the structure of the polysaccharides. Furthermore, a water-insoluble arabinoxyloglucan from the fruit bodies of *G. lucidum* was investigated by Miyazaki *et al.*[3]. During the course of an investigation on polysaccharides in fungi[1], we have now isolated an alkali-soluble glucan (G-A) from the fruit body of *G. japonicum* (Japanese name: mannentake). The present paper deals with the purification, characterization, and structural analysis of G-A.

RESULTS AND DISCUSSION

The dried, fruit body of *G. japonicum* was pulverized, and successively washed with hot dichloromethane and hot methanol. The dried residue was repeatedly extracted with hot water, and the residue was treated with M sodium hydroxide.

---

*Polysaccharides in Fungi, Part X. For Part IX, see ref. 1.

0008-6215/82/0000–0000/$ 02.75,

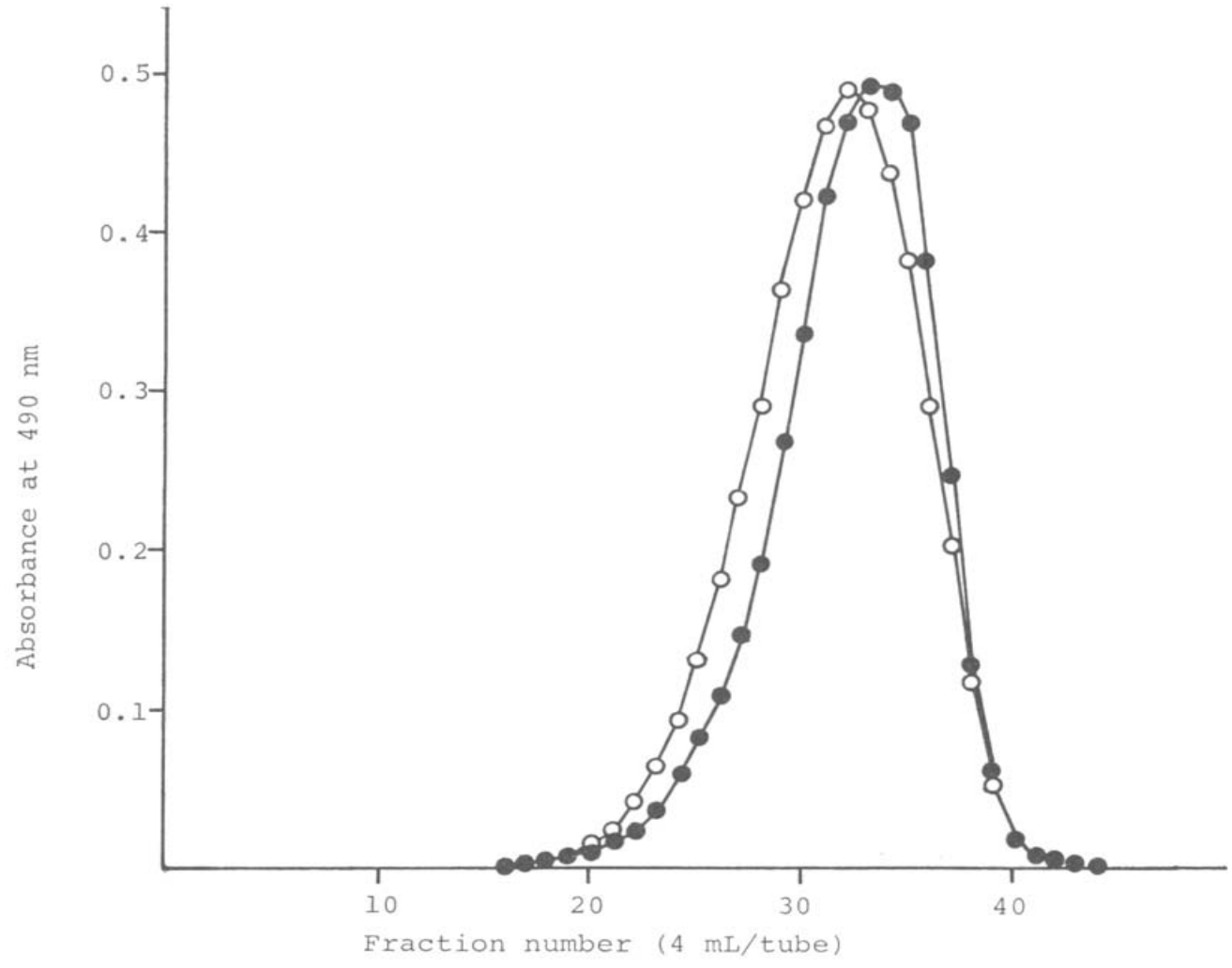

Fig. 1. Chromatograms on Sepharose CL-4B. [The column (1.5 × 96 cm) was eluted with 0.3M sodium hydroxide. Key: —○—, G-A; —●—, G-AS.]

