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Studies on Polysaccharides and their related Glycosidases of Heterobasidiae, *Tremella fuciformis* and *Auricularia auricula-judae*

1978

Yoshiaki Sone

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Chapter 1

Introduction

Polysaccharide occurs in nature as a major component of living matter. In the microbial kingdom, various types of fungal polysaccharides have been found. The feature of fungal polysaccharides that is most striking is that relatively few of them are structurally similar to polysaccharides from other natural sources. Over 75,000 distinct species of fungi are known, and several polysaccharides are often associated with the cell of each species. It is therefore scarcely surprising that new polysaccharides are frequently discovered, especially in view of the techniques that are now available for fractionation of polysaccharides and their fragments.

The properties of fungal polysaccharides have been investigated in relation to biological functions, taxonomy and potential utilizations. In the field of industrial utilization, many extracellular polysaccharides that usually form viscous solutions have been studied because of their unique properties fit to food and pharmaceutical uses.

Among various fungal polysaccharides, a well known polysaccharide is pullulan (1), a linear polymer containing mainly maltotriose residues joined by α-D-(1→6)-linkages, which is produced by the yeast-like fungus Pullularia pullulans. Recently, its useful application to the field of food industry, such as food-packing films appears to be promising. Another example is sclerotial glucans, β-D-glucans consisting of a backbone of β-D-(1→3)-linked D-glucopyranosyl residues, some of which are substituted at C-6 with a D-glucosyl side chain. They are produced by Sclerotium glucanicum (2), Claviceps species (3) and Corticium species (4). Nemerous uses have been suggested for the glucan; these include uses in oil-well

drilling-mud, paper binders, water-soluble material coating and water-based paper thickeners.

On the other hand, chemical and immunological properties of mannans of the pathogenic Candida albicans, the causative agent of thrush, and galactomannans of Trichophyton species, have been investigated in relation to the antigenicity (5, 6).

Generally speaking, previous investigations of the molecular structures and biological properties of the fungal polysaccharides have been restricted to the classes Phycomycetes (water and bread molds) and Ascomycetes (yeast, and mold), and only a few studies of the polysaccharides of the higher fungi of Basidiomycetes have been reported.

A well known polysaccharide of the Basidiomycetes is schizophyllan, of which structure is similar to that of scleroglucan, produced by Schizophyllum commune (7). Recent years, antitumor polysaccharides have been obtained from a number of sources, such as yeast, fungi, bacteria and plants. It is noteworthy that one of the most active polysaccharides, lentinan, was isolated from the Basidiomycetous fungi, Lentinus edodes, which is one of the popular edible mushrooms in Japan (8). Recent studies (9) showed that the structure of lentinan is a branched molecule having a backbone of (1→3)-β-D-glucan and β-D-(1→6)-linked D-glucose residues, together with a few internal β-D-(1→6)-linkages. In addition to the β-glucans of the Basidiomycetes, Bjorndal and Lindberg (10) reported the isolation and characterization of a manno-fuco-galactan and a glucurono-glucan from the fruit bodies of the fungi Polyporus fomentarius and Polyporus igniarius.

The class Basidiomycetes is divided into three sub-classes, the Hemibasidiae, the Heterobasidiae and the Homobasidiae, according to the form of basidium. The fungi mentioned above, Lentinus, Schizophyllum and

Polyporus are belonging to the Homobasidiae. It is well known that many species, which belong to the Heterobasidiae, produce fruit bodies which are waxy or even cartilagious. However, the precise structures of the polysaccharides of the jelly fungi, the Heterobasidiae, have not been elucidated. Therefore, the author attempted to clarify the chemical nature of the polysaccharides which constitute the fruit bodies of Tremella and Auricularia, which are belonging to the Heterobasidiae.

In the course of investigations on the chemical nature of the polysaccharides from the edible fruit bodies of A. auricula-judae and Tremella fuciformis, the author became aware that the haploid cells of T. fuciformis produce an acidic heteropolysaccharide in the culture broth abundantly. Slodki and associates (11) have suggested a possible taxonomic relationship between Tremella and Cryptococcus, based on similar carbon-assimilation patterns, morphology and polysaccharide production. Organisms of the both genera produce partly acetylated heteropolysaccharide containing glucuronic acid, mannose and xylose.

The first investigation on the polysaccharide of Cryptococcus neoformans was made by Evans et al.(12). They reported that the crude polysaccharide of type B cell was separated into two polysaccharides through fractional precipitation using lead acetate, and the precipitated polysaccharide was consisted of D-xylose, D-mannose and D-glucuronic acid, and non-precipitated one was consisted of D-galactose and D-xylose. Abercrombie and et al.(13) investigated on the polysaccharide of C. laurentii NRRL Y-1401, and revealed the presence of two types of polysaccharides in the culture broth, a polysaccharide consisted of D-xylose, D-mannose and D-glucuronic acid and a glucan. The acidic polysaccharide was further elucidated and found to be consisted of D-mannose, D-xylose and D-glucuronic acid, in the

molar ratio of 4:1:1, and partly acetylated (14). Harada et al. (15) reported formation and chemical composition of the extracellular polysaccharides produced by some species of genus Rhodotorula from the view of phylogenic relationship between subgenus Rubrotorula and subgenus Flavotorula. The later subgenus includes Cryptococcus neoformans and C. laurentii.

The present thesis is mainly concerned with two types of polysaccharides, glucurono-xylo-mannan and β-1,3-, 1,6-glucans of Tremella fuciformis and Auricularia auricula-judae, particularly their molecular structures. Glucurono-xylo-mannans were isolated from the culture filtrate of the haploid cells of T. fuciformis and the hot water extracts of the cell wall of the haploid cells of T. fuciformis and the fruit body of A. auricula-judae. β-Glucans were present in the cell wall of T. fuciformis as a part of the alkali-insoluble fraction, and in the fruit body of A. auricula-judae as water soluble and alkali-insoluble fractions.

Chapter 2 deals with the structural studies of the extracellular polysaccharide of T. fuciformis. As described before, a possible taxonomic relationship between Tremella and Cryptococcus was proposed by Slodki et al. Although 2-O-(β-D-glucopyranosyluronic acid)-D-mannose has been obtained from the heteropolymer of Tremella mesenterica (16) and Cryptococcus laurentii NRRL Y-1401 (14), a definite correlation between the two groups of polysaccharides were not clear. Therefore, in the view of the chemical taxonomy and the possible commercial use, the structure and the conditions for the production of the extracellular polysaccharide of T. fuciformis were investigated.

Methylation and periodate oxidation studies indicated that the extracellular polysaccharide of T. fuciformis is built up with α-(1→3)-linked mannan backbone to which β-linked D-glucuronic acid residues, L-fucose

residues, D-mannose residues, and single or short chains of (1→2)-linked D-xylose units are attached by (1→2)-linkages. This structural feature is very similar to that of the extracellular polysaccharide of C. laurentii NRRL Y-1401 (14), suggesting the very close phylogenic relationship between Tremella and Cryptococcus.

Chapter 3 deals with the structural studies of the cell wall polysaccharides of the haploid cells of T. fuciformis. The acidic polysaccharide, which may originate from the outer layer of the cell wall, found to be consisted 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 revealed to comprise two polysaccharide moieties, i.e., β-1,3-1,6-glucan and glucurono-xylo-mannan.

Chapter 4 deals with β-D-glucans and an acidic heteropolysaccharide isolated from the fruit body of Auricularia auricula-judae. Methylation and periodate oxidation analysis showed that both β-glucans consist of a backbone chain of β-(1→3)-linked D-glucose residues, some of which are substituted at the C-6 positions with single or short side chains of D-glucose residues. Methylation analysis suggested that the acidic polysaccharide consists of a backbone chain of (1→3)-linked D-mannose residues, which are attached with D-xylose, D-mannose and D-glucuronic acid residues at the C-2 or C-6 positions.

In chapter 5, in order to clarify the serological relationship between the polysaccharides of T. fuciformis, A. auricula-judae and C. neoformans, immunochemical studies using the antiserum against the haploid cells of T. fuciformis are described. Chemical and immunochemical studies showed that the two strains of T. fuciformis (strain T-7 and T-19) are serologically

different from each other, and the capsular polysaccharide of C. neoformans was highly reactive with the antiserum against T. fuciformis, strain T-7, providing a confirmation of the very close phylogenic relationship between Tremella and Cryptococcus.

In addition to the chemical and immunochemical studies of the polysaccharides, Chapter 6 deals with the purification and characterization of β-D-mannosidase and β-N-acetyl-D-hexosaminidase of T. fuciformis. In the course of the studies on the cell wall polysaccharides of yeast-like cells of T. fuciformis, the author became aware that the cell extract of the cultured T. fuciformis contained only two particular glycosidases, i.e., β-D-mannosidase and β-N-acetyl-D-hexosaminidase, and no appreciable activities of other glycosidases were detected. Since these two glycosidases were thought to be useful tools for investigations of the carbohydrate chains of glycoproteins, an attempt has been made to isolate these two enzymes in highly pure states.

Chapter 2.

Formation and Characterization of Extracellular Polysaccharide of *Tremella fuciformis*.

Introduction

It is known that the fruit body of *Tremella fuciformis*, called "Shirokikurage", an edible mushroom, contains an acidic polysaccharide. In a series of studies on edible fungal polysaccharides, the author became aware that the haploid cells of *T. fuciformis* produce an acidic polysaccharide into the liquid culture medium, and its property is similar to that of the acidic polysaccharide of the fruit body.

Slodki *et al.*(11) have suggested a possible taxonomic relationship between *Tremella* and *Cryptococcus*, based on similar carbon-assimilation patterns, morphology and polysaccharide production. Although the structural studies of the acidic polysaccharide from *Cryptococcus laurentii* NRRL Y-1401 have been reported (13,14), the structural analysis of the acidic polysaccharide of *Tremella* has been limited on *Tremella mesenterica* (16). Therefore, the author attempted to find out the precise structure of the acidic extracellular polysaccharide of *T. fuciformis*.

The present chapter is concerned with the condition for production of the extracellular polysaccharide of *T. fuciformis* and its structure.

Materials and Methods

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

Paper chromatography was carried out using Toyo-roshi No. 50 or Whatman 3MM 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 (2:1:1). 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 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 x 200 cm) packed with 3% ECNSS-M on Gas Chrom Q, at 190° and 180° (methyl sugars). Methylated sugars were also analyzed as their methyl glycosides using a glass column (0.4 x 100 cm) packed with 15% butandiol succinate polyester on Neosorb NC at 175°.

Hydrolysis of polysaccharide was achieved by formolysis followed by heating with 1 N sulfuric acid. A sample (about 10 mg) was heated first, with 90% formic acid (1 ml) at 100° for 12 h in a screw capped vial, and after evaporation, the residue was heated with 1 N sulfuric acid (0.5 ml) further 12 h. The hydrolysate was neutralized with $BaCO_3$; filtered, and the sugars in the filtrate were analyzed by glc or paper chromatography (solvent a).

Determination of carbohydrate was generally made by phenol sulfuric acid method (17). Colorimetric determination of uronic acid was done by the carbazol method (18).

Isolation of the extracellular polysaccharide—— Haploid cells of T. fuciformis (strain T-7 and T-19) was grown in a medium containing 5% glucose, 0.4% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0, at 30° 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 crude polysaccharide was dissolved in water, passed through a column of Amberlite IR-120 (H^+ form), and 10% aqueous solution of cetylpyridinium chloride (CPC) was added to precipitate the acidic polysaccharide as an insoluble complex. The resulting CPC complex was dissolved in 10% sodium chloride solution, centrifuged to remove a trace of impurity, and polysaccharide was precipitated with ethanol. It was then dissolved in water, dialyzed, and precipitated again with acetone. The yield to initial glucose was about 15% with both strain T-7 and T-19.

Methylation analysis——Dry sample (15 mg) of the polysaccharide was dissolved under nitrogen atmosphere into dimethyl sulfoxide (2 ml), and treated at room temperature for 3 h with methylsulfinyl carbanion (0.5 ml). The resulting polysaccharide alkoxide was then methylated with methyl iodide (1.5 ml) at 20°, according to the method of Hakomori (19). The reaction mixture was dialyzed, concentrated to a small volume, and the methylated polysaccharide 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.

A portion of the fully methylated polysaccharide was dissolved in cold 72% sulfuric acid, and after standing at room temperature for 1.5 h, the solution was heated at 100° for 6 h. The hydrolysate was neutralized with $BaCO_3$, filtrated, and the filtrate was concentrated in vacuo to a syrup. A portion of the hydrolysate was examined by paper chromatography (solvent b). The methylated sugars in another portion of the hydrolysate were reduced with sodium borohydride, and converted into their corresponding alditol acetates, and analyzed by glc. Another portion of the methylated

polysaccharide was treated with 3% methanolic hydrogen chloride (0.5 ml) in a sealed tube at 80° for 12 h, and after heating, the methanolysate was neutralized with silver carbonate, and filtrated, concentrated to a syrup, and then analyzed by glc.

