Structures of the Cell Wall Polysaccharides of *Tremella fuciformis*[†]

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The cell wall fraction prepared from the yeast-like cells of *Tremella fuciformis* was fractionated by proteinase digestion, hot-water and alkaline extractions. The acidic polysaccharide, which may originate from the outer layer of the cell wall, was purified from the hot-water extract. It was composed of D-glucuronic acid, D-mannose and D-xylose (molar ratio, 0.5: 3.8: 1.0). The methylated polysaccharide yielded on acid hydrolysis 2,3,4-tri-O-methyl-D-xylose, 2,3,4,6-tetra-O-methyl-, 2,4,6-tri-O-methyl- and 4,6-di-O-methyl-D-mannose, and 2,3,4-tri-O-methyl-D-glucuronic acid (molar ratio, 1.0: 0.8: 2.7: 2.3: 0.5) together with a trace of 3,4-di-O-methyl-D-xylose, suggesting that the polysaccharide consists of a backbone of (1→3)-linked D-mannose residues, some of which are substituted at the C-2 positions with single or short side chains of D-xylose, D-mannose and D-glucuronic acid residues.

The alkali-insoluble residue of the cell wall was composed of D-glucose, D-glucuronic acid, D-mannose and D-xylose (molar ratio, 4.3: 0.6: 2.5: 1.0). Methylation and periodate oxidation studies suggested it to comprise two polysaccharide moieties, i.e., β -D-glucan and glucurono-xylo-mannan, the structure of the latter resembling that of the cell surface acidic polysaccharide. The glucan moiety was shown by methylation and enzymatic degradation to have a branched structure consisting of β -(1→3)- and (1→6)-linkages (approx. ratio, 2: 3). The controlled Smith degradation of the alkali-insoluble polysaccharide yielded an insoluble, degraded, predominantly (1→3)-linked gluco-mannan which may represent the backbone of the cell wall polysaccharide. On the basis of these findings, the constitution of the cell wall is discussed.

Species of the basidiomycetous genus *Tremella* occur in nature in the bisexual (dikaryotic) form, while haploid strains derived from single basidiospores are known to grow by budding to form yeast-like cells. Our previous studies showed that haploid strain of *Tremella fuciformis* produce a large amount of D-glucurono-D-xylo-D-mannan into the culture medium, and its structure was established.¹⁾ The information on the structure of yeast cell wall has hitherto been restricted to *Saccharomyces cerevisiae*, *Candida albicans* and some species of *Schizosaccharomyces*. Only a few members of the group basidiomycetous yeasts were investigated.²⁾

During the isolation of β -D-mannosidase and β -N-acetyl-D-glucosaminidase from the cell

extract of *Tremella fuciformis*,³⁾ our attention has been drawn toward the cell wall composition and structure of the haploid cell of *T. fuciformis*. This paper is concerned with fractionation of the cell wall of *T. fuciformis* and elucidation of detailed structure of the two polysaccharides, i.e., a glucurono-xylo-mannan from the outer cell wall, and an alkali-insoluble glucurono-xylo-manno-glucan which may be responsible for rigidity of the cell wall.

EXPERIMENTAL PROCEDURES

General methods. Evaporations were carried out under diminished pressure at temperatures not exceeding 37°C, unless otherwise stated.

Paper chromatography was carried out using Toyoshiki No. 50 or Whatman 3MM paper (preparative chromatography), with the following solvent systems (v/v): (a) 1-butanol-pyridine-water (6: 4: 3), (b) butanone-water azeotrope, (c) *tert*-amyl alcohol-formic acid-water (4: 1: 1.5), (d) 1-propanol-ethanol-water (6: 1: 3), (e) 1-butanol-acetic acid-water (50: 12: 25).

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Sugars on a paper chromatogram were detected with alkaline-silver nitrate or *p*-anisidine hydrochloride in 1-butanol-saturated water.

Gas-liquid chromatography (glc) of neutral sugars and their methyl derivatives were 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 × 200 cm) packed with 3% ECNSS-M on Gas Chrom Q, at 190°C and 180°C (methyl sugars). Methylated sugars were also analyzed as their methyl glycosides using a column packed with 15% butandiol succinate polyester on Neosorb W at 175°C.