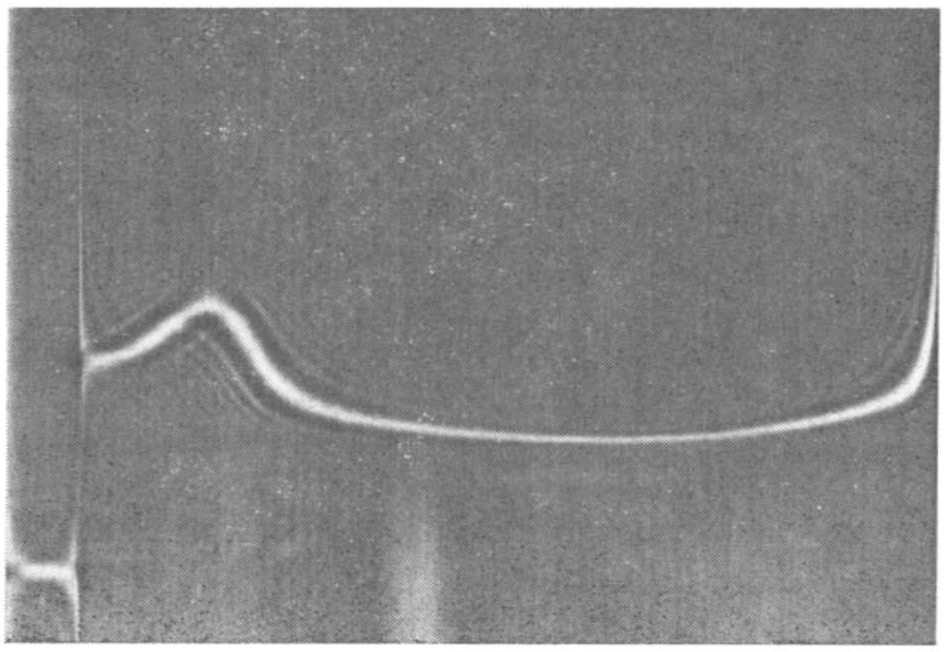

Fig. 2. Ultracentrifugal pattern of G-A. [G-A (5.8 mg/1.5 mL) after 52 min at 60,000 r.p.m.]

The alkaline extract was made neutral, and dialyzed against distilled water, to yield the insoluble material in the internal fraction, which was collected by centrifugation. The crude, insoluble material was dissolved in aqueous sodium hydroxide, and re-dialyzed. The water-insoluble material in the internal fraction was collected, and dried, to afford the polysaccharide (G-A) in 22% yield; this was homogeneous, as

determined by gel filtration on Sepharose CL-4B with sodium hydroxide solution (see Fig. 1). G-A was also found to be pure by Tiselius-type electrophoresis in an alkaline borate buffer, by glass-fiber-paper electrophoresis, and by ultracentrifugal analysis, as shown in Fig. 2.

G-A was soluble in alkaline solutions, but insoluble in neutral or acidic solutions. It slowly dissolved in dimethyl sulfoxide. G-A had a low, positive, specific rotation, $[\alpha]_D^{16}$ +12.8° (*c* 0.4, M sodium hydroxide), and showed characteristic absorbance at 890 cm^{-1} in the infrared (i.r.) spectrum, indicating the presence of the β-D configuration[4]. The polysaccharide was composed solely of D-glucosyl residues, as shown by paper chromatography (p.c.) of the hydrolyzate, by gas–liquid chromatography (g.l.c.) of the alditol acetates prepared from the hydrolyzate, and by the specific rotation of the hydrolyzate. The total sugar content was found to be 99.1% (as hexosyl residues) by the phenol–sulfuric acid method[5]. The glucan contained neither nitrogen nor ash (by elementary analysis). The calibration curve shown in Fig. 3 was made by gel filtration of standard dextrans on Sepharose CL-4B; the molecular weight of G-A thus estimated was ~82,000. The degree of polymerization (d.p.) was estimated to be 330 by the Yamaguchi–Makino method[6].

