Comparative Structural Studies on Acidic Heteropolysaccharides Isolated from "Shirokikurage," Fruit Body of *Tremella fuciformis* Berk, and the Growing Culture of Its Yeast-like Cells*

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Acidic heteropolysaccharides, D-glucurono-D-xylo-D-mannans were isolated from the water- and alkaline extracts of the fruit body of *Tremella fuciformis* Berk. Similar polysaccharides were isolated from the growing culture of the haploid cells of two strains (T-19 and T-7) of *T. fuciformis*, when they were cultured in sucrose or glucose-yeast extract medium. The extracellular polysaccharides contain, D-glucuronic acid, D-xylose and D-mannose [molar ratios, 1.3: 1.0: 3.5 (T-7) and 0.8: 1.0: 2.1 (T-19)], and, in addition, small proportions of L-fucose and O-acetyl groups. Methylation and Smith degradation studies indicated that both fruit body and extracellular polysaccharides are built up of α -(1 $\rightarrow$ 3)-linked D-mannan backbone chain to which β -linked D-glucuronic acid and single or short chains of β -(1 $\rightarrow$ 2)-linked D-xylose residues are attached at the C-2 position. L-fucose residues in the extracellular polysaccharides may form the single branches. The structural features of these polysaccharides are discussed in comparison with the similar polysaccharides from other fungi.

Tremella fuciformis Berk, which belongs to *Heterobasidiaceae*, occurs usually in the bisexual form, and its fruit body, called "Shirokikurage" has long been appreciated as an edible mushroom. In the previous study, the fruit body of *Auricularia auricula-judae* (kikurage), also belonging to *Heterobasidiaceae* was fractionated and a D-glucurono-D-xylo-D-mannan and two types of β -D-(1 $\rightarrow$ 3)-glucans were isolated.¹⁾ During investigations on the relationships between structures and physical or biological properties of edible fungal polysaccharides, we became aware that the yeast-like haploid cells of *T. fuciformis* are capable of producing extracellularly an acidic polysaccharide, and its structure appears to resemble that of the glucurono-xylo-mannan obtained from the hot-

water or alkaline extract of its cell walls.²⁾ The present paper deals with the isolation of the acidic heteropolysaccharides from the fruit body (shirokikurage) and from the growing cultures of the yeast-like cells of *T. fuciformis* (strain T-7 and T-19), and comparison of their detailed structural features. Recently, characterization of the similar polysaccharides from *T. fuciformis* fruit body has been reported by Ukai *et al.*^{3,4)} The isolation of β -D-mannosidase and β -N-acetyl-D-hexosaminidase from the cell-extract of this organism was also reported elsewhere.⁵⁾

MATERIALS AND METHODS

General analytical methods. All evaporations were carried out under diminished pressure at the temperature not exceeding 37°C, unless otherwise stated.

Paper chromatography was performed using Toyo Roshi No. 50 or Whatmann 3 MM paper (preparative chromatography), with the following solvent systems (v/v): (a) 1-butanol-pyridine-water (6: 4: 3), (b) butan-2-one-water azeotrope, and (c) 1-butanol-acetic acid-water (4: 1: 5). Sugars on a paper chromatogram were detected with alkaline silver nitrate or *p*-anisidine hydro-

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chloride in butanol-saturated water.

Gas liquid chromatography (GLC) was carried out with a Shimadzu Gas Chromatograph Model GC-6AM fitted with a flame ionization detector, using a glass column (0.4 cm, i.d.). Methylated sugars were separated as their methyl glycosides on a column (1 m length) of 15% butanediol succinate polyester on Neosorb NC. Retention times (T_R) are quoted relative to tht of 2,3,4,6-tetra-*O*-methyl- β -methyl-D-glucoside. Neutral sugars and their methyl derivatives were usually converted into the corresponding alditol acetates, and separated on a column (2 m length) of 3% ECNSS-M on Gas Chrom Q, at 190°C, and 180°C (methylated sugars), in the same manner as used previously.¹⁾

Hydrolysis of a polysaccharide was achieved by formolysis followed by hydrolysis with 1 N sulfuric acid. A sample (ca. 10 mg) was heated, first, with 90% formic acid (1 ml) at 100°C for 12 hr in a screw capped vial, and after evaporation, the residue was heated with 1 N sulfuric acid (0.5 ml) for additional 8 hr. The hydrolyzate was neutralized (BaCO_3), filtered, and the sugars in the filtrate were analyzed by GLC or paper chromatography.