Hydrolysis of polysaccharide 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 8 hr in a screw capped vial, and after evaporation the residue was further heated with 2 N TFA (0.5 ml) for 6 hr. After TFA was removed by repeated evaporations 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.⁴⁾ Colorimetric determination of uronic acid was done by the carbazole method.⁵⁾

Ultracentrifugation was conducted in a Beckman Model L3-50 ultracentrifuge at 44,800 rpm.

Preparation of the cell wall. The haploid cells of *T. fuciformis* Berk (strain T-7), which was kindly provided by Dr. Tubaki, was grown at 30°C for 4 days in a 30 liters jar fermentor which contained a medium (25 liters) consisting of glucose (2%), yeast extract (0.5%), KH_2PO_4 (0.05%) and K_2HPO_4 (0.05%). The cultured cells were collected by centrifugation, washed

three times with 0.9% aqueous sodium chloride (saline), and disintegrated in the presence of 0.05 M phosphate buffer, pH 7.0, by a Sorvall-Ribi cell fractionator Model RF-1 with the pressure of 40,000 psi, or by sonication at 20 KHz for 30 min at 5°C. The insoluble materials were collected by centrifugation at $12,600 \times g$ for 30 min, and washed three times with water, and re-suspended in water. The suspension was centrifuged again at $7600 \times g$ for 30 min to remove the intact cells, and the cell wall fraction in the supernatant was then collected by centrifugation at $14,000 \times g$ for 20 min and freeze-dried. This cell wall preparation contained about 2% nitrogen. The photograph of the freeze-dried cell wall preparation taken by a scanning electron microscope showed no intact cell (see, Fig. 1).

Fractionation procedure of the cell wall. First, the cell wall preparation (*ca.* 3 g) was suspended in 0.05 M phosphate buffer, pH 7.8 (100 ml), and incubated under gentle shaking, with proteinase (25 mg, Pronase E, Kakenkagaku) in the presence of one drop of toluene at 37°C. After 72 hr, the reaction mixture was heated at 85°C to inactivate the proteinase, and centrifuged. The supernatant was dialyzed against running water for 2 days, and added into 3 volumes of ethanol to precipitate polysaccharide (yield: 16.2 mg, Fraction E-S). The residue was suspended in 0.05 M citrate buffer, pH 7.0 (100 ml) and autoclaved at 120°C for 30 min. The insoluble residue was treated two times by the same procedure. The extracts were combined, dialyzed, and poured into ethanol to precipitate polysaccharide (182 mg). In order to purify the acidic polysaccharide, the water-extracted polysaccharide was dissolved into 0.01 M sodium sulfate (20 ml), to which was added cetylpyridinium chloride (CPC, 540 mg) until no more precipitate formed. The precipitate was collected by centrifugation and dissolved in 10% sodium chloride

FIG. 1. Scanning Electron Photomicrograph of the Cell Wall Preparation of *T. fuciformis*, Enlarged 1500 Times.

solution (40 ml). After removal of a small amount of insoluble matter, the solution was dialyzed and then poured into ethanol (2 volumes), to precipitate acidic polysaccharide (yield: 110 mg, Fraction HW-S). The residue left after proteinase digestion and hot-water extraction was suspended in 1 N sodium hydroxide solution (20 ml) and stirred under nitrogen atmosphere at room temperature for 6 hr, and allowed to stir overnight. The mixture was centrifuged and the supernatant was neutralized with acetic acid to give a small amount of precipitate (7 mg, Fraction AS-I). The extract after neutralization was dialyzed and poured into ethanol to precipitate polysaccharide (yield: 92 mg, Fraction AS-II). The residue left after cold alkaline extraction was extracted with 1 N sodium hydroxide solution (20 ml) at 70°C for 3 hr under nitrogen atmosphere. The residue was collected by centrifugation and washed thoroughly with water and dried *in vacuo* (yield: 2360 mg, Fraction HA-R-I). The extract was neutralized with acetic acid, dialyzed, and the polysaccharide was precipitated with ethanol (yield: 57 mg, Fraction HA-S).