The glucan (G-A) was methylated by the methods of Hakomori[7] and Purdie[8]. The fully methylated glucan was successively hydrolyzed with formic acid and sulfuric acid. The hydrolyzate was analyzed as the alditol acetate derivatives[9] by g.l.c. and g.l.c.–mass spectrometry (g.l.c.–m.s.). The partially methylated alditol acetates were identified by comparing the retention times in g.l.c., and the mass spectra, with those of authentic samples, or with the values in the literature[10]. The results of the methylation analysis are given in Table I; these indicated that G-A is composed of a main chain of (1→3)-linked D-glucopyranosyl residues, and has some branching points

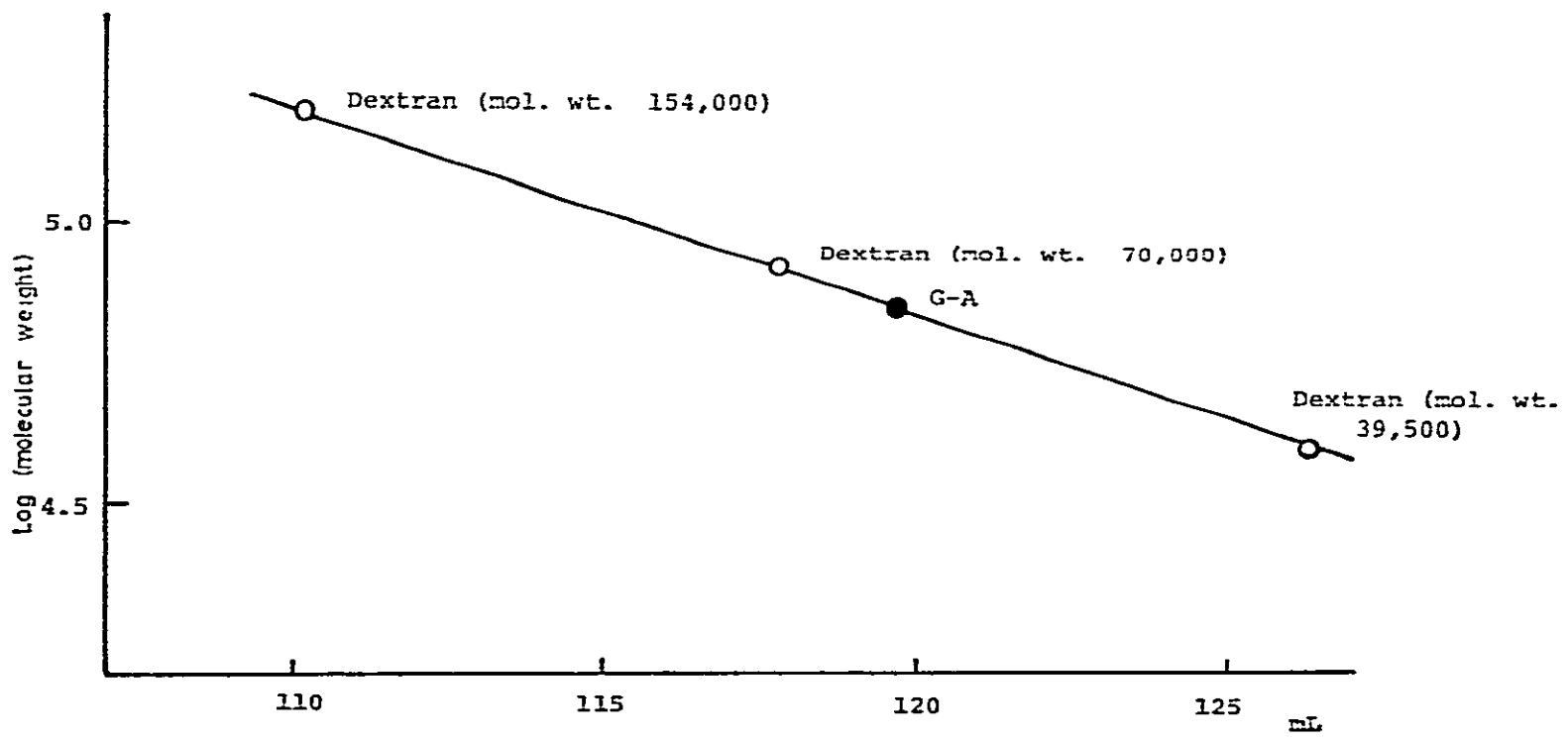

Fig. 3. Determination of molecular weight of G-A by gel filtration on Sepharose CL-4B. [The elution volume plotted against the logarithm of the molecular weight for dextrans T-150, T-70, and T-40.]

