Isolation and Characterization of Polysaccharides of "Kikurage," Fruit Body of *Auricularia auricula-judae*[†]

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Two kinds of β -D-glucans and an acidic heteropolysaccharide were isolated from the fruit body of *Auricularia auricula-judae*, and their structural features were elucidated. A water soluble glucan ($[\alpha]_D -10^\circ$) consists of a backbone chain of β -(1 $\rightarrow$ 3)-linked D-glucose residues, two out of three glucose residues being substituted at the C-6 positions with single glucose units. The other glucan, which was obtained as the hot alkali insoluble residue, is also β -(1 $\rightarrow$ 3)-glucan with single branches at C-6 positions, but it has an extremely highly branched structure; a small proportion of (1 $\rightarrow$ 6)-internal linkages may be situated in the side chains.

An acidic heteropolysaccharide ($[\alpha]_D -20^\circ$), isolated from the hot water extract through insoluble complex formation with cetylpyridinium chloride, contains D-xylose, D-mannose, D-glucose and D-glucuronic acid (molar ratio, 1.0:4.1:1.3:1.3). Methylation followed by acid hydrolysis of the polysaccharide yielded 2,3,4,6-tetra-O-methyl-D-mannose(glucose), 2,3,4-tri-O-methyl-D-xylose, 2,4,6-tri-, 4,6-di-, and 2,4-di-O-methyl-D-mannose, 2,4-di-O-methyl-D-glucose, together with 2,3,4-tri-O-methyl-D-glucuronic acid, suggesting that it consists of a backbone chain of (1 $\rightarrow$ 3)-linked mannose residues, which are attached with D-xylose, D-mannose and D-glucuronic acid residues at the C-2 or C-6 positions.

On the basis of these findings, the constitution of the *A. auricula-judae* was compared with that of *Tremella fuciformis*.

"Kikurage," the fruit body of *Auricularia auricula-judae*, which belongs to the family of *Heterobasidiaceae* has long been used as a popular edible mushroom. In a series of studies on edible fungal polysaccharides, a D-glucurono-D-xylo-D-mannan was isolated from the fruit body of *Tremella fuciformis*, called "Shirokikurage," also an edible mushroom, and also from the culture of its yeast-like haploid cells.^{1,2)} Since *A. auricula-judae* and *T. fuciformis* fall under the same family "*Heterobasidiaceae*," our attention has been drawn toward the chemical nature of their polysaccharides which constitute mainly the fruit bodies of an ear-like shape.

EXPERIMENTAL PROCEDURES

General methods. Evaporations were carried out

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under diminished pressure at temperatures not exceeding 37°C, unless otherwise stated.

Paper chromatography was carried out using Toyo Roshi No. 50 or Whatman 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-propanol-ethanol-water (6:1:3). Sugars on a paper chromatogram were detected with alkaline-silver nitrate or *p*-anisidine hydrochloride in butanol-saturated water.

Gas liquid chromatography (glc) of neutral sugars and their methyl derivatives was usually performed after conversion into their corresponding alditol acetates, with a Shimadzu gas chromatograph Model GC-6AM fitted with a flame-ionization detector using a glass column (0.4 $\times$ 200 cm) packed with 3% ECNSS-M on Gas Chrom Q, at 190°C and 180°C (methyl sugars).

Hydrolysis of polysaccharides was achieved by formolysis followed by heating with trifluoroacetic acid (TFA). A sample (ca. 10 mg) was heated, first, with 90% formic acid (1 ml) at 100°C for 16 hr in a screw capped vial, and after evaporation the residue was heated with 4 N TFA (0.5 ml) further for 6 hr. After TFA was removed by repeated evaporation with water, the sugars in the hydrolyzate were analyzed by glc or paper chromatography.