Methylation analysis. Since the alkali-insoluble polysaccharide (Fraction HA-R-III) was not soluble in dimethyl sulfoxide (DMSO), it was methylated, first, by the method of Muskat in liquid ammonia.⁶³ The dried polysaccharide (700 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 two more treatments with the same reagents, the ammonia was evaporated at room temperature. A portion (20 mg) of the partially methylated polysaccharide was dissolved in DMSO (2 ml) under ultrasonication (20 KHz, 5 min), in nitrogen atmosphere, and then methylated by the method of Hakomori, with fresh methylsulfinyl carbanion (0.5 ml) and methyl iodide (1.5 ml).⁷⁷ After the reaction was completed, 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 hot-water extracted polysaccharide (Fraction HW-S) and the mild Smith degraded polysaccharide, both of which were soluble in DMSO, were methylated by the method of Hakomori.

A portion of the fully methylated polysaccharide was 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 borohydride, and converted into their corresponding alditol acetates, and were analyzed by glc. Another portion of the methylated polysaccharide was treated with 3% methanolic hydrogen chloride (0.5 ml) in a

sealed tube at 100°C for 16 hr. The methanolizate was neutralized with silver carbonate, and filtrated, concentrated to a syrup, and then analyzed by glc.

RESULTS AND DISCUSSION

Fractionation and chemical constitution of the cell wall

For the chemical analysis of the cell wall preparation, it was hydrolyzed with acid, and the acidic and neutral sugars in the hydrolyzate were separated by use of a column of Dowex-I (formate form, 2.0 × 13 cm).

The neutral carbohydrate composition was glucose, mannose and xylose in a molar ratio of 5.6:3.6:1.0, as revealed by glc after conversion into the alditol acetates. The acidic sugar(s) adsorbed on the column was eluted with 1 N formic acid. After evaporation of the eluate, the acidic sugar(s) was converted into barium salt(s), and was reduced to the corresponding aldonic acid(s), which was subsequently converted to its 1,4-lactone(s) according to the method of Perry *et al.*⁸¹ The spot of the resulting lactone coincided in position with that of authentic L-gulonolactone on paper chromatogram using solvent (c). Furthermore, the trimethylsilyl (TMS) derivative of the lactone had the same retention time as that of the TMS derivative of the authentic L-gulonolactone on glc (column; Neopentyl glycol succinate on Chromosorb W) at 175°C. Thus, the acidic component of the cell wall was identified as D-glucuronic acid. The colorimetric determination with carbazole reagent indicated that the content of D-glucuronic acid in the cell wall was 6.7%.

The cell wall preparation of *T. fuciformis* was fractionated according to Scheme 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 yields and chemical compositions of polysaccharide fractions are listed in Table I.

The hot-water extracted fraction (HW-S) was further purified through CPC insoluble

SCHEME 1. Fractionation of the Cell Wall of *Tremella fuciformis*.TABLE I. CARBOHYDRATE COMPOSITION OF THE POLYSACCHARIDE FRACTIONS FROM THE CELL WALL OF *Tremella fuciformis*

The neutral carbohydrate composition was determined by glc as their alditol acetates, and the content of D-glucuronic acid was determined colorimetrically.

Fraction ^a	Yield (%)	Composition (molar ratio)			
		Glucose	Man-nose	Glucuronic acid	Xylose
Whole cell wall	—	5.6	3.6	0.7	1.0
E-S	0.6	0.2	2.1	0.6	1.0
HW-S	4.2	—	3.8	0.5	1.0
AS-I	0.4	16.1	2.2	0.6	1.0
AS-II	3.5	6.2	2.0	0.4	1.0
HA-S	2.2	4.4	2.4	0.4	1.0
HA-R-I	89.3	5.2	2.8	0.7	1.0
HA-R-II	—	4.6	2.7	0.6	1.0
HA-R-III	—	4.3	2.5	0.6	1.0

^a see, SCHEME 1.

complex formation, which gave an acidic heteropolysaccharide consisting of D-xylose, D-mannose and D-glucuronic acid in the molar ratio of 1.0: 3.8: 0.5; no glucose was detected.