TABLE I

G.L.C. AND G.L.C.–M.S. OF ALDITOL ACETATES DERIVED FROM METHYLATED PRODUCT

Methylated sugar (as alditol acetate)	T[a]	*Primary mass fragments*	*Molar percentages*		*Mode of linkage*
			G-A	G-AS	
2,3,4,6-Tetra-*O*-Me-D-Glc[b]	1.00	45, 117, 161, 205	3.2	trace	Glc*p*-(1→
2,4,6-Tri-*O*-Me-D-Glc	1.99	45, 117, 161, 233	93.7	99>	→3)-Glc*p*-(1→
4,6-Di-*O*-Me-D-Glc	4.12	45, 161, 261	trace	trace	→2,3)-Glc*p*-(1→
2,4-Di-*O*-Me-D-Glc	5.21	117, 189	3.1	trace	→3,6)-Glc*p*-(1→

[a]Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[b]2,3,4,6-Tetra-*O*-Me-D-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.

at O-6 of (1→3)-linked D-glucopyranosyl residues. Furthermore, from the results, the average chain-length of G-A was estimated to be 31.

Periodate oxidation of G-A was conducted, with monitoring by the Fleury–Lange method[11]. G-A consumed 0.11 mol of periodate per hexosyl residue. The oxidized polysaccharide was treated with sodium borohydride, and the resulting polyalcohol was hydrolyzed with acid. The hydrolyzate (the Smith-degradation product[12]) was analyzed by g.l.c. as the alditol acetate derivatives, and glycerol and glucose were detected in the molar ratio of 1:29. The glycerol must have arisen from terminal residues, and the occurrence of glucose must be due to the presence of oxidation-resistant D-glucose, such as (1→3)-linked residues. These results are in good agreement with those of the methylation analysis.

The mild, acid hydrolysis of the polyalcohol just described yielded a water-insoluble product (the controlled, Smith-degradation product: G-AS) which was chromatographed on Sepharose CL-4B with sodium hydroxide solution (see Fig. 1). The position of elution of G-AS was slightly delayed, compared with that of the native glucan (G-A). The results of the methylation analysis of G-AS (shown in Table I) showed that it is essentially composed of (1→3)-linked D-glucopyranosyl residues. These results demonstrated that G-A contains a backbone of (1→3)-linked D-glucopyranosyl residues; some of the residues are substituted at O-6, and (1→3)-linked residues are absent from the side chains.

The partial hydrolysis with acid gave glucose and a number of oligosaccharides in p.c. As shown in Fig. 4, a linear relationship existed between the presumed degree of polymerization of the oligosaccharides detected and their log $[R_F/(1 - R_F)]$ values as proposed by French and Wild[13]. Moreover, the partial, acid-hydrolyzate was fractionated by gel filtration on Bio-Gel P-2, and a disaccharide fraction was isolated. The disaccharide was identified as laminarabiose by g.l.c. of the trifluoroacetyl derivative of the disaccharide-alditol[14]. These results indicated that partial, acid hydrolysis of G-A yielded a homologous series of β-(1→3)-linked gluco-oligosaccharides.