Determination of carbohydrate was generally made by phenol sulfuric acid method.³⁾ Colorimetric determination of uronic acid was done by the carbazole method.⁴⁾

Isolation of polysaccharides. Dried fruit body of *A. auricula-judae* (ca. 50 g) available commercially was immersed in 0.9% aqueous sodium chloride (saline, 500 ml) overnight, and cut into pieces, and then homogenized with a Waring blender. The homogenized material was extracted with saline at cold for 24 hr, and centrifuged. To the supernatant solution was added 3 volumes of ethanol to give precipitation of polysaccharide (yield: 0.48 g). The residue was suspended in water (500 ml) and autoclaved at 120°C for 20 min. The insoluble residue was treated two times by the same procedure. The extracts were combined and the polysaccharide in the extracts was precipitated with ethanol (Fraction I, yield: 2.0 g). In order to purify the acidic polysaccharide, the water-extract which contained uronic acid was dissolved into 0.01 M sodium sulfate (1000 ml), to which was added cetylpyridinium chloride (CPC, 6 g) until no more precipitate formed. The precipitate was collected by centrifugation and dissolved in 10% sodium chloride solution (400 ml). After removing a small amount of insoluble matter, the solution was dialyzed, and then poured into ethanol (2 volumes), which gave precipitation of an acidic polysaccharide (Fraction IA, yield: 1.3 g). The supernatant solution of the precipitate was dialyzed and, a neutral polysaccharide in non-dialyzable solution was precipitated by the gradual addition of ethanol (Fraction IB, yield: 0.2 g). The insoluble residue after hot water extraction was extracted with 1 N sodium hydroxide, at 65°C for 2 hr, under nitrogen atmosphere. The extract was neutralized with hydrochloric acid, dialyzed, and the polysaccharide was precipitated with ethanol (Fraction II, yield: 4.7 g). Since Fraction II also contained an acidic polysaccharide, it was purified as CPC-complex as the case of the hot water extract (Fraction I). However, in the supernatant solution of the insoluble complex, no appreciable amount of polysaccharide was precipitated by the addition of ethanol, indicating the absence of a neutral polysaccharide. The residue left after alkaline extraction was washed thoroughly with water and dried *in vacuo* (Fraction III, yield: 41.0 g).

Glc analysis of acidic polysaccharide. The purified acidic polysaccharide (Fraction IA, 10 mg) was hydrolyzed with 90% formic acid followed by 4 N TFA, as described previously. After the acids were distilled off, the hydrolyzate was reduced with sodium borohydride, whereby the neutral monosaccharide and uronic acid were converted into their corresponding alditols and aldonic acid, respectively. A portion (5 mg) of the mixture was acetylated by heating with a mixture of

acetic anhydride and pyridine (1:1, 0.2 ml) at 100°C for 1 hr, and the resulting alditol acetates were analyzed by glc. The remainder of the reduced hydrolyzate was dried up in a vacuum desiccator, and heated with methanol containing dried Amberlite IR 120 H⁺ form resin (ca. 100 mg/ml of methanol) at 100°C for 1 hr, in a sealed vial according to the procedure of Sømme.⁵⁾ After the reaction was completed, the resin was removed, and the solution containing alditols and methyl-ester(s) of aldonic acid(s) was concentrated. The mixture was reduced with sodium borohydride as before. After the excess reagent was decomposed by the addition of Amberlite IR 120 (H⁺-form), boric acid was removed by repeated evaporations with methanol in the usual way. The resulting mixture of alditols was acetylated as before, and analyzed by glc in the same condition as used previously. The uronic acid(s) in the acidic polysaccharide could be identified and simultaneously quantitated by comparison of the proportions of particular alditols in the two hydrolyzates, one with methanolysis followed by reduction and the other without methanolysis. When equal moles of D-glucuronic acid and D-galacturonic acid were treated in the same way described above, the recovery of galacturonic acid as the galactitol was 73% of glucuronic acid (glucitol).