The alkali-insoluble residue (Fraction HA-R-1), which accounted for approximately 89% of the cell wall, gave on acid hydrolysis D-xylose, D-mannose, D-glucose and D-glucuronic acid. Although this fraction was further treated twice with 1 N sodium hydroxide solution at 70°C, the carbohydrate composition of this fraction scarcely changed (see Table I).

Among various fractions, the particular two polysaccharide fractions, Fraction HW-S and Fraction HA-R, seem to represent major constituents of the cell wall of *T. fuciformis*. Therefore, their chemical structures were investigated by methylation and periodate oxidation techniques.

Water-soluble glucurono-xylo-mannan (Fraction HW-S)

The purified acidic heteropolysaccharide from the hot-water extraction of the cell wall with CPC had $[\alpha]_D -26.2^\circ$ ($c=1.0$, water), and was composed of D-xylose, D-mannose and D-glucuronic acid in the molar ratio of 1.0: 3.8:

FIG. 2. Gas-liquid Chromatographic Separation of the Hydrolysis Products of the Methylated Water-soluble Glucurono-xylo-mannan (Fraction HW-S) as Their Alditol Acetates.

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

0.5, as revealed by paper chromatography (solvent a) and glc. The homogeneity of this water-soluble glucurono-xylo-mannan was assessed from the ultracentrifugal pattern. The polysaccharide was methylated by the method of Hakomori. The resulting fully methylated polysaccharide was hydrolyzed with acid, and the partially methylated sugars were analyzed by glc. As shown in Fig. 2, 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 (peak II, T_g 1.0), 2,4,6-tri-*O*-methyl-D-mannose (peak IV, T_g 2.0) and 4,6-di-*O*-methyl-D-mannose (peak V, T_g 3.3) together with a trace of 3,4-di-*O*-methyl-

D-xylose (peak III). The presence of 2,3,4-tri-*O*-methyl-D-glucuronic acid was confirmed by glc as its methyl glycoside methyl ester. Thus, the molar ratios of the methyl sugar fragments are listed in Table II. The methylation data strongly suggest that the polysaccharide has (1→3)-linked mannan backbone chain, two out of five mannose residues are branched at C-2, and D-xylose and D-glucuronic acid residues are both located only at the terminal ends. Figure 3 illustrates one of possible structures of the glucurono-xylo-mannan. Such structural feature may be similar to that of the extracellular polysaccharide of its own strain (T-7).¹⁾

TABLE II. MOLAR RATIOS OF THE HYDROLYSIS PRODUCTS OF THE METHYLATED WATER-SOLUBLE GLUCURONO-XYLO-MANNAN (Fraction HW-S) OF THE CELL WALL OF *T. fuciformis*

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

<i>O</i> -Methyl sugar	Linkage indicated	T_g^a	Molar ratio
2,3,4-Tri- <i>O</i> -methyl-D-xylose	[Xyl]1→	0.67	1.0
2,3,4,6-Tetra- <i>O</i> -methyl-D-mannose	[Man]1→	1.0	0.8
3,4-Di- <i>O</i> -methyl-D-xylose	→2[Xyl]1→	1.54	trace
2,4,6-Tri- <i>O</i> -methyl-D-mannose	→3[Man]1→	2.0	2.7
4,6-Di- <i>O</i> -methyl-D-mannose	→3[Man]1→	3.29	2.3
2,3,4-Tri- <i>O</i> -methyl-D-glucuronic acid	[GlcUA]1→		0.5

^a Retention time relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose.