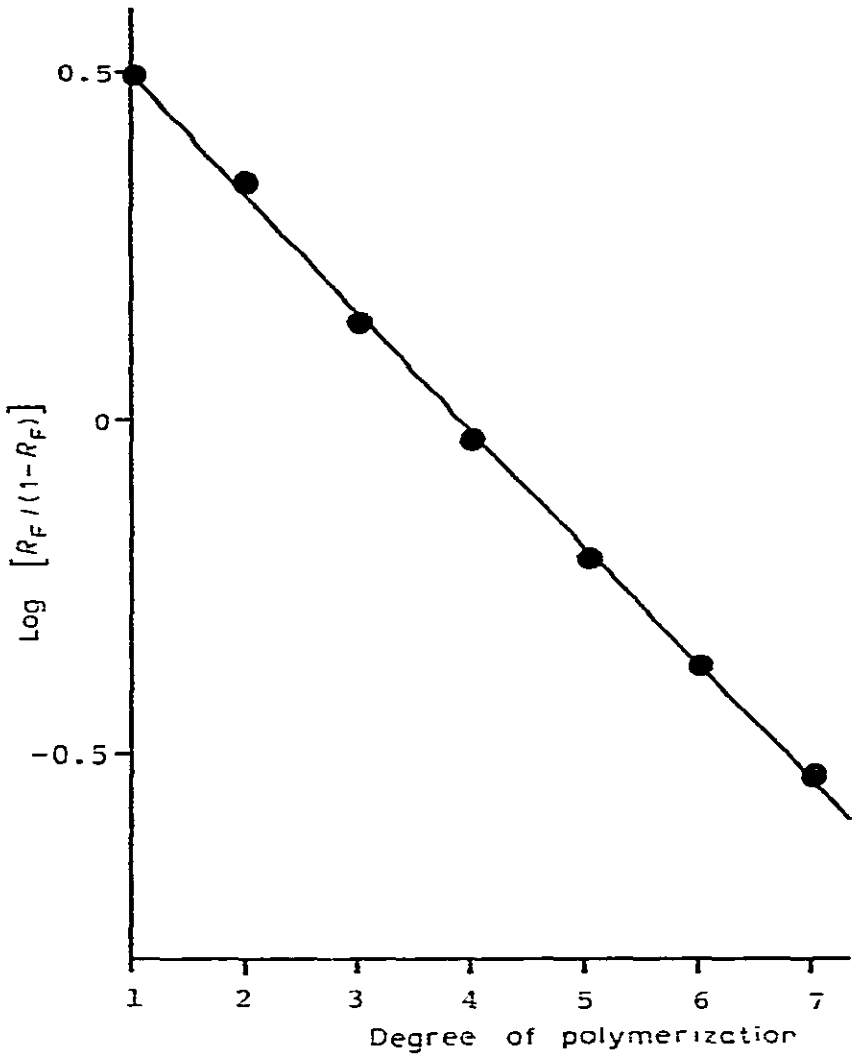

Fig. 4. Relationship between degree of polymerization and log $[R_F/(1 - R_F)]$ values of partial hydrolyzate in p.c.

The proton nuclear magnetic resonance (^{1}H-n.m.r.) spectrum of the methyiated G-A in $CDCl_3$ showed a doublet at δ 4.74, $J_{1,2}$ 6.5 Hz. The anomeric-proton signal for linear β-(1→3)-linked D-glucopyranosyl residues in the spectrum of the methylated laminaran in $CDCl_3$ resonated at δ 4.74, $J_{1,2}$ 8.0 Hz. The result demonstrated that G-A mainly contains β-D-glucosidic linkages.

Chromium trioxide oxidation[15] of G-A resulted in the loss of 88.3% of the D-glucose residues, supporting the presence of the β-D configuration.

The foregoing data indicate that the alkali-soluble and water-insoluble glucan (G-A) from *G. japonicum* is composed of a backbone of β-(1→3)-linked D-glucopyranosyl residues, and has side chains of single, β-(1→6)-linked D-glucopyranosyl groups attached, on average, to every 30th residue of the backbone.

Branched (1→3)-β-D-glucans have been found in several sources, and the structure of the glucan (G-A) from *G. japonicum* was similar to those of the less-branched, alkali-soluble and water-insoluble glucans, such as pachyman from *Poria cocas*[16], curdlan from *Alkaligenes faecalis*[16], and paramylon from *Peranema trichophorum*[17], *Astasis ocellata*[18], and *Euglena gracilis*[19]. However, paramylons possess a lower d.p. (50–150), and curdlan has few, or no, branching points, although the

branches in pachyman occur at every 50th residue. Furthermore, curdlan and pachyman contain a few, internal, (1→6)-D-glucosidic linkages in their molecules. Some (1→3)-β-D-glucans exhibit antitumor activities against transplanted tumors[20,21].