Results

Formation of the extracellular polysaccharide—— To see the effect of the concetration of the yeast extract as a nitrogen source on polysaccharide production and cell growth of T. fuciformis strain T-19, the cells were incubated in the medium containing glucose (5%), KH_2PO_4 (0.05%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%) and various concentrations of yeast extract, at 30° for 5 days. The result is shown in Table II-I, revealing that both cell growth and polysaccharide production were best at the concentration of 0.4%. Table II-II shows the effect of carbon-sources on polysaccharide production of T. fuciformis strain T-19. The cells were grown in the medium containing varied carbon-source (5%), KH_2PO_4 (0.05%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.05%) and yeast extract (0.4%) at 30° for 5 days. It was found that sugar alcohols were not utilized well,while glucose, mannose, xylose and sucrose were good carbon-sources. From these results, the glucose medium described in "Materials and Methods" was employed as the standard medium for polysaccharide production. A typical time course of the extracellular polysaccharide production on a large scale (6 liters) is given in Fig. II-1. The cell growth continued for 80 h, the polysaccharide production increased, and pH of the culture broth gradually decreased to about 4. After 80 h cultivation, neither the cell growth nor pH change of the brith was observed. Under these conditions, the maximum yield of the extracellular polysaccharide reached

POLYSACCHARIDE PRODUCTION AND CELL GROWTH OF T-19

Conc. of yeast extract (%)	pH (final)	Utilization of glucose (%)	Polysaccharide (mg/100 ml)	Weight of cells (mg/100 ml)
0.15	4.3	64	577	744
0.2	4.1	74	635	1,345
0.3	4.2	90	775	1,632
0.4	4.3	95	815	2,050
0.5	4.8	100	734	1,820

Basal medium: glucose 5%, KH_2PO_4 0.05%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%;
Culutural condition: shaken at 30°, for 5 days.

TABLE II-II

EFFECT OF CARBON-SOURCE ON POLYSACCHARIDE PRODUCTION OF T-19.

C-Source	Final pH	Utilization (%)	Polysaccharide mg/ 100 ml 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

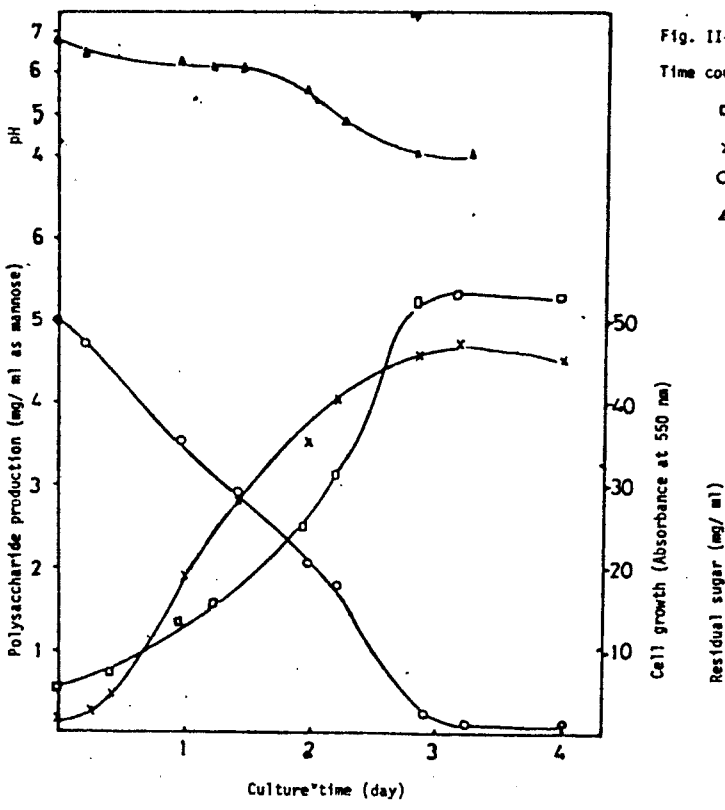


Fig. II-1
Time course of Polysaccharide Production.
□-□ : Polysaccharide
×-× : Cell growth
○-○ : Residual sugar
△-△ : pH

TABLE II-III Comparison of the properties of extracellular polysaccharides of T. fuciformis.

The molar ratio of sugar components was estimated by glc, as the alditol acetates, after hydrolysis as described in "Materials and Methods".

Polysaccharide	$[\alpha]_D$ (water)	s_{20w}	N	O-acetyl-	Carbohydrate(ratio)				
					D-GlcUA	D-Man	L-Fuc	D-Xyl	Man/GlcUA
Extracellular									
T-19	-27°	3.68S	nil	10.36%	0.8	2.1	0.45	1.0	2.62
T-7	-6°	3.08S	nil	8.19%	1.31	3.48	0.11	1.0	2.66

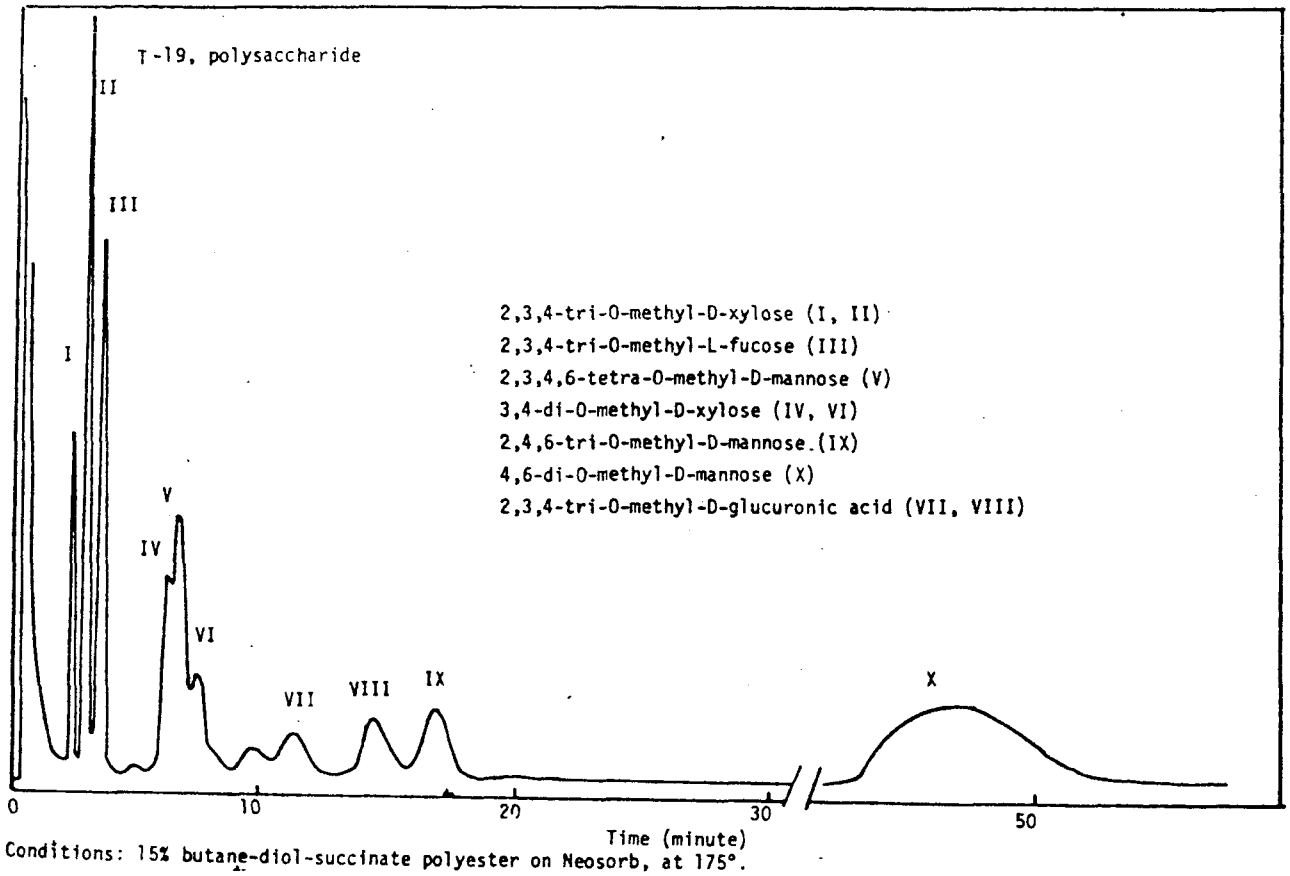
TABLE II-IV Identities and molar ratios of methyl sugar components of polysaccharides of T. fuciformis

The methylated polysaccharides were hydrolyzed and the methyl sugar fragments were analyzed by glc-mass, described in " Materials and Methods".

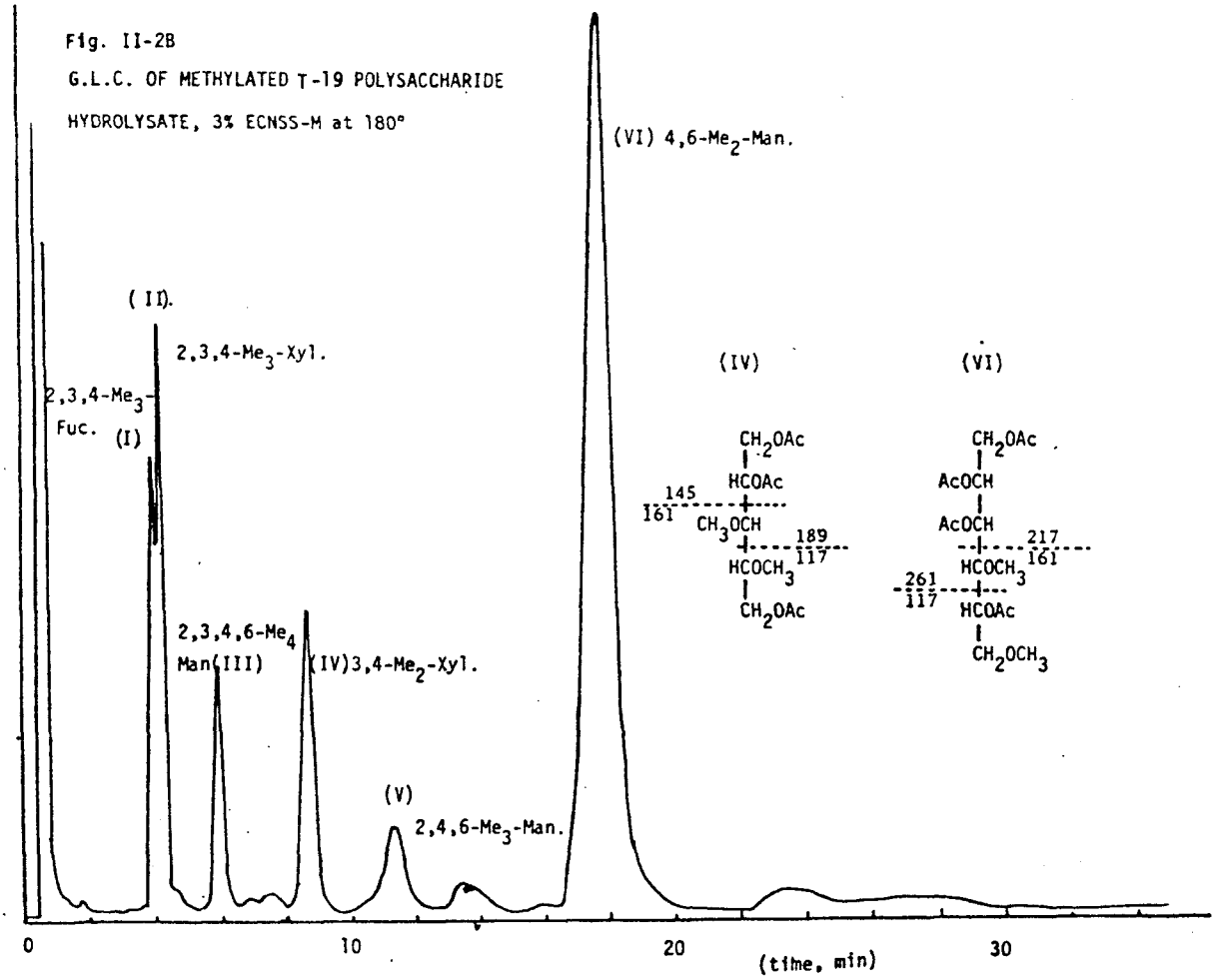
O-methyl sugar	Linkage indicated	Polysaccharide	
		Extracellular	
		T-19	T-7
2,3,4-tri-O-methyl-D-xylose	[Xyl] 1→	1.0	1.0
2,3,4-tri-O-methyl-L-fucose	[Fuc] 1→	0.46	0.1
2,3,4,6-tetra-O-methyl-D-mannose	[Man] 1→	0.77	0.32
3,4-di-O-methyl-D-xylose	→2 [Xyl] 1→	1.15	trace
2,4,6-tri-O-methyl-D-mannose	→3 [Man] 1→	0.46	1.25
4,6-di-O-methyl-D-mannose	→2,3 [Man] 1→	4.0	2.77
2,3,4-tri-O-methyl-D-glucuronic acid	[GlcUA] 1→	1.77	1.30

Fig. II-2A

GLC SEPARATION OF THE METHYL SUGAR COMPONENTS OF T.fuciformis POLYSACCHARIDE, AS THEIR METHYLGLYCOSIDES



Conditions: 15% butane-diol-succinate polyester on Neosorb, at 175°.



to a level of 8 g per liter.