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), 3,4-di-*O*-methyl-D-xylose (Peak III, T_g 1.54), a mixture of 2,4,6-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose (peak IV, T_g 2.0), 2,3,4-tri-*O*-methyl-D-glucose (peak V, T_g 2.47), 4,6-di-*O*-methyl-D-mannose (peak VI, T_g 3.29), and 2,4-di-*O*-methyl-D-glucose (peak VII, T_g 5.15). Since some methyl derivatives of D-mannose and D-glucose, such as 2,3,4,6-tetra-*O*-, 2,4,6-tri-*O*-methyl derivatives have similar retention times, and cannot be separated from

each other, under the present glc condition, there are possibilities that peak II and peak IV contain some proportions of the corresponding methyl derivatives of D-glucose, respectively. The coexistence of 2,4,6-tri-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose was confirmed by glc as their methyl glycosides (column: 15% Butanediol succinate polyester at 175°C: retention times, 3.82: 2,4,6-tri-*O*-methyl-D-mannose, 3.31 and 4.88: 2,4,6-tri-*O*-methyl-D-glucose, respectively). The 2,3,4-tri-*O*-methyl-D-glucuronic acid was also identified by glc

FIG. 4. Gas-liquid Chromatographic Separation of the Hydrolysis Products of the Methylated Alkali-insoluble Polysaccharide (Fraction HA-R-III) as Their Alditol Acetates.

Condition: 3% ECNSS-M on Gas Chrom Q, at 180°C; (I) 2,3,4-tri-*O*-methyl-D-xylose; (II) 2,3,4,6-tetra-*O*-methyl-D-mannose (glucose); (III) 3,4-di-*O*-methyl-D-xylose; (IV) 2,4,6-tri-*O*-methyl-D-mannose (glucose); (V) 2,3,4-tri-*O*-methyl-D-glucose; (VI) 4,6-di-*O*-methyl-D-mannose; (VII) 2,4-di-*O*-methyl-D-glucose.

TABLE III. MOLAR RATIOS OF THE HYDROLYSIS PRODUCTS OF THE METHYLATED ALKALI-INSOLUBLE POLYSACCHARIDE OF THE CELL WALL OF *T. fuciformis*

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

<i>O</i> -Methyl sugar	Linkage indicated	T_g^a	Molar ratio
2,3,4-Tri- <i>O</i> -methyl-D-Xylose	[Xyl]1→	0.67	1.0
2,3,4,6-Tetra- <i>O</i> -methyl-D-Mannose	[Man]1→	1.0	] 1.9
2,3,4,6-Tetra- <i>O</i> -methyl-D-Glucose	[Glc]1→	1.0	
3,4-Di- <i>O</i> -methyl-D-Xylose	→2[Xyl]1→	1.54	0.9
2,4,6-Tri- <i>O</i> -methyl-D-Mannose	→3[Man]1→	2.0	] 3.9
2,4,6-Tri- <i>O</i> -methyl-D-Glucose	→3[Glc]1→	2.0	
2,3,4-Tri- <i>O</i> -methyl-D-Glucose	→6[Glc]1→	2.47	4.1
4,6-Di- <i>O</i> -methyl-D-Mannose	→3[Man]1→	3.29	2.2
2,4-Di- <i>O</i> -methyl-D-Glucose	→3[Glc]1→	5.15	0.6
2,3,4-Tri- <i>O</i> -methyl-D-Glucuronic acid	[GlcUA]1→		1.1

^a Retention time relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose.

as its methyl glycoside methyl ester (retention times relative to that of methyl-2,3,4,6-tetra-*O*-methyl- β -D-glucopyranoside, 2.5 and 3.2). The identities and molar ratios of the methyl sugar fragments are listed in Table III. From the inspection of the table, it is evident that the polysaccharide has a relatively highly branched structure, in which non-terminal units consist of (1 $\rightarrow$ 3)-linked D-mannose residues and (1 $\rightarrow$ 3) linked and (1 $\rightarrow$ 6)-linked D-glucose residues. The presence of 4,6-di-*O*-methyl-D-mannose and 2,4-di-*O*-methyl-D-glucose indicates that (1 $\rightarrow$ 3)-linked D-mannose and D-glucose residues are branched at the C-2 and C-6 positions, respectively, or alternately (1 $\rightarrow$ 6)-linked D-glucose residues may be branched at the C-3 positions. Most D-xylose residues and D-glucuronic acid residues may be located only in the terminal ends; some of the mannose units are also located in the side chains.