EXPERIMENTAL

Materials. — The fruit body of *Ganoderma japonicum* was harvested in Gifu, Japan. Sepharose CL-4B and standard dextrans (dextran T-150, T-70, and T-40) were purchased from Pharmacia Fine Chemicals. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. Laminarabiose was prepared by acetolysis of laminaran.

General. — All evaporations were conducted under diminished pressure at bath temperatures not exceeding 40°. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. I.r. spectra were recorded with a JASCO IRA-1 spectrometer. Ultracentrifugal analysis was conducted in M sodium hydroxide with a MOM 3170/b analytical ultracentrifuge at 60,000 r.p.m. at 20°. P.c. was performed on Toyo No. 51 filter paper with the following solvent-systems: (*A*) 6:4:3 1-butanol–pyridine–water by the double-ascending method, and (*B*) 6:1:3 1-propanol–ethyl acetate–water by the triple-ascending method. Sugars were detected with an alkaline silver nitrate reagent[22]. G.l.c. was performed in a JEOL JGC-1100 apparatus equipped with a flame-ionization detector. Glass columns (0.3 × 200 cm) packed with (*1*) 3% of ECNSS-M on Gaschrom Q (100–120 mesh) were used at 179°, and (*2*) with 2% of GE-XF-1105 on Chromosorb P (80–100 mesh), at 200°, with nitrogen as the carrier gas at a flow rate of 43 mL/min. G.l.c.–m.s. was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 × 100 cm) packed with ECNSS-M (1), at 169°, at a pressure of helium of 0.8 kg/cm^2. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 210°.

The ^{1}H-n.m.r. spectra of the methylated G-A and the methylated laminaran (each, 5%) were recorded at 100 MHz with a JEOL-FX 100 spectrometer at ambient temperature. Tetramethylsilane was used for the analysis as the internal standard, in $CDCl_3$.

Isolation of the polysaccharide. — The fruit body (70 g) was pulverized, successively washed with hot dichloromethane and hot methanol, and the residue was dried, and extracted 25 times with hot water (1.5 L) for 40 h. The residual material was suspended in M sodium hydroxide (1.2 L) for 20 h at room temperature under a nitrogen atmosphere. The alkaline suspension was centrifuged for 40 min at 6,500 r.p.m., and the extract was made neutral with 3M hydrochloric acid, and then dialyzed against distilled water for 7 days. The insoluble material in the internal fraction was collected by centrifugation, and the precipitate was dried *in vacuo*, to give crude material; this was dissolved in 25mM sodium hydroxide, and the solution was redialyzed. The internal fraction was centrifuged, and the precipitate was washed with water, dispersed in water, and lyophilized, to give fibrous material (G-A) (yield: 22%).

Electrophoresis. — Glass-fiber paper-electrophoresis was conducted on What-

man GF-81 glass-fiber paper (4 × 40 cm) with 0.1M sodium hydroxide containing 0.05M sodium tetraborate for 1.5 h at 250 V. The spot was detected with the 1-naphthol-sulfuric acid reagent[23]. G-A gave one spot at a distance of 9.5 cm from the origin.

Tiselius-type electrophoresis was performed with a Hitachi HID-1 boundary electrophoresis apparatus in the aforementioned buffer for 40 min at 25 V. Electrophoretic mobility: $u = 1.0 \times 10^{-4}$ cm^2/V · s.

Gel filtration. — The sample (2 mg) was dissolved in 0.3M sodium hydroxide (0.5 mL), and applied to a column (1.5 × 96 cm) of Sepharose CL-4B. The column was eluted with 0.3M sodium hydroxide at a flow rate of 6 mL/h. Fractions (4 mL each) were collected, and an aliquot of each fraction was analyzed by the phenol–sulfuric acid method[5].

Estimation of molecular weight and degree of polymerization. — Gel filtration on a column of Sepharose CL-4B was conducted with 0.3M sodium hydroxide as already described. A calibration curve was constructed by use of dextran T-150 (mol. wt. 154,000), T-70 (70,000), and T-40 (39,500) as shown in Fig. 3, and the molecular weight was estimated.

G-A was treated with sodium borohydride, and the resulting product hydrolyzed with acid. The aldose and alditol in the hydrolyzate were separated on a column of Dowex-1 X-8 (OH^-) resin, and analyzed by g.l.c. (column *1*) as the alditol acetates[9]; then the d.p. was estimated from the alditol content in the hydrolyzate by the Yamaguchi–Makino[6] method.