The isolation and purification of the polysaccharide are described in "Materials and Methods". The properties of the extracellular polysaccharides of T. fuciformis strain T-7 and T-19 are compared in Table II-III. Both polysaccharides were partly O-acetylated, and consisted of D-xylose, D-mannose, L-fucose and D-glucuronic acid.

Methylation analysis—— The extracellular polysaccharides from strain T-7 and T-19 were methylated and their methyl sugar fragments were analyzed by glc. The complete methylation of the polysaccharide was performed by two treatments with methylsulfinyl carbanion and methyl iodide according to the method of Hakomori. A portion of the methylated polysaccharide was hydrolyzed with acid and the methyl sugar fragments were analyzed by glc, as their corresponding alditol acetates. The other portion of the methylated polysaccharide was methnolyzed with 3% methanolic hydrogen chloride, and resulting methyl glycosides of the methyl sugars were analyzed by glc. Fig. II-2 shows the glc separation of the methyl sugar fragments from the methylated extracellular polysaccharide of T. fuciformis strain T-19, as their methyl glycosides (Fig. II-2A) and as their alditol acetates (Fig. II-2B), revealing the presence of 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, 3,4-di-O-methyl-D-xylose, 2,4,6-tri-O-methyl-D-mannose, 4,6-di-O-methyl-D-mannose and 2,3,4-tri-O-methyl-D-glucuronic acid (as its methyl glycoside methyl ester). The methylated extracellular polysaccharide of strain T-7 gave similar chromatographic separations on glc, except for a trace amount of 3,4-di-O-methyl-D-xylose. Two methyl sugar fragments, 3,4-di-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose were further identified by gas chromatography-mass spectrometry (glc-ms) as their alditol acetates, since 3,4-di-O-methyl-D-xylose and

4,6-di-O-methyl-D-mannose have similar retention times to that of 2,3-di-O-methyl-D-xylose and 2,6-di-O-methyl-D-mannose, respectively, in the present glc condition. As regard to 3,4-di-O-methyl-D-xylose, it was noted that alditol acetates derived from 3,4-di-, and 2,3-di-O-methyl-D-xylose had the same glc-ms patters, but these two sugars could be distinguished by mass spectrometric analysis of the acetates of alditols after borodeuteride reduction.

In Table II-IV, identities and molar ratios of the methyl sugar components of the methylated polysaccharides from T. fuciformis strain T-7 and T-19 are compared. It is evident that two polysaccharides possess similar branched structures. They consist of (1→3)-linked D-mannan backbone chain which are attached with D-xylose, D-mannose and D-glucuronic acid residues at the C-2 positions of the mannose residues. The presence of (1→2)-linked D-xylose short side chains is suggested in the polysaccharide from strain T-19.

Smith degradation—— The presence of the glycosidic linkages suggested by the methylation analysis was supported by the Smith degradation (20), which involves periodate oxidation, borohydride reduction and hydrolysis with acid. The polysaccharide (about 100 mg, from both strains) was oxidized with 0.05 M sodium periodate (100 ml) at 4° in a dark. At suitable time intervals, periodate consumption was determined by arsenite method (21), and formic acid production by titration with 0.01 N sodium hydroxide. On the oxidation (4 days), the polysaccharide from strain T-19 consumed 1.14 mol of periodate with concomitant liberation of 0.7 mol of formic acid per hexose residue, and the polysaccharide from strain T-7 consumed 0.93 mol of periodate with 0.39 mol of formic acid per hexose residue. The oxidized polysaccharide was reduced with sodium borohydride to the polysaccharide

polyalcohol (yield of the reduced polysaccharide, 61 mg and 65 mg from strain T-19 and T-7, respectively). The polysaccharide polyalcohol gave on acid hydrolysis a large proportion of mannose and a small amount of glycerol and a trace of ethylene glycol. This result supports that the backbone of the extracellular polysaccharide of T. fuciformis is (1→3)-linked D-mannan. In another experiment, the polysaccharide polyalcohol was hydrolyzed with 0.1 N sulfuric acid at 25° for 20 h (controlled Smith degradation (22)), and the structure of the resulting insoluble degraded polysaccharide from the mild hydrolysate was examined by methylation. The methylated degraded polysaccharide yielded on acid hydrolysis, predominant amounts of 2,4,6-tri-O-methyl-D-mannose with trace amounts of 2,3,4,6-tetra-O-methyl-D-mannose, and 4,6-di-O-methyl-D-mannose, as revealed by glc as their alditol acetates. In addition to the methylation analysis of the degraded polysaccharide, it showed a specific rotaion of +30.5° (in water, strain T-19). These results show that the backbone of the polysaccharide is α-(1→3)-linked D-mannan.

Partial hydrolysis of the polysaccharide—— The extracellular polysaccharide of T. fuciformis was hydrolyzed with 0.5 N sulfuric acid for 5 h at 95°. The solution was neutralized with $BaCO_3$, filtrated, and the barium ions were removed by passage through Amberlite IR 120 (H^+) ion-exchange resin. The solution was then passed through a bed (2.0 x 13 cm) of Dowex-I (formate form) ion-exchange resin , and the neutral sugars were washed through with water. The acidic components were displaced with 1 N formic acid, and the formic acid solution was concentrated to a syrup. Paper chromatography of the products in solvent c indicated three major components, glucurone, glucuronic acid, and an aldobiouronic acid. The spot of the aldobiouronic acid coincided in the position with that of 2-O-β-(D-glucuronosyl)-D-mannose, which has been tentatively identified from the hydrolysis of Xanthomonas

oryzae (23). The isolation of this type of aldobiouronic acid indicates that D-glucuronic acid residues are β-linked to the C-2 position of D-mannose residues of the backbone.

Discussion

An acidic extracellular polysaccharide was obtained from the culture filtrate of the haploid cells of T. fuciformis strain T-7 and T-19 in high yields, and purified through cetylpyridinium chloride insoluble complex formation. The constituent sugars of the polysaccharide were found to be D-xylose, L-fucose, D-mannose and D-glucuronic acid, and this polysaccharide was partly O-acetylated. These analytical results are in basic agreement with those of the extracellular polysaccharide from other Tremell species previously reported (11) except that L-fucose was found in the present preparation of the polysaccharide.

The methylated polysaccharide was hydrolyzed, and the hydrolysis products following their conversion to alditol acetates derivatives were identified by gas-liquid chromatography mass spectrometry as 2,3,4-tri- (13%, 18%) and 3,4-di-O-methyl-D-xylose (15%, trace), 2,3,4-tri-O-methyl-L-fucose (6%, 2%), and 2,3,4,6-tetra-,(10%, 6%), 2,4,6-tri-,(6%, 23%), and 4,6-di-O-methyl-D-mannose (51%, 51%)(molar ratio, strain T-19 and T-7, respectively). In addition, all the glucuronic acid in the acidic polysaccharide was shown to be end group as the only methylated acidic residue detected in the methanolysis products was 2,3,4-tri-O-methyl-D-glucuronic acid (see, Table II-IV).

The methylation analysis indicates that the acidic polysaccharide is a very highly branched structure having non-reducing end group xylopyranose,

fucopyranose, mannopyranose, and glucopyranosyluronic acid residues. Particularly, the acidic polysaccharide from strain T-19 is very highly branched as the ratio of 2,4,6-tri-O-methyl-D-mannose and 4,6-di-O-methyl-D-mannose is 1:8.5. The absence of 3-O-methyl-substituted mannose derivatives suggests that the polysaccharide has a (1→3)-linked mannose backbone, and this was confirmed by Smith degradation of the polysaccharide. The mild Smith degradation of the polysaccharide polyalcohol produced a periodate-resistant mannan. The positive specific rotation suggests that it is α-linked. These results thus provide a feature of similarity with the acidic polysaccharide of C. laurentii and several other fungal polysaccharides (24).

The fact that all the glucuronic acid residues are β-linked to the C-2 positions of the mannose backbone was established by the isolation of 2-O-β-(D-glucopyranosyluronic acid)-D-mannose.

The presence of 2,3,4-tri-O-methyl-D-xylose, and 3,4-di-O-methyl-D-xylose in the polysaccharide from strain T-19 indicates that the xylose is linked to the mannose backbone in the form of (1→2)-linked xylose side chain .

On the basis of these results, a possible repeating unit of the acidic polysaccharide is shown in Fig. II-3. The lenght of the xylose side chain in the polysaccharide from strain T-19 must await the isolation of xylo-oligosaccharide.

A comparison of the structures of the extracellular polysaccharides of the both strains of T.fuciformis with that of C. laurentii NRRL Y-1401 (14) indicates that the polysaccharide of strain T-7 has a similar structure to that of C. laurentii, while the essential difference between the polysaccharide of strain T-19 and that of C. laurentii is found in the form of the xylose side chain.

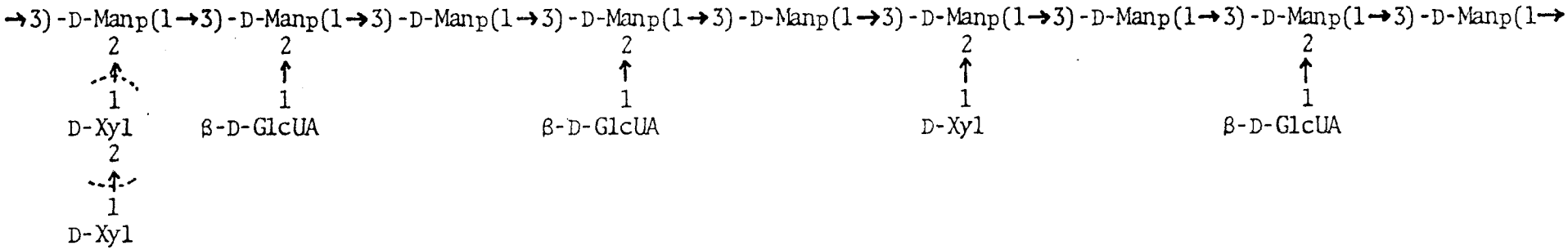


Fig. II-3 A possible repeating unit of the acidic polysaccharide of strain T-7

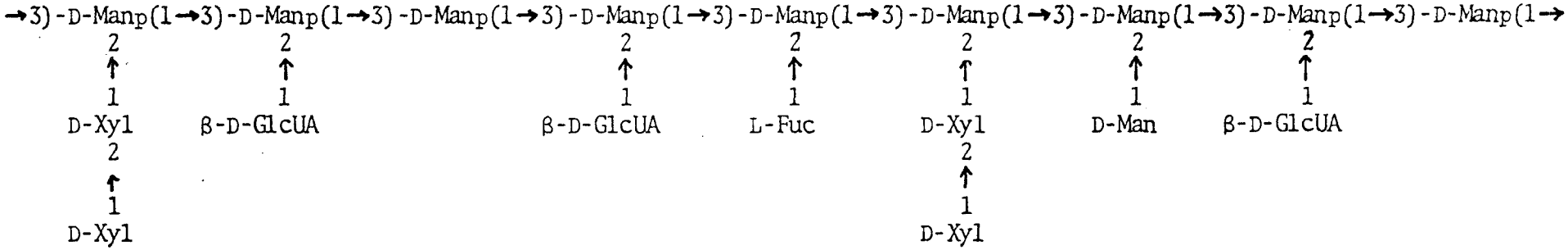


Fig. II-3 A possible repeating unit of the acidic polysaccharide of strain T-19

Summary

An acidic heteropolysaccharide was isolated from the culture filtrate of the haploid cells of Tremella fuciformis (strain T-7 and T-19). The polysaccharide was purified through CPC-insoluble complex formation, and its structural feature was elucidated.

The extracellular polysaccharide ($[\alpha]_D$ -27° in water, strain T-19), elaborated by the haploid cells of T. fuciformis contained D-xylose, L-fucose, D-mannose, and D-glucuronic acid (in the molar ratio, 1.0:0.45:2.1:0.8, strain T-19), and was partly O-acetylated. Methylation and periodate oxidation analysis indicated that the polysaccharide is built up with α-(1→3)-linked mannose backbone to which β-linked D-glucuronic acid, L-fucose, and D-mannose residues, and single or short chains of (1→2)-linked D-xylose units are attached by (1→2)-linkages.

Chapter 3

Structures of the Cell Wall Polysaccharides of Tremella fuciformis.

Introduction

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. As described in the previous chapter, haploid cells of Tremella fuciformis produce a large amount of D-glucurono-D-xylo-D-mannan into the culture medium, and its structure was established. 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 (25).