In order to obtain more knowledges in the sequences of the sugar residues, the polysaccharide was subjected to Smith degradation, which involves periodate oxidation, borohydride reduction and hydrolysis with acid. The polysaccharide (200 mg) was suspended and oxidized with 0.05 M sodium periodate (100 ml) at 4°C in a dark. At suitable time intervals, periodate consumption was determined by arsenite method,¹⁸⁾ and formic acid production by titration with 0.01 N sodium hydroxide. When the oxidation was completed (7 days, consumption of periodate 1.55 mol, and production of formic acid, 0.22 mol per hexose unit), the oxidized polysaccharide, which was still insoluble, was collected by centrifugation and suspended in water and reduced with sodium borohydride (yield, 30 mg). The insoluble polysaccharide polyalcohol yielded by acid hydrolysis (2 N TFA, 100°C, 6 hr) glycerol, mannose and glucose in the molar ratio of 1.0: 2.6: 5.4. To the supernatant solution, ethylene glycol was added and the solution was dialyzed against distilled water, and the non-dialyzable fraction was reduced with sodium borohydride, and neutralized with acetic acid. The neutralized solution was passed through an Amberlite IR-120 (H⁺-

form) column, and the eluate was evaporated to dryness. The boric acid in the residue was removed by repeated evaporations with methanol (yield of the reduced polysaccharide, 110 mg). The soluble polysaccharide polyalcohol gave by acid hydrolysis glycerol, mannose and glucose in the molar ratio of 1.0: 0.4: 0.1. In another experiment, both soluble and insoluble polysaccharide polyalcohols were hydrolyzed with 0.1 N sulfuric acid at 25°C for 20 hr (controlled Smith degradation¹⁹⁾), and structures of the degraded polysaccharides from the mild hydrolyzates were examined by methylation. Both methylated degraded-polysaccharides yielded on acid hydrolysis, predominant amounts of 2,4,6-tri-*O*-methyl-D-mannose (D-glucose) with trace amounts of 2,3,4,6-tetra-*O*-methyl-D-mannose (D-glucose) and 4,6-di-*O*-methyl-D-mannose, as revealed by glc as their alditol acetates. Gas-liquid chromatography as their methyl glycosides showed that the ratio of 2,4,6-tri-*O*-methyl-D-mannose to 2,4,6-tri-*O*-methyl-D-glucose is about 4: 1 and 1: 4, in the methanolizate of the methylated degraded-polysaccharide from the soluble portion and that from the insoluble portion, respectively. These results strongly indicate that (1 $\rightarrow$ 3)-linked D-manno-glucan is the backbone of this polysaccharide.

Additional data were provided by examination of the enzyme digestion using the purified exo- β -1,3-D-glucanase of *Basidiomycete* QM 806,²⁰⁾ 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.²¹⁾ The native polysaccharide (5 mg) was suspended in 0.05 M of acetate buffer, pH 4.8 (1 ml) and incubated in a shaker at 37°C with the β -1,3-glucanase (about 100 units). After 24 hr incubation, the reaction mixture was centrifuged, and the solubilized product in the supernatant solution was examined by paper chromatography (solvent d), which showed two spots corresponding to glucose and gentiobiose, respectively. The insoluble residue after the enzyme digestion gave by acid hydrolysis

D-xylose, D-mannose and D-glucose in the molar ratio of 1.0: 2.6: 1.8, as revealed by glc, and also D-glucuronic acid by paper chromatography. When the Smith degraded polysaccharide (insoluble portion) was treated with the same enzyme, the product in the solubilized digest was only glucose, while the acid hydrolysis of the insoluble residue yielded D-mannose and a trace of D-glucose.