Analysis of component sugars. — G-A (3.8 mg) was heated with 90% formic acid (2 mL) in a sealed tube for 8 h at 100°. After removal of the formic acid by evaporation, the residue was hydrolyzed with 0.25M sulfuric acid for 18 h at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension filtered, the filtrate passed through a column of Amberlite IR-120 (H^+) resin, and the eluate evaporated. The syrupy residue was found by p.c. (solvent *A*) to be glucose. An aqueous solution of the hydrolyzate was reduced with sodium borohydride, the alditol acetylated[9], and the resulting alditol acetate determined as glucitol acetate by g.l.c. (column *1*).

The specific rotation of the hydrolyzate of G-A, obtained by a procedure similar to that already described, was $[\alpha]_D^{20}$ +52.2° (*c* 0.2, 0.1M sulfuric acid). Authentic D-glucose showed $[\alpha]_D^{20}$ +52.5° (*c* 0.3, 0.1M sulfuric acid).

Methylation analysis. — G-A (14.9 mg) was methylated by Hakomori's method[7] and then five times by the Purdie method[8] as previously described[24,25]. The final, methylation product showed no hydroxyl absorption band in the i.r. spectrum. The fully methylated glucan was successively heated with 90% formic acid (2 mL) for 8 h at 100°, and 0.25M sulfuric acid (2 mL) for 18 h at 100°. After neutralizing the acid with Amberlite IR-400 (carbonate) resin, the hydrolyzate was converted into the alditol acetates[9]. The resulting, partially methylated alditol acetates were analyzed by g.l.c. (column *1*) and g.l.c.–m.s.

Partial hydrolysis with acid. — A solution of G-A (4.6 mg) in 50% (v/v) sulfuric acid (2 mL) was kept for 16 h at 4°, and then stirred for 1 h at 35°. After

neutralization of the acid with Amberlite IR-400 (carbonate) resin, the hydrolyzate was analyzed by p.c. (solvent *B*).

A portion (0.2 mL) of the hydrolyzate was applied to a column (1.5 × 97 cm) of Bio-Gel P-2. The column was eluted with water at a flow rate of 4 mL/h, and fractions (1.2 mL) were collected, and then analyzed by the phenol–sulfuric acid method[5]. The hydrolyzate was fractionated into a monosaccharide, a disaccharide, a trisaccharide, and a tetrasaccharide fraction. The disaccharide fraction was isolated, and treated with sodium borohydride, followed by (trifluoroacetyl)ation[14] with 1 : 1 trifluoroacetic anhydride–*N*,*N*-dimethylformamide for 5 min at room temperature. The resulting trifluoroacetate of the disaccharide-alditol was analyzed by g.l.c. (column *2*). The retention time of the derivatives of the sample and of laminarabiose was 25.8 min.

Periodate oxidation and Smith degradation. — The pH of a solution of G-A (29.6 mg) in M sodium hydroxide (1 mL) was adjusted to 4.85 with 0.5M hydrogen chloride, and 10mM sodium metaperiodate (50 mL) was added. The mixture was kept in the dark, with stirring, for 12 days at 4°. An aliquot (3 mL) was taken at different periods, and the periodate consumption was determined by the Fleury–Lange method[11]. The oxidation was stopped by addition of ethylene glycol, the mixture dialyzed, and the contents reduced with sodium borohydride (25 mg) for 24 h at room temperature. The mixture was treated with acetic acid, dialyzed, and then lyophilized, to give the polyalcohol; this (2.1 mg) was successively treated with 90% formic acid for 6 h at 100°, and 0.25M sulfuric acid for 17 h at 100°. The hydrolyzate was converted into the corresponding alditol acetates as already described, and analyzed by g.l.c., using the dual columns of 3% of ECNSS-M (column *1*). The column temperature was increased by 4°/min from 40 to 180°. The retention times of the acetates of glycerol and glucitol were 22.4 and 61.4 min.

Controlled, Smith degradation. — The polyalcohol (36.7 mg) obtained by a procedure similar to that just described was hydrolyzed with 50mM sulfuric acid (5 mL), with stirring, for 24 h at 25°, the mixture centrifuged for 40 min at 9,500 r.p.m., and the precipitate washed with water, and dried, to give the controlled-Smith-degradation product (G-AS) (29.8 mg). An alkaline solution of G-AS was applied to a column of Sepharose CL-4B. A portion (12.8 mg) of G-AS was methylated by Hakomori's method[7] and 9 times by the Purdie method[8]. The fully methylated product was hydrolyzed, the sugars were reduced with sodium borohydride, the alditols acetylated, and the acetates analyzed by g.l.c. (column *1*) as already described.