During the isolation of β-D-mannosidase and β-N-acetyl-D-hexosaminidase from the cell extract of Tremella fuciformis (Chapter 6), the author was interested in the cell wall composition and structure of the haploid cells of T. fuciformis to compare with other yeast cell walls.

This chapter is concerned with fractionation of the cell wall of T. fuciformis and elucidation of detailed structures 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.

Materials and Methods

General methods—— Paper chromatography was carried out as described

in Chapter 2, with the following solvent systems in addition to those in Chapter 2 (V/V): (f) tert-amyl alcohol-formic acid-water (4:1:1.5),(g) 1-propanol-ethanol-water (6:1:3), (h) 1-butanol-acetic acid-water (50:12:25). Sugars on a paper chromatogram were detected as described in the previous chapter.

Gas-liquid chromatography (glc) of neutral sugars and their methyl derivatives were usually performed as described in the previous chapter.

Hydrolysis of polysaccharide was achieved by formolysis followed by heating with trifluoroacetic acid (TFA). A sample (about. 10 mg) was heated, first, with 90% formic acid (1 ml) at 100° for 8 h in a screw capped vial, and after evaporation the residue was further heated with 2 N TFA (0.5 ml) for 6 h. After TFA was removed by repeated evaporations with water, the sugars in the hydrolysate were analyzed by glc or paper chromatography.

Determination of carbohydrate and colorimetric determination of uronic acid were made by the methods described in the previous chapter.

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

Preparation of the cell wall—— The cultivation of the haploid cells of T. fuciformis strain T-7 was carried out as described in the previous chapter. 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°. The insoluble materials were collected by centrifugation at 12,600 x g for 30 min, and washed three times with water, and re-suspended in water. The suspension was centrifuged again at 7,600 x 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 x 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. III-1).

Fractionation procedure of the cell wall—— First, the cell wall preparation (about 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°. After 72 h, the reaction mixture was heated at 85° 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° 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 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 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 h, 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 after cold alkaline extraction was extracted with 1 N sodium hydroxide solution (20 ml) at 70° for 3 h 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 (26). The dried polysaccharide (700 mg) was dispersed in liquid ammonia by stirring at -60°, and methylated by addition of metallic sodium (500 mg) and methyl iodide (12 ml) for 1.5 h. 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, then methylated by the method of Hakomori, with fresh methylsulfinyl carbanion (0.5 ml) and methyl iodide (1.5 ml) as described in the previous chapter. 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° for 12 h, and after the formic acid was distilled off, the residue was further heated with 2 N TFA at 100° for 5 h. The hydrolysate 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° for 16 h. The methanolysate 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 hydrolysate were separated by use of a column of Dowex-I (formate form, 2.0 x 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. (27). The spot of the resulting lactone coincided to the position with that of authentic L-gulonolactone on paper chromatogram using solvent (f). Furthermore, the trimethylsilyl (TMS) derivative of the lactone had the same retention time as that of the TMS derivative of authentic L-gulonolactone on glc (column; Neopentyl glycol succinate on Chromosorb W) at 175°. 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

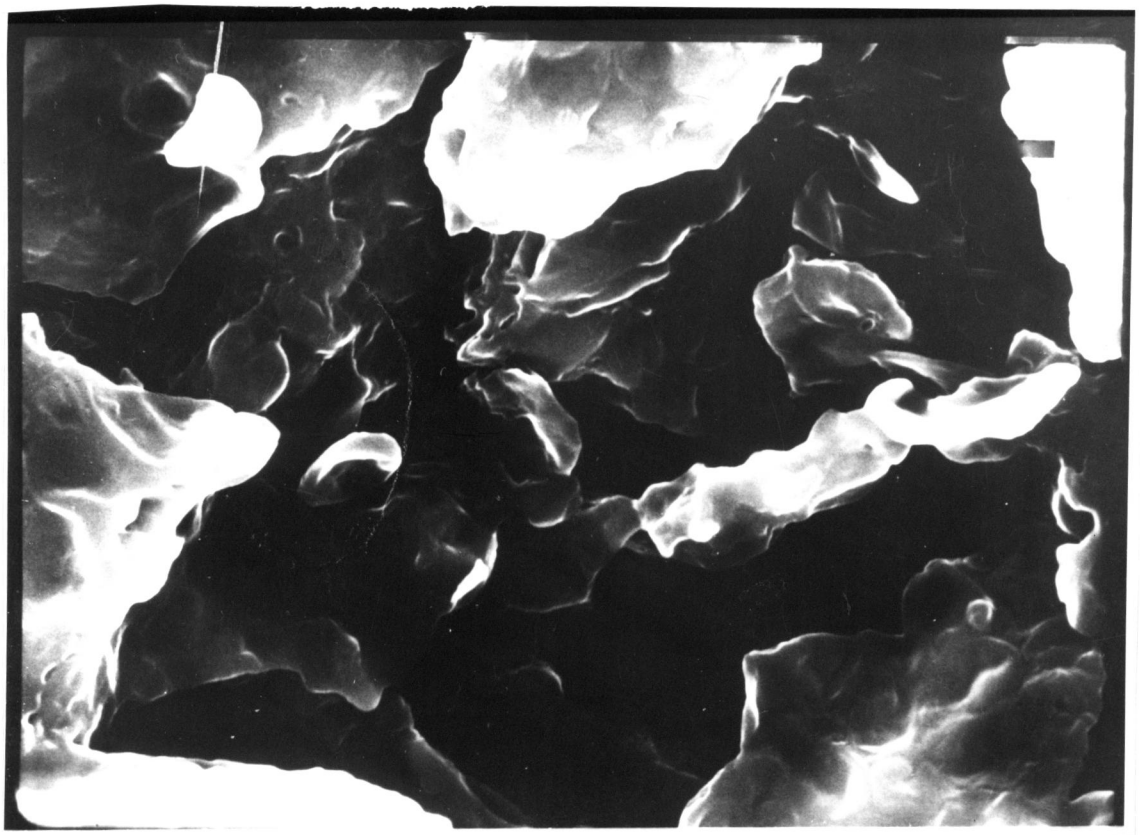


Fig. III-1. Scanning electron photomicrograph of the cell wall preparation of T. fuciformis, enlarged 1,500 times.

SCHEME III-I Fractionation of the Cell Wall of Tremella fuciformis.

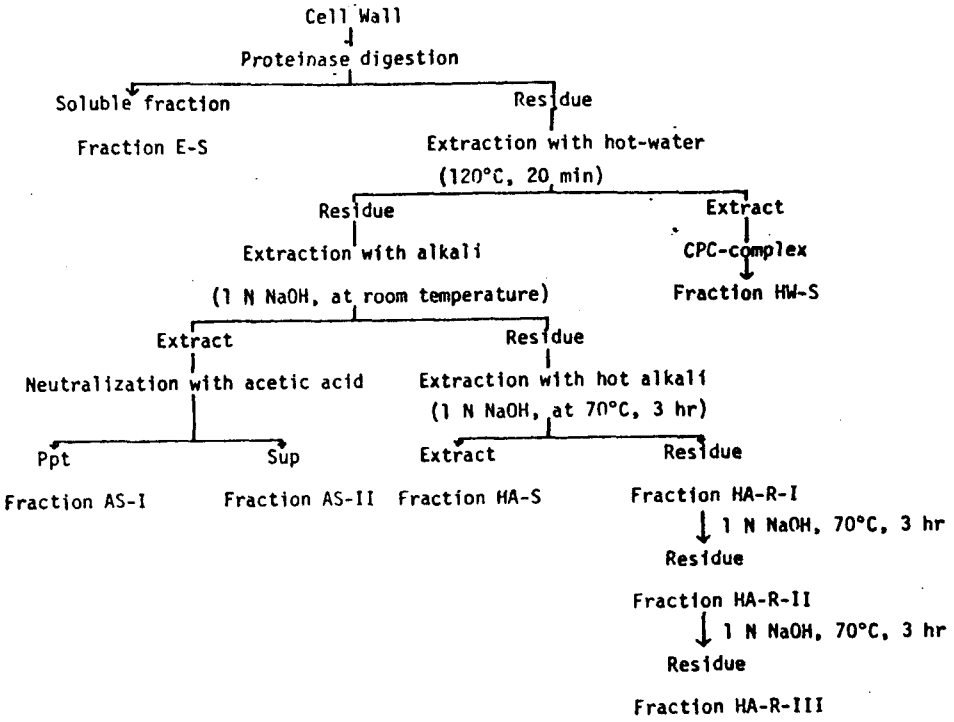


TABLE III-I . Carbohydrate composition of the polysaccharide fractions from the cell wall of Tremella fuciformis.
The neutral carbohydrate composition was determined by glc as their alditol acetates, and the content of D-glucuronic acid was determined colorimetrically.

Fraction [a]	Yield (%)	Composition (molar ratio)			
		Glucose	Mannose	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 I.

to Scheme III-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 III-I.

The hot-water extracted fraction (HW-S) was further purified through CPC insoluble 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°, the carbohydrate composition of this fraction scarcely changed (see, Table III-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 I. 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 has $[\alpha]_D$ -26.2° (c=1.0, water), and was composed of D-xylose, D-mannose and D-glucuronic acid in the molar ratio of 1.0:3.8:0.5, 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. III-2, glc 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 III-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. Fig. III-3 illustrates one of possible structures of the water soluble glucurono-xylo-mannan.

The structural similarity between the polysaccharide from the hot-water extract and the extracellular polysaccharide was also provided by the immunochemical studies, using rabbit antiserum against the formalin-killed yeast-like cells of T. fuciformis (see, Chapter 5). This strongly reacted with the present glucurono-xylo-mannan from the hot-water extract. This suggests that this polysaccharide must be located on the outer layer of the cell wall and responsible for the serological properties of T. fuciformis. It is interesting that the extracellular polysaccharide of T-7 strain also reacted to this antiserum to give the similar quantitative pricipitin curve as that of the cell wall glucurono-xylo-mannan, indicating that both polysaccharides have similar structures, or at least the antigenic determinant must be the same.

The acidic polysaccharide isolated from the fruit body of Auricularia auricula-judae was shown to have a similar structure to that of the extracellular and cell wall polysaccharides of T. fuciformis as revealed by methylation and immunochemical studies (see Chapter 4 and Chapter 5). It has been reported that several fungal heteropolysaccharides contain α-(1→3)-linked mannan backbone chains (28-32), but the occurrence of

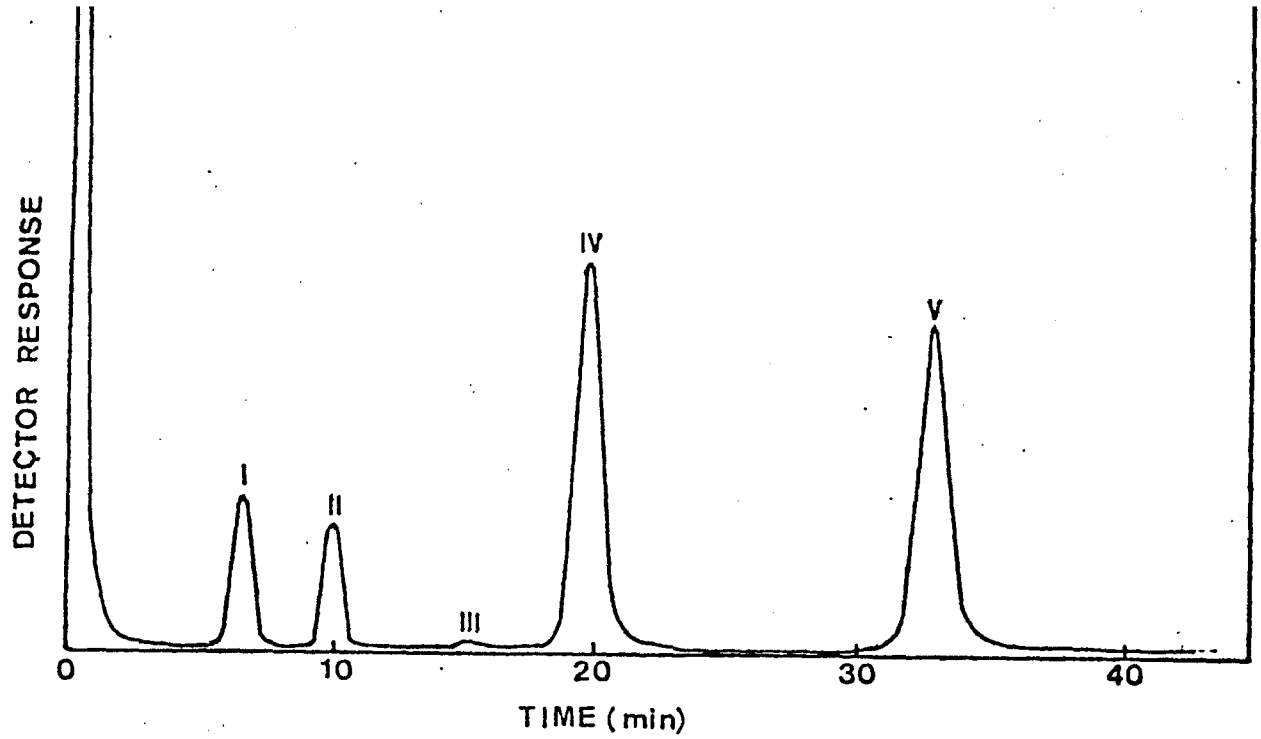


Fig. III-2. Gas-liquid chromatographic separation of the hydrolysis products of the methylated water-soluble glucurono-xylo-mannan (Fraction HW-S) as their alditol acetates.
Condition: 3% ECNSS-M on Gas Chrom Q, at 180°C; (I) 2,3,4-tri-O-methyl-D-xylose; (II) 2,3,4,6-tetra-O-methyl-D-mannose; (III) 3,4-di-O-methyl-D-xylose; (IV) 2,4,6-tri-O-methyl-D-mannose; (V) 4,6-di-O-methyl-D-mannose.