Determination of carbohydrate content of the polysaccharide was generally made by phenol-sulfuric acid method.⁶⁾ Colorimetric determination of uronic acid was done by the carbazole method.⁷⁾

Fractionation of polysaccharides of Fruit body. Dried commercial "Shirokikurage" (ca. 60 g), which was harvested in Formosa was immersed in 0.9% aqueous sodium chloride (saline) (1500 ml) overnight, and homogenized with a Waring blender, and centrifuged. The homogeneous suspension was stirred at 5°C for three days, and centrifuged. To the supernatant solution was added 3 volumes of ethanol to give precipitation of a polysaccharide (Fraction WS). The residue was autoclaved two times in the aqueous suspension (700 ml) at 120°C for 20 min. The mixture of polysaccharides precipitated from the extract by addition of ethanol was subjected to fractionation using cetyl pyridinium chloride (CPC), after being dissolved into 0.01 M sodium sulfate (1000 ml) followed by gradual addition of 10% aqueous CPC solution until no more precipitate was formed. The insoluble polysaccharide CPC-complex was collected by centrifugation, and dissolved into 10% sodium chloride (400 ml). After removal of a small amount of insoluble matter, the solution was dialyzed, and the acidic polysaccharide in the non-dialyzable solution was precipitated with ethanol (Fraction HWS-A). To the supernatant solution of the precipitation of CPC-complex was gradually added ethanol to give a neutral polysaccharide (Fraction HWS-N). The insoluble residue after extraction with hot water was then treated with 1 N sodium hydroxide (250 ml) at 65°C for 2 hr in the presence of sodium borohydride (1 g), under the nitrogen atmosphere. After one more treatment with alkali, the

extracts were combined, neutralized with 1 N hydrochloric acid, dialyzed, and the mixture of polysaccharides was precipitated with ethanol. It was further fractionated through CPC-complex formation in the manner described above, to give an acidic polysaccharide (Fraction HAS-A) and a neutral polysaccharide (Fraction HAS-N). The residue after alkaline extraction was washed with water and then lyophilized. Each polysaccharide fraction was purified by repeated precipitations with ethanol. The procedure of polysaccharide fractionation is shown in Scheme I.

SCHEME 1. Fractionation Procedure of Fruit Body Polysaccharides.

Production and isolation of extracellular polysaccharides. The haploid cells of *T. fuciformis* (T-7 and T-19 strain²⁾) were usually grown in media composed of 6% glucose, 0.4% yeast-extract, 0.05% KH_2PO_4 , 0.05% K_2HPO_4 and 0.05% MgSO_4 , pH 7.0, at 30°C for 5 days. After removal of the cells and insoluble materials by centrifugation, the crude polysaccharide was precipitated from the clarified broth by addition of 2 volumes of acetone. For purification, the above polysaccharide preparation was dissolved in water, passed through a column of Amberlite IR-120 (H^+ -form), and 10% aqueous solution of the CPC was added, in the manner similar to that used for fruit body polysaccharides. The CPC-complex was dissolved in 10% sodium chloride, centrifuged, and the acidic polysaccharide was precipitated by the addition of acetone or ethanol. The purified polysaccharide was obtained by dissolving into water, followed by repeated precipitations with acetone. The yield of polysaccharide was approx. 15% of the initial glucose in the medium.

Methylation analysis of polysaccharides. Dry samples of the polysaccharides (15 mg) were dissolved in dehydrated dimethyl sulfoxide (2 ml) and treated for

3 hr at room temperature with methylsulfinyl carbanion (0.5 ml), and the polysaccharide alkoxide, so formed, was treated with methyl iodide (1.5 ml) at 20°C, according to the method of Hakomori.⁸⁾ The reaction mixture was dialyzed, concentrated to a small volume, and the methylated product was extracted. If methylation was not complete, as judged from the infrared spectrum (OH-band at 3400~3600 cm⁻¹), the methylation procedure was repeated.