Methylation analysis. Since the alkali-insoluble polysaccharide (Fraction III), which contains only glucose, was not soluble in dimethyl sulfoxide, it was methylated, first, by the method of Muskat in liquid ammonia.⁶⁾ The dried polysaccharide (600 mg) was dispersed in liquid ammonia by stirring at -60°C, and methylated by addition of metallic sodium (500 mg) and methyl iodide (12 ml) for 1.5 hr. After one more treatment with the same reagents, the ammonia was evaporated at room temperature. A portion (20 mg) of the partially methylated polysaccharide was then dissolved in dimethyl sulfoxide (2 ml), and methylated with methylsulfinyl carbanion (0.5 ml) and methyl iodide (1.5 ml) according to the procedure of Hakomori.⁷⁾ The reaction mixture was dialyzed, concentrated to a small volume, and the methylated product was extracted with chloroform. If methylation was not complete, as judged from the infrared spectrum (OH-band at 3400~3600 cm⁻¹), the methylation procedure was repeated. The water soluble glucan obtained from the neutral fraction of the hot water extract (Fraction I), and also the acidic polysaccharide were methylated by the method of Hakomori.

The methylated polysaccharides were heated with 90% formic acid at 100°C for 12 hr, and after formic acid was distilled off, the residue was further heated with 2 N TFA at 100°C for 5 hr. The hydrolyzate was repeatedly evaporated in the presence of water, and the methylated sugars were reduced with sodium boro-

hydride, and converted into their corresponding alditol acetates, and were analyzed by glc.

RESULTS AND DISCUSSION

Isolation of polysaccharides

The dried fruit body of *A. auricula-judae* (Kikurage) was homogenized in saline, and polysaccharides were extracted successively with saline at a cold, hot water at 120°C (Fraction I), and 1 N sodium hydroxide at 65°C under a nitrogen atmosphere (Fraction II). The polysaccharides in the water extract were further fractionated by treatment with CPC to give an acidic polysaccharide (Fraction IA) and a neutral polysaccharide (Fraction IB, designated glucan I). The polysaccharide in each fraction was purified generally by repeated fractional precipitations with ethanol, and its carbohydrate components were examined by glc, after hydrolysis with acid.

The alkali-insoluble residue (Fraction III), which accounts for approximately 80% of the fruit body, gave on acid hydrolysis only glucose, and designated glucan II. The procedure for fractionation is shown in Scheme I. The three kinds of polysaccharides, glucan I and II, and the acidic polysaccharide from water extract seem to represent major constituents of fruit body, therefore, their structural features were investigated.

β -D-Glucans

The water-soluble glucan (glucan I), isolated from the hot-water extract had $[\alpha]_D -10^\circ$ ($c=1.5$, 0.5 N NaOH), and showed an absorption band at 890 cm^{-1} in the infrared spectrum characteristic of β -D-configuration. The alkali-insoluble glucan (glucan II) was also shown by its infrared spectroscopy to have β -D-glucosidic linkages.

Both glucans were methylated and their methyl sugar fragments were analyzed by gas liquid chromatography-mass spectrometry (glc-ms). The complete methylation of the water-soluble glucan was performed by two treatments with methylsulfinyl carbanion and methyl iodide by the method of Hakomori. Methylation of glucan II, which was not soluble in dimethyl sulfoxide, was achieved by treatments with metallic sodium and methyl iodide in liquid ammonia by the method of Muskat, and then twice methylation by the method of Hakomori. The methylated glucan I and glucan II were hydrolyzed with acid and the methyl sugar fragments were analyzed by glc-ms, as their corresponding alditol acetates. Figure 1 shows the glc profile of the hydrolysis products of the methylated alkali-insoluble glucan (glucan II), revealing the presence of 2,3,4,6-tetra-, 2,4,6-tri- and 2,4-di-*O*-methyl-D-glucose, together with a small amount of 2,3,4-tri-*O*-methyl-D-glucose. The methylated

SCHEME I. Fractionation of Polysaccharides from Fruit Body of "Kikurage"

FIG. 1. Gas-liquid Chromatographic Separation of the Hydrolysis Products of Methylated Glucan II as Their Alditol Acetates.

Condition: 3% ECNSS-M, at 180°C; (I) 2,3,4,6-tetra-*O*-methyl-D-glucose; (II) 2,4,6-tri-*O*-methyl-D-glucose; (III) 2,3,4-tri-*O*-methyl-D-glucose; (IV) 2,4-di-*O*-methyl-D-glucose.