TABLE III-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.

O-methyl sugar	Linkage indicated	T_g*	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	[Man]1→	1.0	0.8
3,4-di-O-methyl-D-xylose	→2[Xyl]1→	1.54	trace
2,4,6-tri-O-methyl-D-mannose	→3[Man]1→	2.0	2.7
4,6-di-O-methyl-D-mannose	→2,3[Man]1→	3.29	2.3
2,3,4-tri-O-methyl-D-glucuronic acid	[GlcUA]1→		0.5

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

polysaccharides containing glucuronic acid residue, in addition to mannose and xylose residue seems to be limited to Tremella sp., Auricularia sp., and Cryptococcus sp.(24). The extracellular polysaccharide of a non-pathogenic Cryptococcus laurentii NRRL Y-1401 contains α-(1→3)-linked mannan main chain, and D-xylosyl and D-glucuronosyl side chains are attached to the C-2 position of the mannose residue (13). Immunochemical studies described in Chapter 5 showed that the purified capsular polysaccharide of pathogenic C.neoformans is highly reactive with the antiserum against T. fuciformis. These results provided by chemical and immunochemical studies may lead to a tentative conclusion that Tremella fuciformis and other Tremella species and Auricularia auricula-judae, both groups belonging to the same Heterobasidiae, and also the group of Cryptococcus species have very close phylogenic relationships.

Alkali-insoluble polysaccharide (Fraction HA-R)—— The residue after extensive extractions, with hot-water and alkali, which did not dissolve even in DMSO, seemed to constitute a rigid structure of the cell wall. Since this fraction contained in addition to D-glucose, the same components of the extracellular or cell surface polysaccharide (xylose, mannose and glucuronic acid), two more treatments with hot alkali were carried out, but the carbohydrate composition in the residue scarcely changed. In addition to the hot alkaline extractions, an attempt to separate the glucurono-xylo-mannan from the glucan by treatment with DMSO, which was successfully used for the Saccharomyces cell wall glucan, was not successful. This result suggests that the glucan and glucurono-xylo-mannan moieties (ratio, 1:1) are covalently joined to form a complicated rigid structure.

The residual polysaccharide (Fraction HA-R-III, N = 0.57%) after repeated alkaline treatments was methylated and their methyl sugar fragments were

analyzed by glc. Fig. III-4 shows the glc profile of the hydrolysis products of the methylated alkali-insoluble polysaccharide, revealing 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), 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°: 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 as its methyl glycoside methyl ester (retention times relative to that of methyl-2,3,4,6-β-D-glucopyranoside, 2.5 and 3.2). The identities and molar ratios of the methyl sugar fragments are listed in Table III-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→3)-linked D-mannose residues and (1→3)-linked and (1→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→3)-linked D-mannose and D-glucose residues are branched at the C-2 and C-6 positions, respectively, or alternately (1→6)-linked D-glucose residues may be branched at the C-3 positions. Most D-xylose

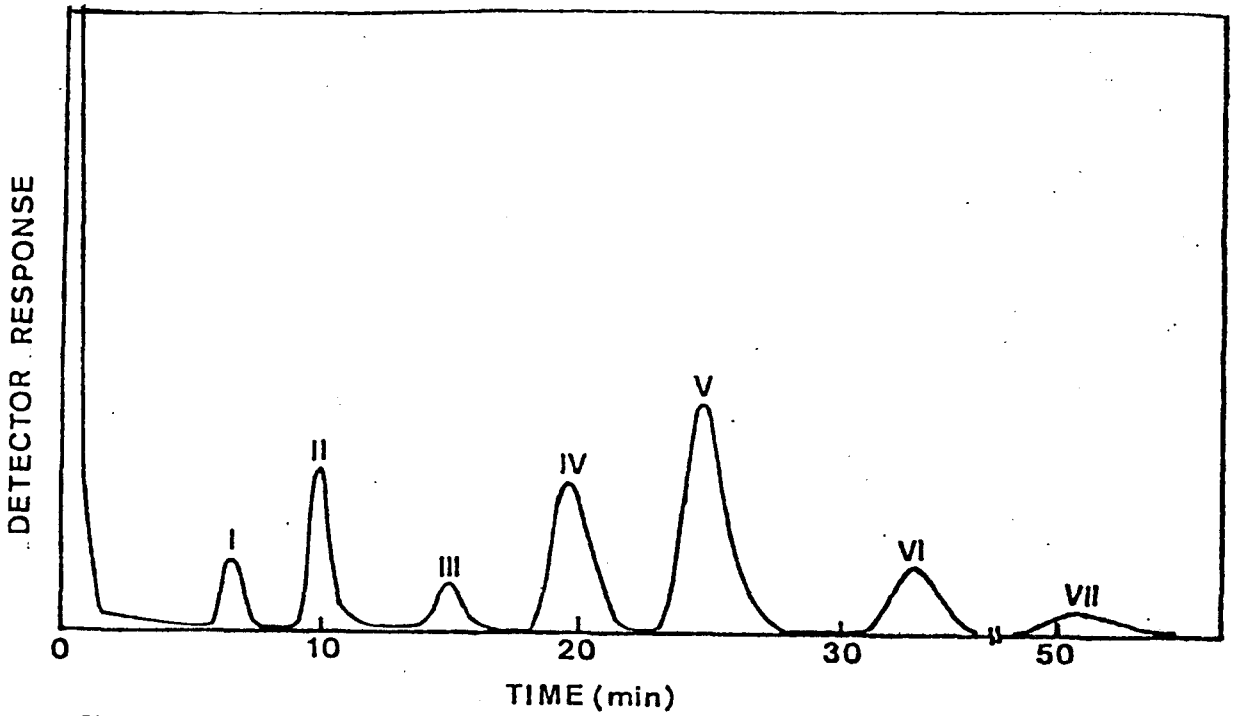


Fig. III-4. Gas-liquid chromatographic separation of the hydrolysis products of the methylated alkali-insoluble polysaccharide (Fraction HA-R-III) as their alditol acetates.
Condition: 3% ECNSS-M on Gas Chrom Q, at 180°C; (I) 2,3,4-tri-O-methyl-D-xylose; (II) 2,3,4,6-tetra-O-methyl-D-mannose (glucose); (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-III. Molar ratios of the hydrolysis products of the methylated alkali-insoluble polysaccharide of the cell wall of I. fuciformis. The methylated polysaccharide was hydrolyzed and the methyl sugar fragments were analyzed by glc.

O-methyl sugar	Linkage indicated	T_g*	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	[Man]1→	1.0	1.9
2,3,4,6-tetra-O-methyl-D-Glucose	[Glc]1→	1.0	
3,4-di-O-methyl-D-Xylose	→2[Xyl]1→	1.54	0.9
2,4,6-tri-O-methyl-D-Mannose	→3[Man]1→	2.0	3.9
2,4,6-tri-O-methyl-D-Glucose	→3[Glc]1→	2.0	
2,3,4-tri-O-methyl-D-Glucose	→6[Glc]1→	2.47	4.1
4,6-di-O-methyl-D-Mannose	$\rightarrow^{2}_{3}$[Man]1→	3.29	2.2
2,4-di-O-methyl-D-Glucose	$\rightarrow^{3}_{6}$[Glc]1→	5.15	0.6
2,3,4-tri-O-methyl-D-Glucuronic acid	[GlcUA]1→		1.1

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

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° in a dark. At suitable time intervals, periodate consumption by arsenite method (21), and formic acid production by titration with 0.01 N sodium hydroxide were determined. 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°, 6 h) glycerol, mannose and glucose in the molar ration 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 on acid hydrolysis, glycerol, mannose and glucose in the molar ration 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° for 20 h (controlled Smith degradation (22)), and structures of the degraded polysaccharides from the mild hydrolysates were examined by methylation analysis. 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 methanolysate of the methylated degraded-polysaccharide from the soluble portion and that from the insoluble portion, respectively. These results strongly indicate that (1→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 (33), 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 (34). 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° with the β-1,3-glucanase (about 100 units). After 24 h incubation, the reaction mixture was centifuged, and the solubilized product in the supernatant solution was examined by paper chromatography (solvent g), 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→3)- and (1→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 I. fuciformis as illustrated in Fig. III-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→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 (see Chapter 2).

Although the structural features of the glucan moiety may be somewhat similar to that of the yeast (Saccharomyces cerevisiae) cell wall glucan (35), the glucan moiety of I. fuciformis cell wall appears to contain a higher proportion of (1→6)-glucosidic linkage 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→3)-linked sugar residues which are

attached with (1→6)-linked side chains, however, a possibility that (1→6)-linked glucose units are also located in the backbone chain cannot be ruled out (see, Fig. III-5). In relation to the content of (1→6)-linked glucose units, Manners et al. reported the presence of β-(1→3)- and β-(1→6)-glucans, separately, in baker's yeast (36). However, Konishi et al. re-investigated to show that the highly purified homogeneous β-glucan contains both (1→3)- and (1→6)-D-glucosidic linkages (37).

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 (38 , Chaper 4). Such a difference of the constitutions between the cell wall in the yeast-like haploid cells and fruit body may reflect the neccessity of particular rigid architecture of the cell envelope during the growth of the cells in the liquid enviroments.

The recent work by Reid et al.(39) suggested the presence of α-(1→3)-glucan in the cell wall of the yeast-like cells of Tremella mesenterica. Bacon et al. (40) also reported the occurrence of α-(1→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.,

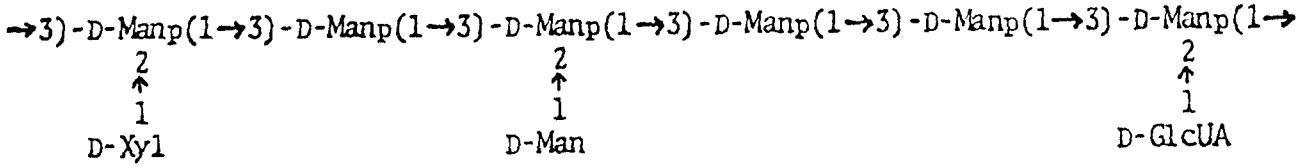
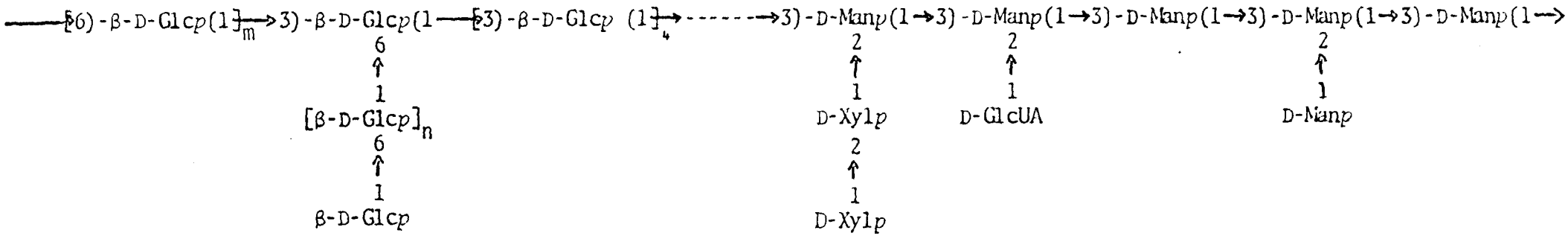


Fig. III-3. A possible structure of the water-soluble glucurono-xylo-mannan.



$n + m = 7$

$n = 0 \sim 7$

Fig. III-5. A possible structure of the alkali-insoluble polysaccharide.

Fraction AS-I and Fraction AS-II, of the cell wall of T. fuciformis are being undertaken.

Summary

The cell wall fraction prepared from the yeast-like cells of T. 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.

Chaper 4.

Isolation and Characterization of the Polysaccharides of "Kikurage", Fruit Body of Auricularia auricula-judae.