The fully methylated polysaccharide was dissolved in cold 72% sulfuric acid, and after standing at room temperature for 1.5 hr, the solution was diluted with 7 volumes of water and heated at 100°C for 6 hr. The hydrolyzate was neutralized (BaCO₃), filtered, and the filtrate was concentrated *in vacuo* to a syrup. The methylated sugars in the hydrolyzate were examined by paper chromatography (solvent b) and by GLC, as their methyl glycosides and also as their alditol acetates. For preparation of the methyl glycosides, the mixture of sugars was heated with 3% methanolic hydrogen chloride at 80°C for 12 hr. The alditol acetates were prepared from the mixture of sugars by reduction with sodium borohydride and subsequent acetylation with pyridine and acetic anhydride (1:1) at 100°C for 2 hr.

Periodate oxidation. Samples of polysaccharides (100 mg, each) were oxidized with 0.05 M sodium periodate (100 ml) at 4°C in the dark. At suitable intervals, periodate consumption was determined by arsenite method, and formic acid production by titration with 0.01 N sodium hydroxide. After complete oxidation, the oxidized polysaccharide was reduced with sodium borohydride by the usual manner, and a portion of the resulting polysaccharide polyalcohol was hydrolyzed with 0.5 N sulfuric acid at 100°C for 8 hr.⁹⁾ The hydrolyzate was neutralized (BaCO₃), filtered, and the sugar and sugar-alcohols were analyzed by GLC, after borohydride reduction followed by acetylation. In another experiment, the polysaccharide polyalcohol was subjected to mild acid hydrolysis with 0.1 N sulfuric acid at 25°C for 20 hr. The degraded polysaccharide precipitated as an insoluble matter was collected, and its

structure was examined by methylation analysis.

RESULTS

Fractionation of the fruit body polysaccharides and their chemicals constitutions

The dried fruit body of *Tremella fuciformis* Berk was homogenized in saline, and polysaccharides were extracted successively with saline at cold temperature, hot water at 120°C, and 1 N sodium hydroxide at 65°C under nitrogen atmosphere, in the way similar to that used for fractionation of the fruit body of *Auricularia auricula-judae*,¹⁾ as shown in Scheme I. The polysaccharide fraction obtained by hot-water extraction and also by alkali extraction were further fractionated, respectively, by treatments with CPC to separate acidic and neutral polysaccharide fractions. After purification, each polysaccharide was hydrolyzed with acid, and its carbohydrate components were examined by GLC, after conversion into their alditol acetates. The presence of D-glucuronic acid residues in the acidic polysaccharides was confirmed also by GLC, after conversion into the acetylated glucitol, by a series of reactions which involve borohydride reduction, methyl esterification of the aldonic acid derived from the uronic acid, retreatment with borohydride, followed by acetylation, in the manner reported previously.¹⁾ The yields, optical rotations and chemical compositions of the polysaccharides are listed in Table I.

The acidic polysaccharides (Fraction HWS-A, and HAS-A) which account for approxi-

TABLE I. FRACTIONATION AND CARBOHYDRATE COMPOSITIONS OF POLYSACCHARIDES OF *T. fuciformis* FRUIT BODY

Polysaccharide	Weight (g)	[α] _D (c, 1.5 N NaOH)	Composition (molar ratio)				
			Galactose	Glucose	Mannose	Xylose	Glucuronic acid
Whole fruit body	60		tr.	0.65	2.96	1.0	
Fraction WS	7.62	-25.5°	—	8.1	0.33	1.0	0.97
Fraction HWS-A	5.58	-23.0°	—	—	2.85	1.0	1.27
Fraction HWS-N	0.15	+35.9°	1.0	3.4	3.3	1.0	
Fraction HAS-A	13.7	-4.5°	—	—	4.9	1.0	2.77
Fraction HAS-N	0.34	+10.2°	1.7	22.8	1.7	1.0	
Residue	16.56		0.1	4.5	2.5	1.0	1.96
Total	48.95						

mately 70% of the total extractable material, gave on acid hydrolysis, D-xylose, D-mannose and D-glucuronic acid, in the molar ratios of 1.0: 2.85: 0.95 (Fraction HWA-A), and 1: 4.9: 1.27 (Fraction HAS-A). No glucose was detected in both fractions. These results suggested that their constitutions resemble those of the acidic heteropolysaccharides of *A. auricular-judae*, except that the latter contain D-glucose. The presence of acetyl groups in water-extractable fraction was suggested by its infrared spectrum (absorption bands at 1725~1730, and 1240 cm^{-1}). The fractionation of the polysaccharides also indicated that the fruit body contains only small proportions of neutral polysaccharides, which comprise xylose, mannose and galactose, in addition to glucose (Fraction HWS-N, and HAS-N, in Table I). The cold saline extract (Fraction WS) also appears to be a mixture of neutral and acidic polysaccharides, though they were not further investigated.