TABLE I. IDENTITIES AND MOLAR RATIOS OF THE HYDROLYSIS PRODUCTS OF METHYLATED GLUCAN I AND GLUCAN II

The methylated glucans were hydrolyzed and the methyl sugar fragments were analyzed by GLC, as described in EXPERIMENTAL PROCEDURES.

<i>O</i> -Methyl-D-glucose	Linkage indicated	Molar ratio	
		Glucan I	Glucan II
2,3,4,6-tetra-	[Glc]1→	1.0	1.0
2,4,6-tri-	→3[Glc]1→	0.5	0.3
2,3,4-tri-	→6[Glc]1→	—	0.1
2,4-di-	→6[Glc]1→	1.1	0.9
	3 ↑		

glucan I also gave a similar glc profile except for the absence of 2,3,4-tri-*O*-methyl-D-glucose. In Table I, the identities and molar ratios of the D-glucosidic linkages in the two types of glucans are compared. From Table I, it is evident that the basic structural features of the two glucans are similar to each other. They consist of backbone chains of β -(1→3)-linked glucose residues attached with many branches at the C-6 position. However, the alkali-insoluble glucan II appears to have unusual

structural features with an extremely high degree of branches. The methylation data suggested that three out of four (1→3)-linked D-glucose residues are substituted mostly with single glucose units, although small proportion of internal (1→6)-D-glucosidic linkages are also present.

In order to obtain more knowledges in the sequences of sugar residues in glucan II, it was subjected to Smith degradation. The glucan II (500 mg) was oxidized with 0.05 M sodium periodate (125 ml) at 4°C in the dark. At suitable time intervals, periodate consumption was determined by arsenite⁸⁾ and formic acid production by titration with 0.01 M sodium hydroxide. On the oxidation (8 days), the glucan consumed 0.92 mol of periodate with concomitant liberation of 0.42 mol of formic acid per glucose residue, in fairly good agreement with the result of methylation analysis. The oxidized glucan was reduced with sodium borohydride to the glucan polyalcohol, which yielded by complete acid hydrolysis (2 N TFA, at 100°C, 6 hr) an approximately equal molar proportion of glycerol and glucose, as ex-

aminated by glc. When the glucan polyalcohol was hydrolyzed with 0.1 N sulfuric acid at 25°C for 20 hr (controlled Smith degradation⁹⁾), only glycerol was detected in the soluble fraction, leaving an insoluble degraded polysaccharide which accounted for 61% of the parent glucan. The degraded-glucan, which became soluble in dimethyl sulfoxide, was methylated by Hakomori's method, and after hydrolysis the hydrolyzate was examined by glc. The methylated degraded-glucan yielded on acid hydrolysis, predominant amounts of 2,4,6-tri-*O*-methyl-D-glucose, with traces of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose. This result is a strong indication that the consecutive β -(1 $\rightarrow$ 3)-linked D-glucose residues are the backbone chain of this highly branched molecule. The facts that the β -(1 $\rightarrow$ 3)-linked degraded glucan is still a large molecule, and that there was only glycerol and no β -D-glucosylglycerol or any other glycerol-D-glucoside was detected in the supernatant solution, suggest that the (1 $\rightarrow$ 6)-linked glucose units are located mostly in the side chains. These findings provide unambiguous evidence for β -(1 $\rightarrow$ 3)-linked backbone with numerous branches attached by (1 $\rightarrow$ 6)-linkages.

A possible structural model of the alkali insoluble glucan can be illustrated as Fig. 2B. The water-soluble glucan has a less branched β -(1 $\rightarrow$ 3)-linked chain, with a repeating unit of five glucose residues, where two out of three β -(1 $\rightarrow$ 3)-linked D-glucose residues of the backbone chain are attached with single D-glucose units at the C-6 positions, as shown in Fig. 2A.

FIG. 2. Structure of Repeating Unit of (A) Glucan I and (B) Glucan II.

There was no internal (1 $\rightarrow$ 6)-linkages in the side chains.