Introduction

"Kikurage", the fruit body of Auricularia auricula-judae, which belongs to the family of Heterobasidiae 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"(38), also an edible mushroom, and also from the culture of its yeast-like haploid cells (Chapter 2). Since A. auricula-judae and T. fuciformis fall under the same family "Heterobasidiae", the author attempted to find out the chemical nature of their polysaccharides which constitute mainly the fruit bodies of an ear-like shape.

Materials and Methods

General methods——Paper chromatography was carried out as described in the previous chapters.

Gas-liquid chromatography (glc) of neutral sugars and their methyl derivatives were usually performed as described in Chaper 2.

Hydrolysis of polysaccharide was achieved by formolysis followed by heating with trifluoroacetic acid(TFA). A sample (about 10 mg) was heated, first, with 90% formic acid (1 ml) at 100° for 16 h in a screw capped vial, and after evaporation , the residue was heated with 4 N TFA (0.5 ml) further for 12 h. After TFA was removed by repeated evaporations with water,

the sugars in the hydrolysate were analyzed by glc or paper chromatography.

Determination of carbohydrate and colorimetric determination of uronic acid were made by the methods described in Chapter 2.

Isolation of polysaccharides—— Dried fruit body of A. auricula-judae (about 50 g) available commercially was immersed in 0.9% aqueous sodium chloride (saline, 500 ml), and cut into pieces, and then homogenized with a Waring blender. The homogenized material was extracted with saline at cold for 24 h, and centrifuged. To the supernatant solution was added 3 volumes of ethanol to give precipitate of polysaccharide (yield: 0.48 g). The residue was suspended in water (500 ml), and autoclaved at 120° 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 removal of 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° for 2 h, 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 throughly with water and dried in vacuo (Fraction III, yield: 41.0 g).

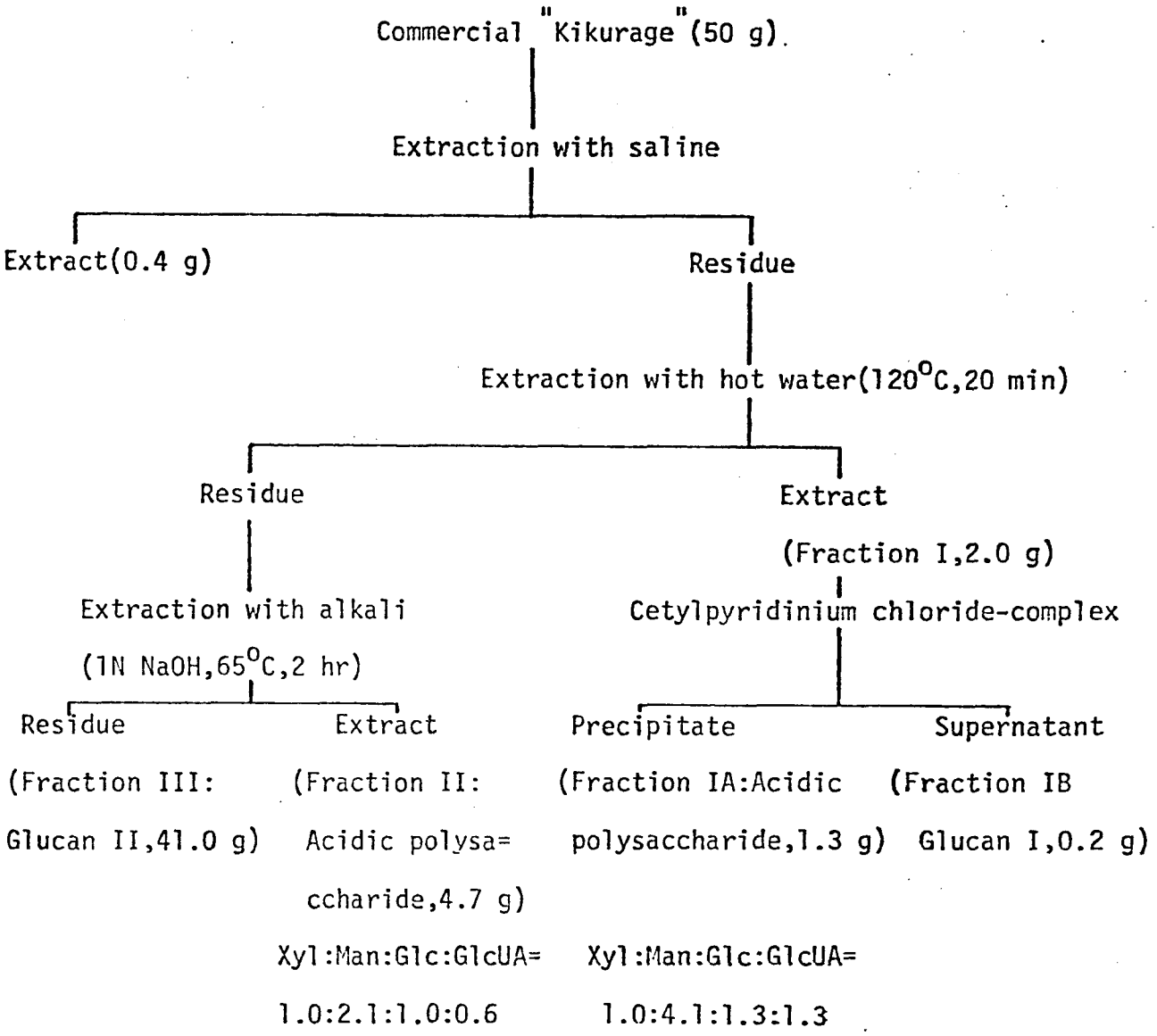
Methylation analysis—— Since the alkali-insoluble polysaccharide (Fraction III), which contains onyl glucose, was not soluble in dimethyl sulfoxide (DMSO), it was methylated, first, by the method of Muskat in liquid ammonia (26) as described in Chapter 3. The dried polysaccharide (600 mg) was dispersed in liquid ammonia by stirring at -60°, and methylated by addition of metallic sodium (500 mg) and methyl iodide (12 ml) for 1.5 h. 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 DMSO (2 ml), and fully methylated by the method of Hakomori as described in Chapter 2. 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.

Hydrolysis of the methylated polysaccharide was performed as described in Chapter 3, and the methylated sugars were reduced with sodium borohydride, and converted into their corresponding alditol acetates, and 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

SCHEME IV-I Fractionation of Polysaccharides from Fruit Body of "Kikurage"



successively with saline at cold, hot water at 120° (Fraction I), and 1 N sodium hydroxide at 65° under 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 IV-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° (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 DMSO, 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 glcuan II were hydrolyzed with

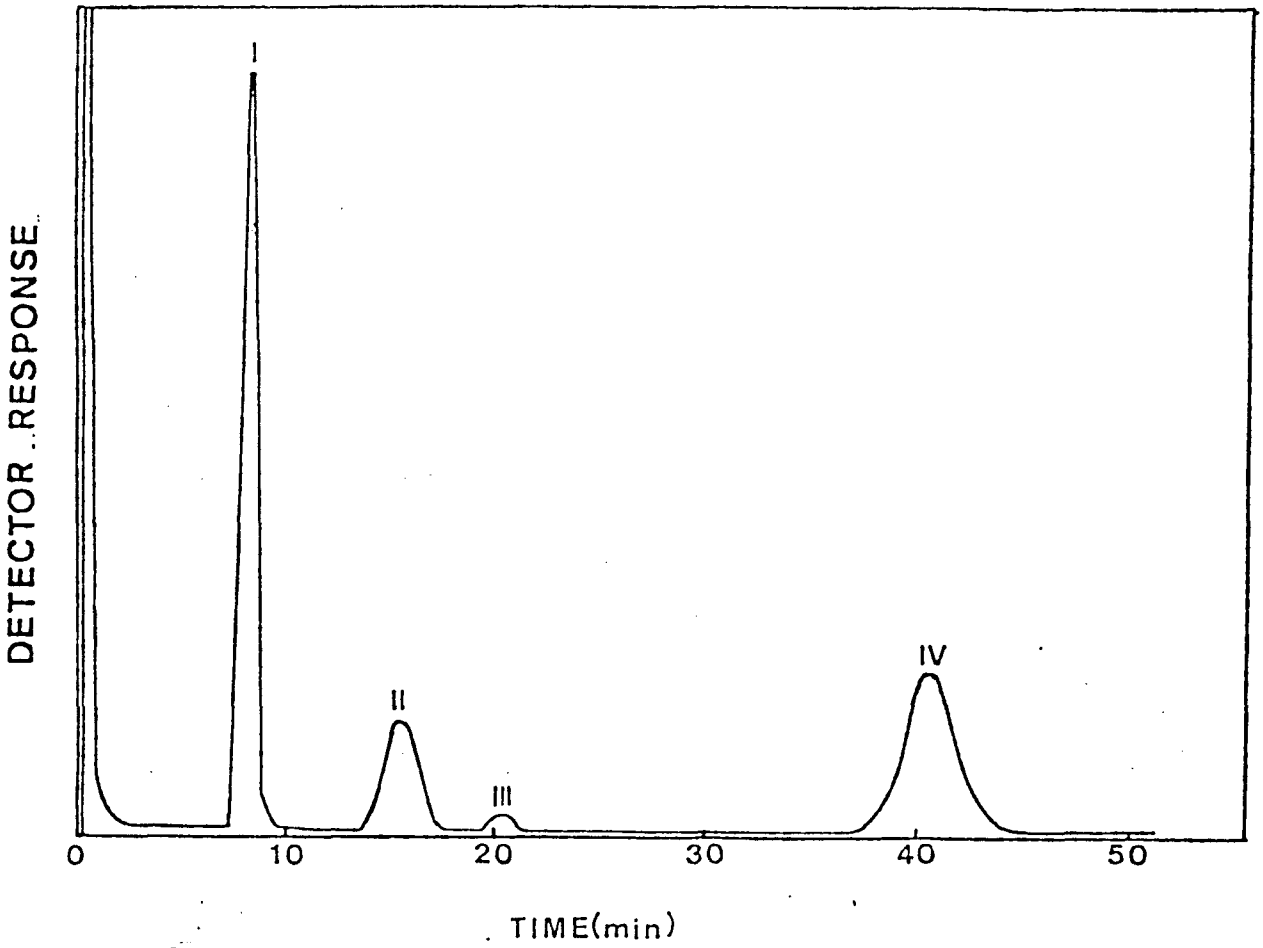


Fig. IV-1. Gas-liquid chromatographic separation of the hydrolysis products of methylated glucan II as their alditol acetates. Condition: 3% ECNSS-M, at 180°C; (I) 2,3,4,6-tetra-O-methyl-D-glucose; (II) 2,4,6-tri-O-methyl-D-glucose; (III) 2,3,4-tri-O-methyl-D-glucose; (IV) 2,4-di-O-methyl-D-glucose.

TABLE IV-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.

O-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→ (3 ↑)	1.1	0.9

acid, and the methyl sugar fragments were analyzed by glc-ms, as their corresponding alditol acetates. Fig. IV-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 glucan I also gave a similar glc profile except for the absence of 2,3,4-tri-O-methyl-D-glucose. In Table IV-I, the identities and molar ratios of the D-glucosidic linkages in the two types of glucans are compared. From Table IV-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. 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-glucose 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° in the dark. At suitable time intervals, periodate consumption was determined by arsenite (21), and formic acid production by titration with 0.01 N 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°, 6 h) an approximately equal molar proportion of glycerol and glucose, as examined by glc. When the glucan polyalcohol was hydrolyzed with 0.1 N sulfuric acid at 25° for 20 h (controlled Smith degradation (22)), 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 DMSO, was methylated by Hakomori's method, and after hydrolysis the hydrolysate 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-O-methyl-D-glucose and 2,4-di-O-methyl-D-glucose. This result is a strong indication that the consecutive β-(1→3)-linked D-glucose residues are the backbone chain of this highly branched molecule. The facts that the β-(1→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→6)-linked glucose units are located mostly in the side chains. These findings provide unambigous evidence for β-(1→3)-linked backbone with numerous branches attached by (1→6)-linkages.

A possible structural model of the alkali insoluble glucan can be illustrated as Fig. IV-2B. The water-soluble glucan has a less branched β-(1→3)-linked chain, with a repeating unit of five glucose residues, where two out of three β-(1→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. IV-2A. There was no internal (1→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 (33), 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 (34). Glucan II (5 mg) was suspended in 0.05M acetate buffer, pH 4.8 (1 ml),and incubated in a shaker at 37° with β-1,3-glucanase (about 100 units). After 24 h

incubation, the solubilized digest was collected by centrifugation, and the products were examined by paper chromatography (solvent g), 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 the molar ratio of gentiobiose to glucose 2.5:1.0, and fairly good agreement with the value calculated from Fig. IV-2B.