The glucan moiety consists of β -(1 $\rightarrow$ 3)- and (1 $\rightarrow$ 6)-linked glucose residues, as indicated by methylation analysis and degradation by exo- β -1,3-glucanase digestion. In the methylation analysis, the ratio of 2,4,6-tri-*O*-methyl-D-mannose to 2,4,6-tri-*O*-methyl-D-glucose was estimated to 1: 2 by glc as their methyl glycosides. Although the amount of 2,3,4,6-tetra-*O*-methyl-D-glucose cannot be estimated by glc, on the assumption that the proportion of 2,3,4,6-tetra-*O*-methyl-D-glucose coincides with that of 2,4-di-*O*-methyl-D-glucose, the molar ratio of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri- and 2,4-di-*O*-methyl-D-glucose should be 1.0: 4.3: 6.8: 1.0.

The foregoing experimental findings may provide a possible structural model of the alkali-insoluble polysaccharide of the cell wall of *T. fuciformis* as illustrated in Fig. 5. The cell wall polysaccharide appears to comprise two moieties, *i.e.*, β -glucan and glucurono-

xylo-mannan. The glucurono-xylo-mannan may consist of (1 $\rightarrow$ 3)-linked D-mannan chain to which single or short side chains of D-xylose, D-mannose and also D-glucuronic acid residues are attached at the C-2 positions of the mannose residues. This structure is similar to those of the glucurono-xylo-mannans isolated from the water-extract of the cell wall, and also produced extracellularly during growth of the same strain in a glucose medium.¹⁾ Although the structural features of the glucan moiety may be somewhat similar to that of the yeast (*Saccharomyces cerevisiae*) cell wall glucan,²²⁾ the glucan moiety of *T. fuciformis* cell wall appears to contain a higher proportion of (1 $\rightarrow$ 6)-glucosidic linkages than ordinary yeast β -glucan. As regards the overall arrangements of D-glucosidic linkages in the glucan moiety, the knowledges obtained from Smith degradation and the enzymatic degradation appear to be in favour of a branched structure consisting of a backbone chain of β -(1 $\rightarrow$ 3)-linked sugar residues which are attached with (1 $\rightarrow$ 6)-linked side chains, however, a possibility that (1 $\rightarrow$ 6)-linked glucose units are also located in the backbone chain cannot be ruled out (see, Fig. 5). In relation to the content of (1 $\rightarrow$ 6)-linked glucose units, Manners *et al.* reported the presence of β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 6)-glucans, separately, in baker's yeast.²³⁾

FIG. 5. A Possible Structure of the Alkali-insoluble Polysaccharide.

However, our recent reinvestigation showed that the highly purified homogeneous β -glucan contains both (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 6)-D-glucosidic linkages.²⁴⁾

With regard to a possibility of the molecular heterogeneity of the alkali-insoluble polysaccharide, the facts that the carbohydrate composition scarcely changed by repeated treatments with hot alkali, and that the glucan and glucurono-xylo-mannan could not be separated by extraction with DMSO, indicate the two moieties are covalently bound or at least joined by some strong bonds to form a rigid structure of the cell wall of *T. fuciformis*. Moreover, it was noted that the methylated polysaccharide dissolved in DMSO gave a single peak in ultracentrifugation. It is interesting that in fractionation of fruit-bodies of *T. fuciformis*, and also of *Auricularia auricula-judae*, the complete separation of the acidic polysaccharide from the glucan was readily achieved by dilute alkaline extraction.^{1,9)} Such a difference of the constitutions between the cell wall in the yeast-like haploid cells and the fruit-body may reflect the necessity of particular rigid architecture of the cell envelope during the growth of the cells in the liquid environments.

The recent work by Reid *et al.*²⁵⁾ suggested the presence of α -(1 $\rightarrow$ 3)-glucan in the cell wall of the yeast-like cells of *Tremella mesenterica*. Bacon *et al.*²⁶⁾ also reported the occurrence of α -(1 $\rightarrow$ 3)-glucan in the cell wall of *Cryptococcus*, *Schizosaccharomyces* and *Polyporus* sp. In the view of chemical taxonomy, further investigations on the other fractions, *e.g.*, Fraction AS-I and Fraction AS-II, of the cell wall of *T. fuciformis* are being undertaken.

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