Since the alkaline-extractable acidic polysaccharide seems to represent the major constituent of the fruit body, its structure was elucidated, in comparison with those of the extracellular polysaccharides.

Production and isolation of the extracellular polysaccharides

When the yeast-like haploid cells of *T. fuciformis*, strain T-7 and T-19, were grown under aerobic conditions in synthetic media

which contain glucose or sucrose and yeast-extract, the media became viscous because of the production of polysaccharide. In order to know the most effective cultural condition for the polysaccharide production, T-19 was incubated in the medium containing 6% glucose, 0.05% KH_2PO_4 , 0.05% K_2HPO_4 , 0.05% MgSO_4 plus different concentrations of yeast-extract, using shaking culture, at 30°C for 5 days. As shown in Table II, both cell growth and production of polysaccharide reached to maxima at 0.4% yeast-extract. When the cells were grown in the media containing 6% of different sugars and sugar alcohols, with 0.4% of yeast-extract, it was found that common sugars, such as glucose, sucrose, mannose and xylose were effectively utilized for both cell growth and the biosynthesis of polysaccharide (700~750 mg per 100 ml of broth), whereas none of the polyalcohols were utilized, as shown in Table III. From these results, the medium comprising 6% glucose, 0.4% yeast-extract, and each 0.05% KH_2PO_4 , K_2HPO_4 and MgSO_4 , was employed for preparation of the polysaccharide, and strain T-19 was grown in the submerged culture using a small fermentor (10 liter volume; 6 liter medium), with aeration of 5~6.5 liter per min, and agitation, 300 rpm. The glucose in the medium was rapidly utilized during 3 days after incubation, with the increases in the cell-growth and ac-

TABLE II. EFFECT OF THE CONCENTRATION OF NITROGEN-SOURCE ON POLYSACCHARIDE PRODUCTION AND CELL GROWTH OF *T. fuciformis* T-19

Yeast extract (%)	Utilization of glucose	Yield of polysaccharide (mg/100 ml broth)	Dry weight of cells (mg/100 ml broth)
0.15	64	577	744
0.2	74	635	1,345
0.3	90	775	1,632
0.4	95	815	2,050
0.5	100	734	1,820

Composition of basal medium; 6% glucose, each 0.05% of K_2HPO_4 , KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, shaken at 30°C for 5 days.

TABLE III. EFFECT OF CARBON-SOURCE ON POLYSACCHARIDE PRODUCTION OF *T. fuciformis* T-19

Carbon-source (5% conc.)	Final pH of broth	Utilization (%)	Yield of polysaccharide (mg/100ml broth)
Ethylene glycol	3.7	—	83
Propylene glycol	6.7	—	180
Glycerol	6.7	—	415
Glucose	6.4	100	750
Galactose	6.4	92	540
Mannose	6.4	98	735
Xylose	6.1	96	778
Maltose	6.4	76	360
Sucrose	6.5	—	720

Basal medium; 0.4% yeast extract, each 0.05% of K_2HPO_4 , KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

cumulation of the polysaccharide. Under this condition, the yield of the polysaccharide reached to the level of 8 g per liter of the broth. After removal of the cells and insoluble matters by centrifugation, the crude polysaccharide was precipitated from the clarified cultural broth by addition of 2 volumes of acetone. The polysaccharide was dissolved in water, and the acidic polysaccharide was purified through the formation of insoluble CPC-complex, in the same manner as described for purification of the fruit body polysaccharide. Strain T-7 was also cultivated in the similar condition, and the polysaccharide was isolated from the cultural filtrate (yield, approximately 15% of the initial glucose in the medium).