Additional evidence for such a structure was provided by the use of the purified exo-type β -D-glucanase of *Basidiomycete* species QM 806,¹⁰⁾ which was kindly provided by Prof. S. Kirkwood of University of Minnesota. This enzyme can hydrolyze the scleroglucan-type polysaccharide from the end of chain, with liberation of glucose and gentiobiose.¹¹⁾ Glucan II (5 mg) was suspended in 0.05 M acetate buffer, pH 4.8 (1 ml) and incubated in a shaker at 37°C with β -1,3-glucanase (about 100 units). After 24 hr incubation, the solubilized digest was collected by centrifugation and the products were examined by paper chromatography (solvent c), which showed two spots corresponding to glucose and gentiobiose. After extraction of these sugars with water from the paper, they were colorimetrically determined by phenol-sulfuric acid method. The result showed that the molar ratio of gentiobiose to glucose 2.5:1.0, is in fairly good agreement with the value calculated from Fig. 2B.

Since Johnson *et al.* first reported the structure of β -D-glucan from *Sclerotium glucanicum*,¹²⁾ glucans containing β -D-(1 $\rightarrow$ 3)- and β -D-(1 $\rightarrow$ 6)-linkages have been recognized as a common type of glucans of fungi, in the fruit bodies or in a culture broth, *e.g.*, *Schizophyllum commune*,¹³⁾ *Lentinus edodes*¹⁴⁾ and *Helvella lacunosa*.¹⁵⁾ However, the water-soluble and alkali-insoluble glucans having the structures such as Fig. 2A and 2B are more branched than sclerotial β -D-glucan, one out of three β -(1 $\rightarrow$ 3)-linked D-glucose residue of the backbone having single branches.

With regard to the physical properties of these glucans, it is noteworthy that the alkali-insoluble glucan (glucan II) having highly branched structure is neither soluble in alkali nor dimethyl sulfoxide, whereas the less branched glucan (glucan I) is readily soluble in water. There may be an explanation that numerous single branches attached to most of (1 $\rightarrow$ 3)-linked glucose residues of the backbone chain would provide certain structural re-

gularity which develops strong hydrogen-bondings between neighboring side chains to form a stable frame work. Alternatively, such insolubility characteristic of highly branched glucan would be attributable to its huge molecular size.

In connection with this, it is interesting that the Smith-degraded polysaccharide which has no branch is not soluble in cold water, but became soluble either in alkali or dimethyl sulfoxide. Thus, the degree of substitutions of (1→3)-linked D-glucose residues with single branches may affect the solubility characteristics of such a type of β -D-glucan, as already reported.¹³⁾

There have been several reports concerning antitumor activities of β -1,3-, 1,6-glucans, such as scleroglucan¹⁶⁾ and lentinan.¹⁷⁾ In relationship between the structures and activities of this type of glucans, it is interesting that the water-soluble glucan I exhibited potent activity, while the alkali-insoluble glucan II appeared to be less active.¹⁸⁾

Acidic polysaccharide

Successive extractions of the fruit body of *A. auricula-judae* with hot water and with warm dilute alkali yielded acidic heteropolysaccharides, containing uronic acid residue. The purified polysaccharide from the hot water extract through CPC complex formation had $[\alpha]_D -20^\circ$, and was composed of xylose, mannose, glucose and glucuronic acid, as revealed by paper chromatography and gas liquid chromatography, (molar ratio; 1.0: 4.1: 1.3: 1.3). The D-glucuronic acid in the hydrolyzate was identified by glc as the trimethylsilyl derivative of the corresponding methyl glycoside of methyl ester. For quantitative glc analysis of the component sugars, both neutral and acidic sugars as their corresponding alditol acetates, the complete acid hydrolyzate (90% formic acid, 12 hr, and 4 N TFA 12 hr at 100°C) was divided into two portions. One was treated by the usual way, which involved borohydride reduction and acetylation by heating with dry pyridine and acetic anhydride. The remainder was subjected to, borohydride

FIG. 3. Gas-liquid Chromatographic Separation of the Hydrolysis Products of Acidic Polysaccharide (Fraction IA), as Alditol Acetates.