Since Johnson et al., first, reported the structure of β-D-glucan from Sclerotium glucanicum (2), glucans containing β-D-(1→3)- and β-D-(1→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 (7), Lentinus edodes (8), and Helvella lacunosa (41). However, the water-soluble and alkali-insoluble glucans having the structure such as Fig. IV-2A and 2B are more branched than the sclerotial β-D-glucan, one out of three β-(1→3)-linked D-glucose residues 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 DMSO, 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→3)-linked glucose residues of the backbone chain would provide certain structural regularity 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

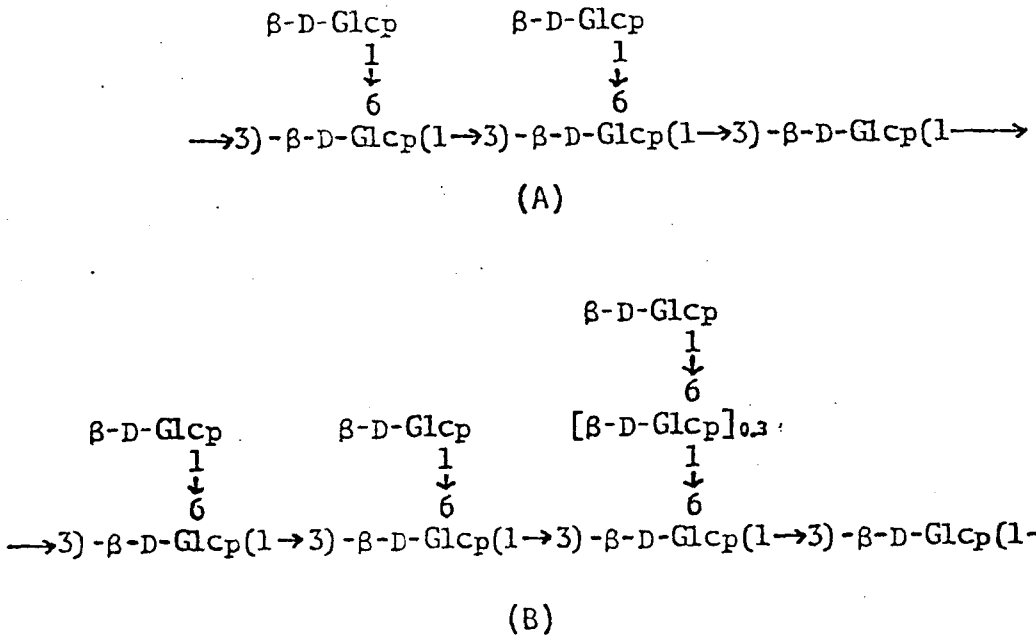


Fig. IV-2. Structure of repeating unit of (A) Glucan I and (B) Glucan II

polysaccharide which has no branch is still not soluble in cold water, but became soluble either in alkali or DMSO. 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 reported already (2).

There have been several reports concerning antitumor activities of β-1,3-, 1,6-glucans, such as scleroglucan (42) and lentinan (9). In relationship between the structure and activity 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 (43).

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°, and was composed of xylose, mannose, glucose and glucuronic acid, as revealed by paper chromatography and glc, (molar ratio; 1.0:4.1:1.3:1.3). The D-glucuronic acid in the hydrolysate was identified by glc as its trimethylsilyl derivative of the corresponding methyl glycoside of methyl ester.

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. IV-3, glc-ms 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

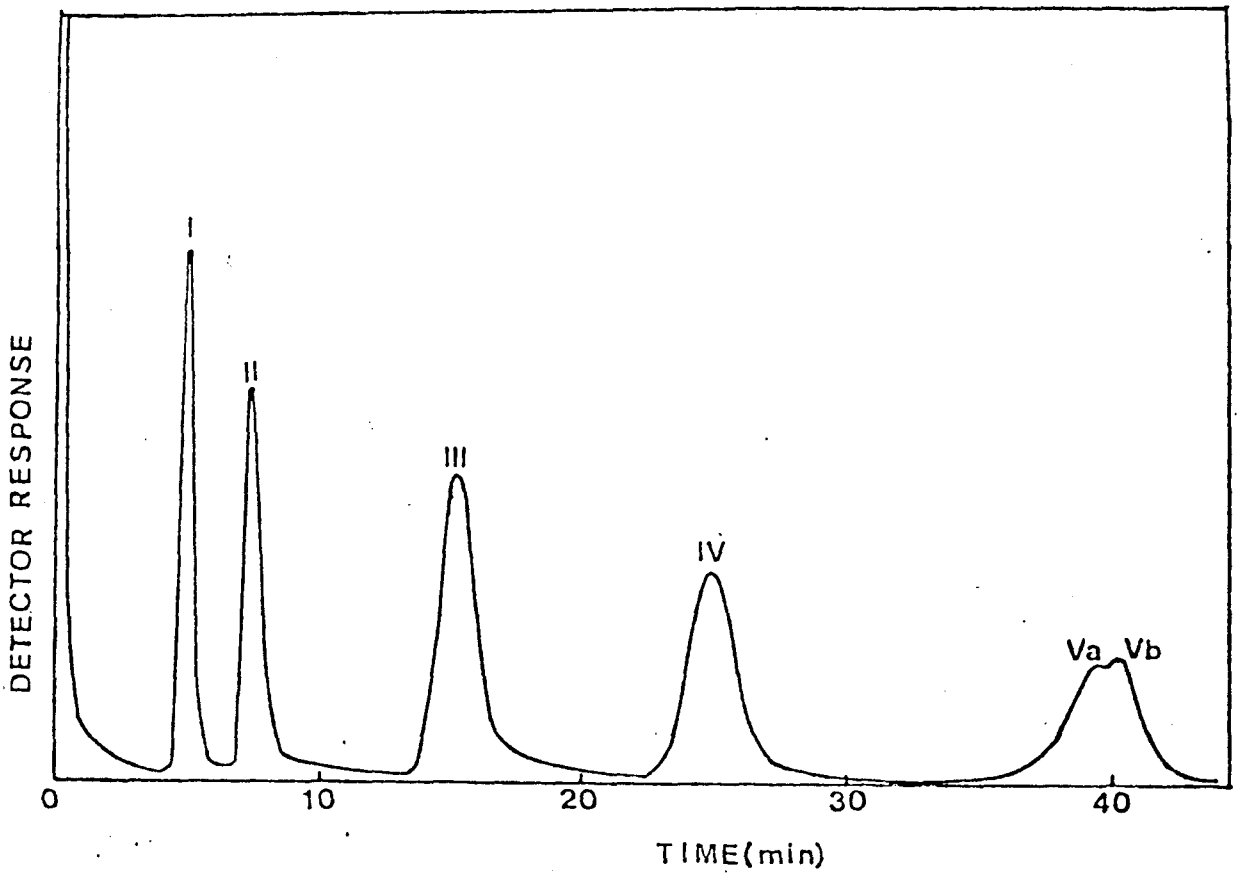


Fig. IV-3. Gas-liquid chromatographic separation of the hydrolysis products of the methylated acidic polysaccharide (Fraction IA), as their alditol acetates.
Condition: 3% ECNSS-M, at 180°; (I) 2,3,4-tri-O-methyl-D-xylose, (II) 2,3,4,6-tetra-O-methyl-D-mannose (glucose), (III) 2,4,6-tri-O-methyl-D-mannose, (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 IV-II Molar ratios of the hydrolysis products of methylated acidic polysaccahride. The methylated acidic polysaccharide was hydrolyzed and the methyl sugar fragments were analyzed by glc.

O-Methyl sugar	Linkage indicated	T_g*	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	$\rightarrow^{2}_{3}$[Man]1→	3.31	1.6
2,4-di-O-methyl-D-Glucose	$\rightarrow^{3}_{6}$[Glc]1→	5.10	1.8
2,4-di-O-methyl-D-Mannose	$\rightarrow^{3}_{6}$[Man]1→	5.37	
2,3,4-tri-O-methyl-D-Glucuronic acid	[GlcUA]1→		1.3

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

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 its methyl glycoside methyl ester on a column of 15% butanediol succinate polyester at 175° (retention times relative to that of methyl 2,3,4,6-β-D-glucopyranoside, 2.5 and 3.2). Thus, the molar ratios of the methyl sugar fragments are listed in Table IV-II. From the inspection of the table, it is evident that the polysaccharide has a relatively highly 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 residues 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 feature appears to resemble those of the fruit body of T. fuciformis, the extracellular polysaccharide and also cell wall polysaccharide of its haploid cells, as established in the previous chapters. They are built up with α-(1→3)-linked mannan backbone to which 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 hydrolysate 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 of (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.

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 (see, Chapter 5). This suggests that antigenic determinants of both polysaccharides, involving either 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 (28), and Armillaria mellea (29), fucosylxylomannans from the

fruit body of Polyporus pinicola (30). The xylomannan type polysaccharides are produced by some yeasts belonging to Trichosporon species (31). 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 (32). However, the occurrence of polysaccharides containing glucuronic acid residues, in addition to mannose and xylose residues seems to be limited to Tremella sp.,Aurulia sp., and Cryptococcus sp. (24). A structural investigation of the extracellular polysaccharide of a non-pathogenic 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-xylose and D-glucuronosyl side chains are attached to C-2 of the mannan (13). As described in Chapter 5, the purified capsular polysaccharide of C. neoformans is highly reactive with antisera against T. fuciformis.

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 Heterobasidiae, and also the group of Cryptococcus species have very close phylogenic relationships.

Summary

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°) consists of a backbone chain of β-(1→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→3)-glucan with single branches at C-6 positions, but it has an extremely highly branched structure; a small proportion of (1→6)-internal linkages may be situated in the side chain.

An acidic heteropolysaccharide ($[\alpha]_D$ -20°), isolated from the hot water extract through CPC insoluble complex formation 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 (D-glucose), 2,3,4-tri-O-methyl-D-xylose, 2,4,6-tri-O-methyl-, 4,6-di-O-methyl- and 2,4-di-O-methyl-D-mannose, 2,4-di-O-methyl-D-glucose, togeher with 2,3,4-tri-O-methyl-D-glucuronic acid, suggesting that it consists of a backbone chain of (1→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.

Chapter 5.

Immunochemical Relationship between Polysaccharides of *Tremella fuciformis*, *Auricularia auricula-judae*, and *Cryptococcus neoformans*

Introduction

Chemical and immunochemical studies on the cell wall polysaccharides have been widely used to classify microorganisms including yeasts and molds (44). In the previous chapters, the author described the structural similarities of the three kinds of acidic heteropolysaccharides, *i.e.*, the extracellular and cell wall polysaccharides of *T. fuciformis*, and the hot-water extracted polysaccharide of *A. auricula-judae* by use of chemical methods.

The present chapter deals with the chemical and immunochemical relationship between the three acidic heteropolysaccharides mentioned above and the capsular polysaccharide of pathogenic *Cryptococcus neoformans* using the antiserum against the hapliod cells of *T. fuciformis* in order to reveal a possible taxonomic relationship between *Tremella*, *Auricularia*, and *Cryptococcus*.

Materials and Methods

Polysaccharides—— The isolation and purification of the acidic extracellular polysaccharide of *T. fuciformis* have been described in Chapter 2. The cell wall polysaccharide of *T. fuciformis* and the acidic polysaccharide of *A. auricula-judae* were isolated by hot water extraction and purified

through CPC insoluble complex formation as described in Chapter 3 and Chapter 4, respectively. The capsular polysaccharide of Cryptococcus neoformans was provided by Dr. I. Azuma, Medical School of Osaka University

Analysis—— Sugars were determined as alditol acetates as described in previous chapters. Methylation analysis and identification of O-methyl sugars were performed as described in Chapter 3.

Immunochemical techniques—— Quantitative precipitin and inhibition assay were performed according to the method of Kabat (45). Antibody nitrogen in specific precipitates was determined by the ninhydrin method after digestion with sulfuric acid as described by Schiffman et al. (46). Generally, 30 µl of serum was taken for each analysis in a total volume of 430 µl. Double diffusion in agar was performed in 1% agarose in 0.9% sodium chloride solution according to the Ouchterlony technique (47) with some modifications. Rabbit antisera were prepared as follows. The haploid cells of T. fuciformis (strain T-7 and T-19), grown as described in Chapter 2, were killed by 0.5% formalinized saline, washed with sterilized saline, and suspended in saline (40 mg wet cells per ml). Five rabbits (2.5 to 3.0 kg) were immunized by injection into the ear vein with 0.5 ml of the suspension twice a week for a month. Nine days after the last injection, blood (about 60 ml) was taken by cardiac puncture. Antisera called No. 250, 262 and 263 were prepared against strain T-19, and No. 260 and 261 were against strain T-7.

Results

Methylation analysis of the capsular polysaccharide of C. neoformans and the cell wall polysaccharide of T. fuciformis strain T-19——

TABLE V-I. Identities and molar ratios of methyl sugar components of the methylated cell wall polysaccharide of T. fuciformis strain T-19 and the capsular polysaccharide of C. neoformans.