Comparison of properties of the fruit body and extracellular polysaccharides

The optical rotations, sedimentation constants, and carbohydrate compositions of three preparations of acidic polysaccharides, isolated from the alkaline extract of fruit body and two different polysaccharides produced by strain T-7 and T-19, respectively, are compared in Table IV. All of the purified polysaccharides gave single and symmetrical peaks in ultracentrifugal analyses, with $S_{20,w}$ between 2 and 3.3, indicating that they are homogeneous. All polysaccharides were essentially nitrogen free, and composed mainly of D-mannose, D-glucuronic acid and D-xylose. Interestingly, however, the extracellular polysaccharides contain small but significant proportions of L-fucose, in addition to the above

sugars. Although their molar ratios vary, depending on their origins, their basic structures appear to be similar to each other and they are partially *O*-acetylated. The fruit body polysaccharide may also contain acetyl groups, which should be removed during alkaline extraction from the fruit body.

Mode of glycosidic linkages

The polysaccharides were methylated by the method of Hakomori,⁸⁾ and their methyl sugar fragments formed by acid hydrolysis were examined by GLC-MS analysis. The methylated uronic acid which could not be detected as the acetyl derivative after borohydride reduction was identified as the methyl ester of methyl glycoside. Figure 1 (A, B) shows GLC separation of the methylated sugars from the methylated T-19 polysaccharide, revealing 2,3,4-tri-*O*-methyl-L-fucose, 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,4,6-tri-*O*-methyl-D-mannose, 4,6-di-*O*-methyl-D-mannose, 3,4-di-*O*-methyl-D-xylose, and 2,3,4-tri-*O*-methyl-D-glucuronic acid. It is noted that the alditol acetates of 4,6-di- and 2,6-di-*O*-methyl-D-mannose give close retention times under the condition employed. However, they could be distinguished from each other by mass spectrometric analysis. Similarly, the alditol acetates derived from 2,3- and 3,4-di-*O*-methyl-D-xylose had very close retention times, and gave the same mass spectrum. However, these two sugars could be distinguished by reduction with borodeutride followed by acetylation (see, Fig. 1 B).

TABLE IV. COMPARISON OF THE PROPERTIES OF EXTRACELLULAR AND FRUIT BODY POLYSACCHARIDES OF *T. fuciformis*

Polysaccharide	[α] _D (water)	$S_{20,w}$	Intrinsic viscosity (25°C)	Carbohydrate composition ^b				
				<i>O</i> -acetyl	Mannose	Fucose	Xylose	Glucuronic acid
Fruit body (HAS-S) ^a	-4.5°	2.18S	5.42	—	4.9	0	1.0	2.77
Extracellular								
T-19	-27°	3.12S	9.10	10.36%	2.1	0.45	1.0	0.8
T-7	-6°	2.28S	6.31	8.19%	3.48	0.11	1.0	1.31

^a Extracted with alkali.

^b Molar ratios of neutral sugars were estimated by GLC, as the alditol acetates, and glucuronic acid was estimated by carbazole method.⁷⁾

FIG. 1. Gas-liquid Chromatographic Profiles of the Hydrolysis Products of the Methylated acidic polysaccharide of *T. fuciformis* strain T-19.

(A) Separated as the methyl glycosides. Condition: 15% butane-diol succinate polyester on Neosorb N. C., at 175°C. (I) and (II) 2,3,4-tri-*O*-methyl-D-xylose; (III) 2,3,4-tri-*O*-methyl-L-fucose; (IV) and (VI) presumably, 3,4-di-*O*-methyl-D-xylose; (V) 2,3,4,6-tetra-*O*-methyl-D-mannose; (VII) and (VIII) methyl ester of 2,3,4-tri-*O*-methyl-D-glucuronic acid; (IX) 2,4,6-tri-*O*-methyl-D-mannose; (X) 4,6-di-*O*-methyl-D-mannose.

(B) Separated as the alditol acetates. Condition: 3% ECNSS-M on Gas Chrom Q, at 180°C. (I) 2,3,4-tri-*O*-methyl-L-fucose; (II) 2,3,4-tri-*O*-methyl-D-xylose; (III) 2,3,4,6-tetra-*O*-methyl-D-mannose; (IV) 3,4-di-*O*-methyl-D-xylose; (V) 2,4,6-tri-*O*-methyl-D-mannose; (VI) 4,6-di-*O*-methyl-D-mannose. The figure also shows the primary mass fragments of 1(D),2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-D-xylitol.