(a) After borohydride reduction and acetylation, and (b) after borohydride reduction, methylesterification with cation resin, borohydride reduction and acetylation.

Condition: 3% ECNSS-M, at 190°C; (I) D-xylitol, (II) D-mannitol, (III) D-glucitol. The molar ratio of each component is indicated under the corresponding peak.

reduction, methyl esterification of the aldonic acid derived from uronic acid by heating with dry cation resin in methanol, re-treatment with borohydride, and then acetylation. By a series of above reaction, an uronic acid is converted into the corresponding alditol acetate, and easily analyzed by glc. The reliability of this procedure was confirmed with known amounts of authentic uronic acid, such as D-glucuronic acid and D-galacturonic acid. The details of this improved method for glc determination of uronic acids will be reported elsewhere. Figure 3 shows the glc separation of the polysaccharide hydrolyzate with and without methyl esterification. From the quantification of each component, the increase in the relative amount of glucitol in (a) compared with that in (b) should correspond to the presence of D-glucuronic acid. Thus, the molar ratio of xylose: mannose: glucose: glucuronic acid = 1.0: 4.1: 1.3: 1.3. The content of D-glucuronic acid being in a good agreement that estimated by colorimetric determination with carbazole reagent (uronic acid 18%).

The purified polysaccharide was methylated by the method of Hakomori, and the fully

methylated polysaccharide was hydrolyzed with acid, and partially methylated sugars were analyzed by glc-ms. As shown in Fig. 4, gas liquid chromatography revealed the presence of 2,3,4-tri-*O*-methyl-D-xylose (peak I, T_g 0.67), 2,3,4,6-tetra-*O*-methyl-D-mannose or D-glucose (peak II, T_g 1.0), 2,4,6-tri-*O*-methyl-D-mannose (peak III, T_g 2.0), 4,6-di-*O*-methyl-D-mannose (peak IV, T_g 3.3), and a mixture of 2,4-di-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-mannose (peak V_a and V_b, T_g 5.1 and 5.4). As regards peak V, glc-ms indicated both the faster (V_a) and slower moving part (V_b) were 2,4-di-*O*-methyl hexoses, and their retention times corresponded to 2,4-di-*O*-methyl-D-glucose (T_g , 5.1) and 2,4-di-*O*-methyl-D-mannose (T_g 5.4), respectively. Since some methyl derivatives of D-glucose and D-mannose have similar retention times, and cannot be separated from each other, under the present glc condition, there are possibilities that peak II and III contain small proportion of 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-D-glucose, respectively. The presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid, which could not be resolved under this condition, was confirmed by glc as

their methyl glycoside methyl ester on a column of 15% butanediol succinate polyester at 175°C (retention times relative to β -methyl-D-glucoside, 2.5 and 3.2). Thus, the molar ratios of the methyl sugar fragments are listed in Table II. From the inspection of the table, it is evident that the polysaccharide has relatively high branched structure. The fact that (1→3)-linked D-mannose residues as only non-terminal units, some of which are branched by (1→2) and also (1→6)-linkages, strongly suggests that the polysaccharide has (1→3)-linked mannan backbone chain, though there is a possibility that a small proportion of (1→3)-linked glucose units are also present. The D-xylose and D-glucuronic acid are both located only in the terminal ends, by attaching to the mannan backbone at C-2 or C-6 position, to form a highly branched structure. Thus, its major basic features appear to resemble those of the acidic polysaccharide from the fruit body of *T. fuciformis*, the extracellular polysaccharide and also cell wall polysaccharide of its haploid cells, as established by our previous works.^{1,2)} They are built up with α -(1→3)-linked mannan backbone to which

FIG. 4. Gas-liquid Chromatographic Separation of the Hydrolysis Products of the Methylated Acidic Polysaccharide (Fraction IA), as Their Alditol Acetates.