Chromium trioxide oxidation. — A solution of G-A (500 mg) in formamide (15 mL) was treated with acetic anhydride (15 mL)–pyridine (15 mL) for 23 h at room temperature, and then for 3 h at 65–70°. Part of the partially acetylated G-A thus obtained was acetylated with 1 : 1 acetic anhydride–pyridine (2 mL) for 48 h at room temperature. A mixture of the resulting, fully acetylated G-A with *myo*-inositol acetate (11 mg), as the internal standard, was dissolved in chloroform (3 mL). Part (1 mL) of the solution was kept as a control. The rest was dissolved in glacial

acetic acid (0.5 mL), dry chromium trioxide (50 mg) was added to the solution, and the mixture was sonicated in an ultrasonic bath for 1 h at 50°. The resulting solution was poured into water, and the mixture extracted 5 times with chioroform. The extracts were combined, and evaporated to dryness. The control sample was treated similarly, but without adding chromium trioxide. The hydrolyzates of the products thus obtained were converted into the alditol acetates, and analyzed by g.l.c. (column *1*).

ACKNOWLEDGMENTS

The authors are grateful to Mr. M. Kurita, Kubota Medical Appliance Supply Corp., for the ultracentrifugal analyses, and to Mr. H. Eto, Faculty of Pharmacy, Meijo University, for measurement of the ^{1}H-n.m.r. spectra. The authors also thank Dr. M. Mizuno of this College for identifying the fruit body of the fungus, and Mr. K. Hirai for supplying the fruit body.

REFERENCES

1 S. Ukai, C. Hara, and T. Kiho, *Chem. Pharm. Bull.*, 30 (1982) in press.
2 H. Ito, S. Naruse, and K. Shimura, *Mie Med. J.*, 26 (1977) 147–152.
3 M. Nishijima and T. Miyazaki, *Proc. Annu. Meet. Pharm. Soc. Jpn.*, (1978) 468.
4 H. Spedding, *Methods Carbohydr. Chem.*, 1 (1962) 539–550.
5 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
6 H. Yamaguchi and K. Makino, *J. Biochem. (Tokyo)*, 81 (1977) 563–569.
7 S.-I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
8 T. Purdie and J. C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021–1037.
9 J. H. Sloneker, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.
10 H. Björndal, C. G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
11 P. F. Fleury and J. Lange, *J. Pharm. Chim.*, 17 (1933) 196–206.
12 J. K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, 78 (1956) 5907–5909.
13 D. French and G. M. Wild, *J. Am. Chem. Soc.*, 75 (1953) 2612–2616.
14 H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.*, 18 (1970) 2314–2321.
15 J. Hoffman, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, 26 (1972) 661–666.
16 H. Saito, A. Misaki, and T. Harada, *Agric. Biol. Chem.*, 32 (1968) 1261–1269.
17 A. R. Archibald, W L. Cunningham, D. J. Manners, and J. R. Stark, *Biochem. J.*, 88 (1963) 444–451.
18 D. J. Manners, J. F. Ryley, and J. R. Stark, *Biochem. J.*, 101 (1966) 323–327.
19 A. E. Clarke and B. A. Stone, *Biochim. Biophys. Acta*, 44 (1960) 161–163.
20 R. L. Whistler, A. A. Bushway, P. P. Singh, W. Nakahara, and R. Tokuzen, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 235–275.
21 T. Usui, Y. Iwasaki, K. Hayashi, T. Mizuno, M. Tanaka, K. Shinkai, and M. Arakawa, *Agric. Biol. Chem.*, 45 (1981) 323–326.
22 W. E. Trevelyan, D. F. Procter, and J. S. Harrison, *Nature*, 166 (1955) 444–445.
23 H. Jacin and A. R. Mishkin, *J. Chromatogr.*, 18 (1965) 170–173.
24 S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.*, 25 (1977) 338–344.
25 S. Ukai, T. Kiho, C. Hara, and K. Hirose, *Chem. Pharm. Bull.*, 26 (1978) 1707–1712.