TABLE V. IDENTITIES AND MOLAR RATIOS OF METHYL SUGAR COMPONENTS OF POLYSACCHARIDES OF *T. fuciformis*

The methylated polysaccharides were hydrolyzed and the methyl sugar fragments were analyzed by GLC-MS.

<i>O</i> -methyl sugar	Linkage indicated	Polysaccharide		
		Fruit body	Extracellular	
			T-19	T-7
2,3,4-Tri- <i>O</i> -methyl-D-xylose	[Xyl] 1→	1.0	1.0	1.0
2,3,4-Tri- <i>O</i> -methyl-L-fucose	[Fuc] 1→	—	0.46	0.1
2,3,4,6-Tetra- <i>O</i> -methyl-D-mannose	[Man] 1→	0.1	0.77	0.32
3,4-Di- <i>O</i> -methyl-D-xylose	→2 [Xyl] 1→	0.4	1.15	trace
2,4,6-Tri- <i>O</i> -methyl-D-mannose	→3 [Man] 1→	1.45	0.46	1.25
4,6-Di- <i>O</i> -methyl-D-mannose	→ ₃ ² [Man] 1→	3.44	4.0	2.77
2,3,4-Tri- <i>O</i> -methyl-D-glucuronic acid	[GlcUA] 1→	1.61	1.77	1.30

The presence of the primary fragments, *m/e* 190 and 117, indicates that peak IV corresponds to 1 (D), 2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-D-xylitol.

In Table V, identities and molar ratios of the methylated sugar components of three polysaccharides are compared. It is evident that these three polysaccharides possess similar branched structures, consisting of a back bone

chain of (1→3)-linked D-mannose residues, some of which are branched at the C-2 positions with D-xylose, D-mannose and D-glucuronic acid residues. In addition, T-7 and T-19 polysaccharides have probably single L-fucosyl side chains. It may be noteworthy from the immunochemical view point that T-19 and fruit body polysaccharides have short side chains of (1→2)-linked D-xylose

residues, which are absent in T-7 polysaccharide. (Sone and Misaki, unpublished results)

Smith degradation

The glycosidic linkages suggested by the methylation analysis were supported by the results of Smith degradation, which involves periodate oxidation, borohydride reduction and acid hydrolysis.⁹⁾ Before oxidation, the extracellular polysaccharides were deacetylated by treatment with 0.1 N sodium hydroxide at room temperature. Each of three polysaccharides was oxidized with 0.05 M sodium periodate at 5°C. The consumption of periodate and production of formic acid with the polysaccharides are listed in Table VI. On com-

TABLE VI. PERIODATE OXIDATION OF THE EXTRACELLULAR AND FRUIT BODY POLYSACCHARIDE OF *T. fuciformis*

Polysaccharide of	Consumption of periodate (mol. prop. ^a)	Production of formic acid (mol. prop.)
Fruit body (HAS-A)	0.91	0.35
Extracellular (T-19)	0.89	0.34
Extracellular (T-7)	0.72	0.28

^a Moles per hexose unit. After the oxidation was completed (20°C, 4 days) the plots on a graph were extrapolated at the time zero.

plete oxidation, the oxidized polysaccharides were reduced with sodium borohydride to the corresponding polyalcohols (yields, 60~65 mg from 100 mg of polysaccharide). All polysaccharide polyalcohols gave on complete acid hydrolysis mannose as the main products, together with glycerol and a small amount of ethylene glycol, and propane-1,2-diol (T-7 and T-19), the latter originating from the terminal L-fucose residues. When the polysaccharide-polyalcohol from T-19 was subjected to mild acid hydrolysis with 0.1 N sulfuric acid at 25°C for 20 hr (mild Smith degradation,¹⁰⁾ the degraded polysaccharide was obtained as insoluble residue. It was methylated, and on acid hydrolysis, 2,4,6-tri-*O*-methyl-D-mannose was found to be in a predominant amount, with traces of 2,3,4,6-tetra-, and 4,6-di-*O*-methyl-D-mannose, confirming the presence of

(1→3)-linked D-mannan back-bone chain. As to the anomeric configuration of the mannose residues, the high optical rotations of (1→3)-linked mannans both from T-19 (+76°) and T-7 (+71.1°) indicate that the mannosidic linkages are in α -type.