Condition: 3% ECNSS-M, at 180°C; (I) 2,3,4-tri-*O*-methyl-D-xylose, (II) 2,3,4,6-tetra-*O*-methyl-D-mannose (glucose), (III) 2,4,6-tri-*O*-methyl-D-mannose, (IV) 4,6-di-*O*-methyl-D-mannose, (V_a) 2,4-di-*O*-methyl-D-glucose, (V_b) 2,4-di-*O*-methyl-D-mannose.

TABLE II. MOLAR RATIOS OF THE HYDROLYSIS PRODUCTS OF METHYLATED ACIDIC POLYSACCHARIDE

The methylated acidic polysaccharide was hydrolyzed and the methyl sugar fragments were analyzed by GLC, as described in EXPERIMENTAL PROCEDURES.

O-Methyl sugar	Linkage indicated	T _g ^{a)}	Molar ratio
2,3,4-Tri-O-methyl-D-xylose	[Xyl]1→	0.67	1.0
2,3,4,6-Tetra-O-methyl-D-mannose(glucose)	[Man]1→	1.0	1.1
2,4,6-Tri-O-methyl-D-mannose(glucose)	→3[Man]1→	2.03	1.8
4,6-Di-O-methyl-D-mannose	→3[Man]1→	3.31	1.6
2,4-Di-O-methyl-D-glucose	→3[Glc]1→	5.10	} 1.8
2,4-Di-O-methyl-D-mannose	→3[Man]1→	5.37	
2,3,4-Tri-O-methyl-D-glucuronic acid	[GlcUA]1→		1.3

^{a)} Retention time relative to that of 2,3,4,6-tetra-O-methyl-D-glucose

β -linked D-glucuronic acid residues and single or short chains of (1→2)-linked D-xylose units are attached by (1→2)-linkages.

Unlike the acidic polysaccharide of *T. fuciformis* fruit body, the acidic polysaccharide of *A. auricula-judae*, purified through the insoluble CPC-complex formation, contained a small but significant proportion of D-glucose residues. The identification of 2,4-di-O-methyl-D-glucose in the hydrolyzate of the methylated polysaccharide indicates at least the presence of the branch points by double substitutions at the C-3 and C-6. In addition, there may be small proportions of (1→3)-internal glucosidic linkages and non-reducing terminal groups. Although the location of these glucose residues in the polysaccharide are not clear from the present data available. In this connection, it may be noteworthy to point out that the alkali-insoluble cell wall polysaccharide of haploid cell of *T. fuciformis* contains the acidic polysaccharide moiety consisting α -(1→3)-linked mannan backbone, with branches of D-xylose and D-glucuronic acid side chains, and the branched β -glucan moiety consisting of (1→3) and (1→6)-glucosidic linkages.²⁾

This structural resemblance between polysaccharides of *A. auricula-judae* and *T. fuciformis* was supported by the immunochemical studies. When the present polysaccharide was reacted with rabbit antisera against the haploid cell of *T. fuciformis*, precipitation occurred, and the quantitative precipitin curve was very similar to that of the extracellular heteropolysaccharide of *T. fuciformis*.

This suggests that antigenic determinants of both the polysaccharides, involving either the terminal xylose or glucuronic acid residues, must be the same.

In addition to *T. fuciformis* polysaccharide, the isolation of several fungal heteropolysaccharides containing α -(1→3)-linked mannan backbone chains have been reported, e.g., xylomannans from the mycelia of *Polyporus tumulosus*¹⁹⁾ and *Armillaria mellea*,²⁰⁾ fucosyl-xylomannans from the fruit body of *Polyporus pinicola*.²¹⁾ The xylomannan type polysaccharides are produced by some yeasts belonging to *Trichosporon* species.²²⁾ The alkali extract of *Fomes marginatus* (Fr.) Gill contains a polysaccharide composed of glucose, mannose, xylose and fucose residues, where (1→3)-linked mannosyl main chain is substituted with xylosyl, mannosyl, fucosyl side chains.²³⁾ However, the occurrence of polysaccharides containing glucuronic acid residues, in addition to mannose and xylose residue seem to be limited to *Tremella* sp., *Aurulia* sp., and *Cryptococcus* sp.²⁴⁾ A structural investigation of the extracellular polysaccharide of a non-pathogen *Cryptococcus laurentii* NRRL Y-1401 suggested that the heteropolymer containing D-xylose, D-mannose and D-glucuronic acid in the ratios of 2:6:1 consists of (1→3)-linked mannan main chain, and D-xylosyl and D-glucuronosyl side chains are attached to at C-2 of the mannan.²⁵⁾ Our recent studies showed that the purified capsular polysaccharide of *C. neoformans* is highly reactive with antisera against *T. fuciformis*.²⁶⁾