Partial acid hydrolysis

In order to know the arrangements of D-glucuronic acid residues in the polysaccharide chain, the extracellular polysaccharide of T-7 was hydrolyzed with 0.5 N sulfuric acid for 5 hr at 95°C. The hydrolyzate was neutralized (BaCO₃), and the filtrate was decationized with Amberlite IR 120 (H⁺-form). The solution was then passed through a column (2×13 cm) of Dowex-I resin (formate form), and after elution of the neutral sugars with water, the acidic components were eluted with 1 N formic acid, after which the formic acid in the eluate was distilled off by evaporation. Paper chromatography (solvent C) revealed the presence of three components, which corresponded, respectively, to glucuronolactone, glucuronic acid and aldobiouronic acid. The aldobiouronic acid ([α]_D−33° (*c*, 2.0, water), extracted from the paper with water, gave on heating with cationic resin (Amberlite IR-120), glucuronic acid and mannose together with aldobiouronic acid. Methylation, and acid hydrolysis of this compound yielded 2,3,4-tri-*O*-methylglucuronic acid and 3,4,6-tri-*O*-methyl-D-mannose (ratio, 0.85:1.0), identified by GLC as their methylglycosides. Thus, the aldobiouronic acid was identified as 2-*O*- β -(D-glucuronosyl)-D-mannose, which was already isolated from the polysaccharides of *Xanthomonas oryzae*,¹¹⁾ and also other species of *Tremella*.¹²⁾

DISCUSSION

It is well known that the fungi belonging to *Heterobasidiaceae* form waxy or even cartilaginous fruit bodies, but the structures of polysaccharides which are major constituents of the jelly-like mushrooms have not been fully elucidated. As reported in our previous paper, two types of polysaccharides, *i.e.*, a β -(1→3)-

D-glucan with many single branches and an acidic polysaccharide which contains α -(1 $\rightarrow$ 3)-linked D-mannan back-bone with side chains of D-glucuronic acid and D-xylose residues, are present in the fruit body of *Auricularia auricula-judae* (Kikurage).¹⁾ The present study has revealed that the fruit body of *Tremella fuciformis* Berk (Shirokikurage), also belonging to the same group, contains a water-soluble D-glucurono-D-xylo-D-mannan whose structure appears to resemble that of Kikurage polysaccharide. However, unlike Kikurage fruit body, careful fractionation afforded very small amounts of neutral polysaccharides (Fractions, HWS-N and HAS-N; see Table I) both composed of glucose, xylose and mannose, suggesting that it is essentially free from β -D-glucan.

Acidic extracellular polysaccharides were also obtained from the culture filtrate of the haploid cells of two strains of *Tremella fuciformis* (strain T-7 and T-19), in high yields, when grown in the synthetic medium containing appropriate carbon sources, *e.g.*, glucose, mannose, xylose or sucrose, and yeast-extract. These polysaccharides were purified through formation of an insoluble cetylpyridinium chloride complex. It is interesting that these extracellular polysaccharides contain a small proportion of L-fucose, in addition to D-glucuronic acid, D-xylose and D-mannose, and that they are partially acetylated (see, Table IV).

These three polysaccharides isolated from the fruit body and from the cultural filtrates may have different molecular sizes as suggested from their sedimentation constants and intrinsic viscosities (see, Table IV). However, their basic structural features must be very similar as suggested by the results of methylation analysis. The acid hydrolyzates of methylated polysaccharides contain commonly 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl- and 4,6-di-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-xylose and also 2,3,4-tri-*O*-methyl-D-glucuronic acid. In addition, the extracellular polysaccharides contain terminal L-fucose residues, while T-19 and fruit body

polysaccharides contain (1 $\rightarrow$ 2)-linked D-xylose residues. These polysaccharides have highly branched structures consisting of (1 $\rightarrow$ 3)-linked D-mannose residues to which D-xylose, D-glucuronic acid, D-mannose and L-fucose (T-19 and T-7) residues are attached at their C-2 positions. These L-fucose, D-xylose and D-xylose and D-glucuronic acid residues are usually located as single unit branches or as short chains, in cases of the fruit body and T-19 extracellular polysaccharides where D-xylose residues may form (1 $\rightarrow$ 2)-linked short side chains and act as strain specific immunodeterminants.