From these chemical and immunochemical

studies, it may be reasonable to conclude that *Auricularia auricula-judae*, and *Tremella fuciformis* and other *Tremella* species, both groups belonging to the same *Heterobasidiaceae*, and also the group of *Cryptococcus* species have very close phylogenetic relationships. In the view of chemical taxonomy, further investigations on the more precise structures of polysaccharides of *A. auricula-judae* and *T. fuciformis*, involving fragmentation analyses, are being undertaken.

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REFERENCES

- 1) A. Misaki and K. Tubaki, Abstracts of Papers, Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April, 1974, p. 83.
- 2) Y. Sone and A. Misaki, Abstracts of Papers, Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, April, 1976, p. 223.
- 3) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 4) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).
- 5) R. Sømme, *Carbohydr. Res.*, **43**, 145 (1975).
- 6) I. E. Muskat, *J. Am. Chem. Soc.*, **56**, 693, 2449 (1934).
- 7) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
- 8) P. F. Fleury and J. Lange, *J. Pharm. Chem.*, **17**, 107, 196 (1933).
- 9) I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, Abstracts of Papers, American Chemical Society, 135, 3D (1959).
- 10) F. I. Huotari, T. E. Nelson, F. Smith and S. Kirkwood, *J. Biol. Chem.*, **243**, 952 (1968).
- 11) T. E. Nelson, J. Johnson, E. Jantzen and S. Kirkwood, *ibid.*, **244**, 5972 (1969).
- 12) J. Johnson, Jr., S. Kirkwood, A. Misaki, T. E. Nelson, J. V. Scaletti and F. Smith, *Chem. Ind. (London)*, **1963**, 820.
- 13) S. Kikumoto, T. Miyajima, T. Yoshizumi, S. Fujimoto and K. Kimura, *Nippon Nôgeikagaku Kaishi*, **44**, 337 (1970).
- 14) M. Shida, T. Mase, Y. Sasakawa and K. Matsuda, *ibid.*, **45**, 454 (1971).
- 15) K. Kawaguchi, and A. Misaki, Abstracts of Papers, Annual Meeting of the Agricultural Chemical Society of Japan, Kyoto, April, 1976, p. 220.
- 16) P. P. Singh, R. L. Whistler, R. Tokuzen and W. Nakahara, *Carbohydr. Res.*, **37**, 245 (1974).
- 17) T. Sasaki and N. Takasuka, *ibid.*, **47**, 99 (1976).
- 18) Sasaki and Misaki, unpublished result.
- 19) B. J. Ralph and V. J. Bender, *Chem. Ind. (London)*, **1965**, 1181.
- 20) H. O. Bouveng, R. N. Fraser and B. Lindberg, *Carbohydr. Res.*, **4**, 20 (1967).
- 21) R. N. Fraser, S. Karacsonyi and B. Lindberg, *Acta Chem. Scand.*, **21**, 1783 (1967).
- 22) P. A. J. Gorin and J. F. T. Spencer, *Can. J. Chem.*, **46**, 2299 (1968).
- 23) J. Rosik, V. Zitko, S. Bauer and J. Kubala, *Collection Czech. Chem. Commun.*, **31**, 3751 (1966).
- 24) J. Gorin and J. F. T. Spencer, *Adv. Carbohydr. Chem.*, **23**, 410 (1968).
- 25) M. J. Abercrombie, J. K. N. Jones, M. V. Lock, M. B. Perry and R. J. Stoodley, *Can. J. Chem.*, **38**, 1617 (1960).
- 26) Sone, Torii and Misaki, unpublished result.