In connection to these branched structures, Ukai *et al.* recently fractionated the fruit body polysaccharides of *T. fuciformis* Berk, harvested in China.^{3,4)} From the methylated polysaccharides, they obtained by GLC analysis, 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-glucose, 4,6-di-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-glucuronic acid, together with small proportions of 3,4-, 2,4- and 2,3-di-*O*-methyl-D-xyloses.⁴⁾ However, our polysaccharide preparations purified by cetylpyridinium complex formation contain no glucose, and the presence of (1 $\rightarrow$ 2)-linked D-xylose units could be confirmed by means of GLC-MS analysis of the di-*O*-methyl-D-xylose as the alditol acetate using sodium borodeuteride reduction. The presence of α -(1 $\rightarrow$ 3)-linked D-mannan back bone was confirmed by the examination of the products from the mild Smith degradation. The degraded polysaccharide fraction ($[\alpha] + 76^\circ$) was solely composed of D-mannose, and methylation analysis showed it to be a linear (1 $\rightarrow$ 3)-linked D-mannan, as those from D-glucurono-D-xylo-D-mannans of Kikurage,¹⁾ and of *Cryptococcus laurentii*.¹³⁾

The isolation of 2-*O*- β -(D-glucuronosyl)-D-mannose from the partial acid hydrolyzate of the polysaccharide indicates that β -linked D-glucuronic acid residues are attached to the C-2 positions of the mannan back bone chain to form single unit-long branches. As regards the anomeric configuration of D-xylose re-

FIG. 2. Possible Features of Repeating Units of Acidic Heteropolysaccharides of *Tremella fuciformis*.

(A) Fruit body polysaccharide.

(B) Extracellular T-19 and T-7 polysaccharide. (The inside of parentheses [] shows the lack of sugar residue in T-7 polysaccharide.)

sidues, comparison of the optical rotations of the native polysaccharides (fruit body, $[\alpha]_D -2^\circ$; T-7, $[\alpha]_D -6^\circ$) and the back-bone mannan ($[\alpha]_D +76^\circ$) suggests that the (1 $\rightarrow$ 2)-linked D-xylose residues are in the β -type and D-mannose residues are in the α -type, respectively.

Thus, in Fig. 2 repeating units of the three polysaccharides are depicted. Although the basic structures of these polysaccharides are essentially the same, the modes of attachments of side chains may vary depending on the individual fungus. Such a basic structure appears to be also common to the polysaccharide of cell-walls of the haploid cells of *T. fuciformis*,²⁾ as well as to the capsular polysaccharide of *Cryptococcus neoformans* and the related *Cryptococcus* species, as revealed by chemical and immunochemical investigations.^{13, 15)}

In the view of immunochemical specificity of this type of polysaccharides, it has been known that the acidic polysaccharides of *C. laurentii* and *T. mesenterica* are cross reactive with Type II Pneumococcal capsular polysaccharide, having the immunodeterminant of terminal D-glucuronosyl units.¹⁴⁾ The acidic polysaccharides of the fruit body of *T. fuci-*

formis was also reported to react with the anti-type II Pneumococcal serum.⁴⁾ Our recent immunochemical investigation clearly indicated that D-glucurono-(D-fuco)-D-xylo-D-mannans of *Tremella* species contain two types of immunodeterminants, i.e., the β -D-glucuronic acid units which are common to all the polysaccharides, and β -(1 $\rightarrow$ 2)-linked D-xylosyl side chains which appear to be specific to particular strains of *Tremella* species.¹⁵⁾ Thus, species of *Cryptococcus* seem to be very close phylogenically to *T. fuciformis* T-7 whose polysaccharide is devoid of β -(1 $\rightarrow$ 2)-linked D-xylosyl side chains, whereas many of species of *Tremella* are rather close to *T. fuciformis* T-19. More detailed structural and immunochemical relationships between *Tremella* and *Cryptococcus* species are being investigated.

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