O-methyl sugar	Linkage indicated	Polysaccharide	
		T. fuciformis	C. neoformans
2,3,4-tri-O-methyl-D-xylose	[Xyl]1→	1.0	1.0
2,3,4-tri-O-methyl-L-fucose	[Fuc]1→	0.5	-
2,3,4,6-tetra-O-methyl-D-mannose	[Man]1→	1.2	0.1
3,4-di-O-methyl-D-xylose	→2[Xyl]1→	0.4	-
2,4,6-tri-O-methyl-D-mannose	→3[Man]1→	0.2	0.8
4,6-di-O-methyl-D-mannose	$\overset{\rightarrow 2}{\underset{\rightarrow 3}{}}$[Man]1→	3.2	3.7
2,3,4-tri-O-methyl-D-glucuronic acid	[GlcUA]1→	0.5	2.6

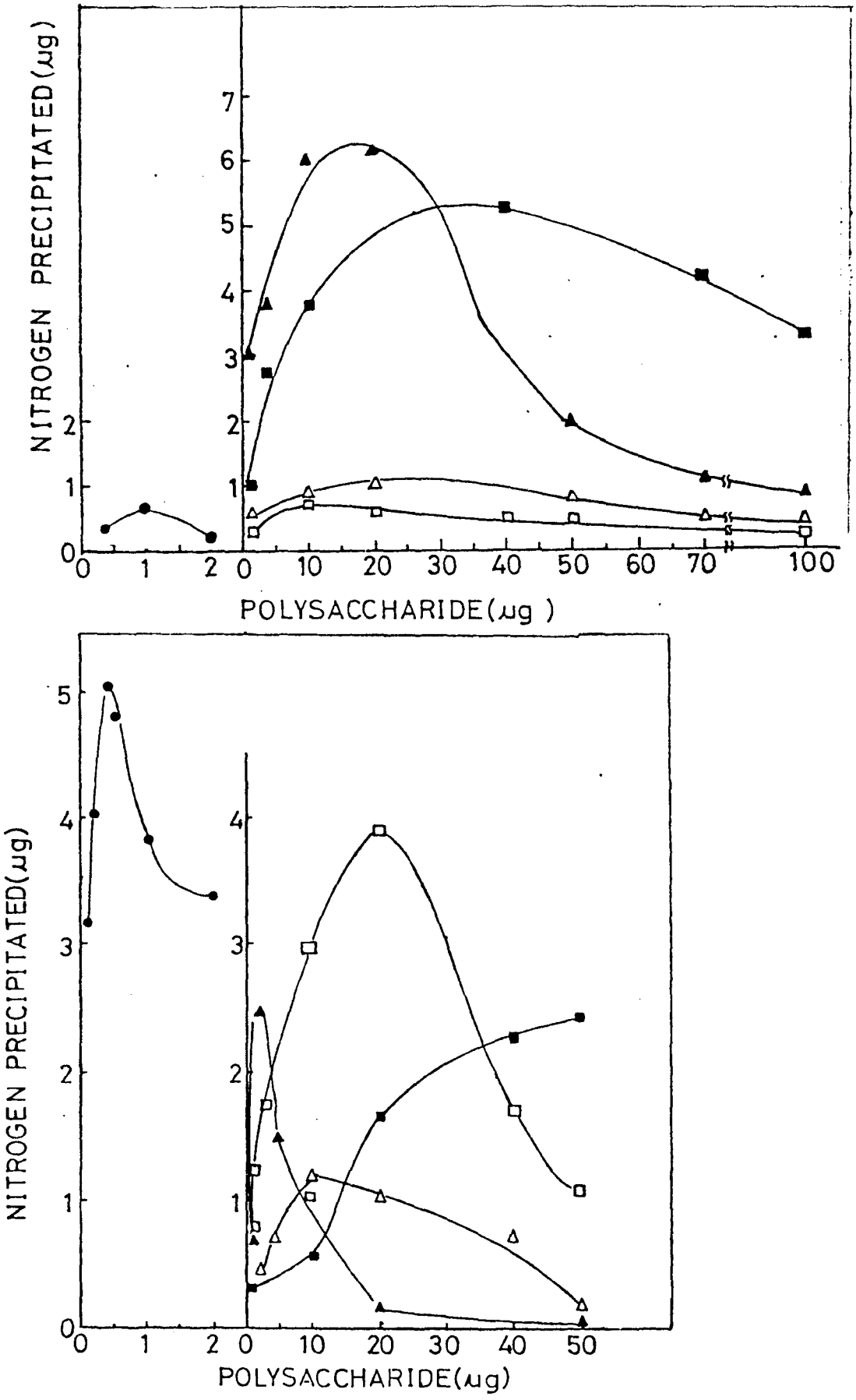


Fig. V-1. Quantitative precipitin curves obtained the serum 262 (top), the serum 261 (bottom) and the cell wall (■) and extracellualr (▲) polysaccharide of *T. fuciformis* strain T-19, the cell wall (□) and the extracellular (△) polysaccharide of *T. fuciformis* strain T-7, the capsular polysaccharide (●) of *C. neoformans*.

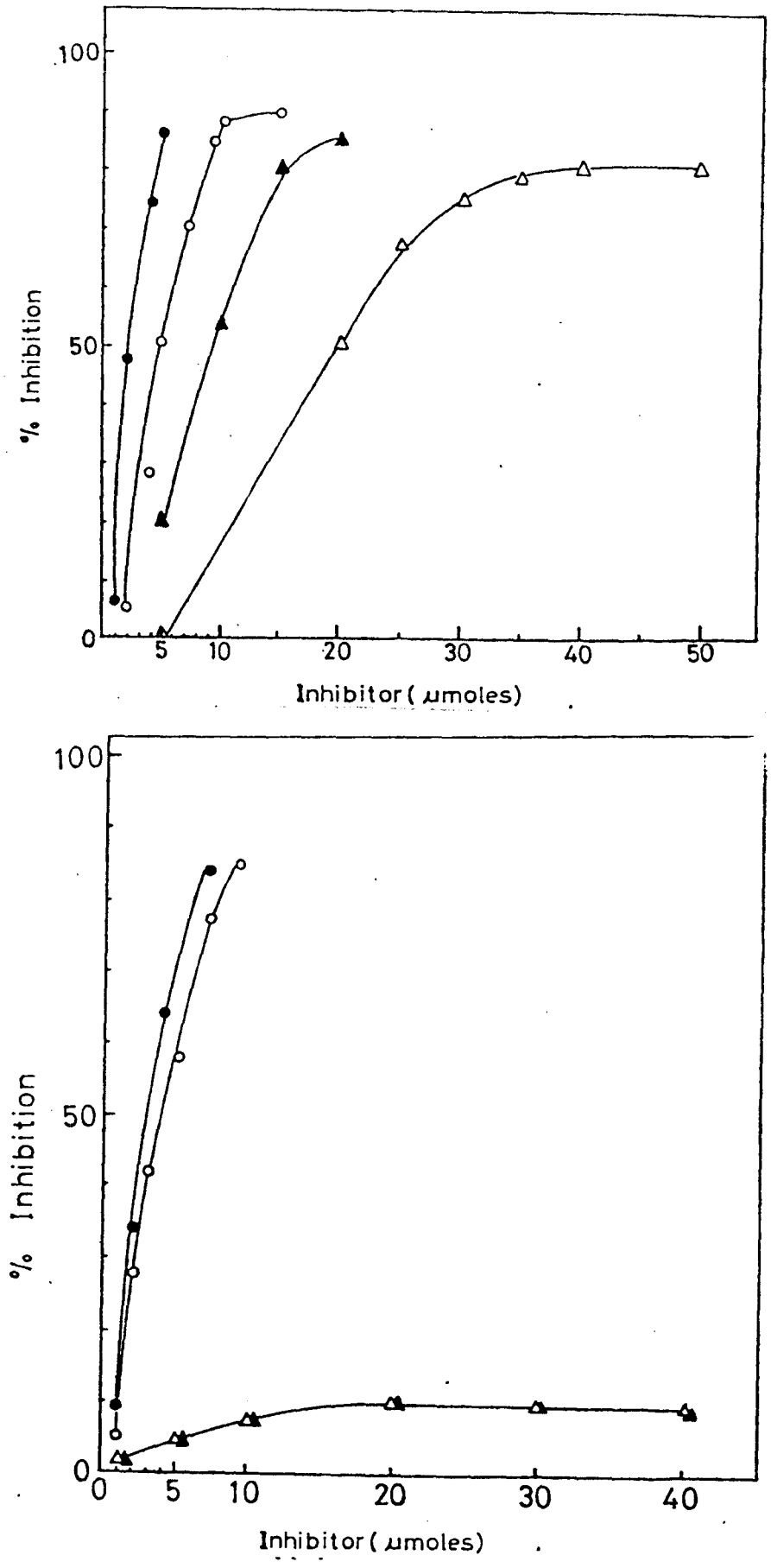


Fig. V-2. Hapten inhibition of the extracellular polysaccharide of strain T-19-the serum 262(top), and the capsular polysachharide-serum 261 (bottom) systems. p-nitrophenyl-β-D-glucuronide (●), sodium glucuronate (○), p-nitrophenyl-β-D-xyloside (▲), and D-xylose (△).

The capsular polysaccharide of C. neoformans and the cell wall polysaccharide of T. fuciformis strain T-19 were methylated by the method of Hakomori and O-methyl sugars were identified as described in previous chapters. The molar ratios of the methyl sugar fragments are listed in Table V-I. From the inspection of the table, it is evident that the capsular polysaccharide of C. neoformans has (1→3)-linked mannan backbone chain, and both D-xylose and D-glucuronic acid residues are located in the terminal ends. This structural feature is very similar to that of the cell wall and extracellular polysaccharide of T. fuciformis strain T-7, except for the high content of D-glucuronic acid. The cell wall polysaccharide of T. fuciformis strain T-19 has a very similar structure to that of the extracellular polysaccharide of its own strain (see Chapter 2).

Precipitation studies—— In the study of ability of two antisera, No. 261 and 262, against cells of T. fuciformis strain T-7 and T-19, respectively, to precipitate with five acidic heteropolysaccharides (Fig. V-1), serum 262 was found to precipitate very strongly with the cell wall and extracellular polysaccharides from the immunizing strain (T-19), but very pooly with the cell wall and extracellular polysaccharides of strain T-7, and the capsular polysaccharide of C. neoformans. Serum 261 reacted strongly with the capsular polysaccharide of C. neoformans and the cell wall polysaccharide of strain T-7, but the extracellular polysaccharide of strain T-7 precipitated about 25% of the maximum amount of antibody precipitated by the cell wall polysaccharide of the same strain. The cell wall and extracellular polysaccharides of strain T-19 reacted with the serum 261, and precipitated about 60% of the maximum amount of antibody precipitated by the homologous cell wall polysaccharide. Quantitative precipitin curves of the acidic heteropolysaccharide of A. auricula-judae with both serum 261 and

262, were very similar to that of the extracellular polysaccharide of T. fuciformis strain T-7.

In double-diffusion in agar, with serum 261 the lines formed by the cell wall polysaccharides of strain T-7 and T-19 showed complete fusion, on the other hand, with serum 262 the homologous reaction line spurred over the reaction line of the cell wall polysaccharide of strain T-7. With serum 261, the lines formed by the cell wall and extracellular polysaccharides of strain T-7, and the capsular polysaccharide of C. neoformans showed complete fusion.

Quantitative hapten inhibition studies—— Quantitative hapten inhibition data for the extracellular polysaccharide of strain T-19—serum 262, and the capsular polysaccharide of C. neoformans—serum 261 systems are shown in Fig. V-2. Significant inhibition of precipitation by the extracellular polysaccharide of strain T-19 and serum 262 was obtained with p-nitrophenyl-β-D-glucuronide and sodium glucuronate (Fig. V-2, top). p-Nitrophenyl-β-D-glucuronide was the most effective inhibitor of those tested, 2 μmol of the inhibitor gave 50% inhibition. Sodium glucuronate was less potent than p-nitrophenyl-β-D-glucuronide and 50% inhibition was obtained with 4 μmol . p-Nitrophenyl-β-D-xyloside and D-xylose also inhibited the reaction, 10 μmol and 20 μmol of these inhibitors gave 50% inhibition, respectively. p-Nitrophenyl-β-D-glucoside, methyl-α-D-mannoside and L-fucose failed to inhibit even in amounts up to 50 μmol. Similar inhibition pattern was obtained in the cell wall polysaccharide of strain T-19—serum 262 system. Inhibition of serum 261 by the capsular polysaccharide of C. neoformans is shown in Fig. V-2 (bottom). p-Nitrophenyl-β-D-glucuronide and sodium glucuronate were comparable in molar inhibitory ability, giving 50% inhibition with 3 μmol and 4 μmol, respectively. D-Xylose and p-nitrophenyl-β-D-xyloside

gave less than 10% inhibition when tested in amounts varing from 1 μmol to 40 μmol in this cross reaction. p-Nitrophenyl-β-D-glucoside, methyl-α-D-mannoside and L-fucose failed to inhibit the reaction.

Discussion

The antisera prepared against T. fuciformis strain T-7 and strain T-19 have specificities directed against D-glucuronic acid, D-xylose or L-fucose residues containing determinants, as would be expected from the methylation analysis, however, that serum 261 prepared against cells of strain T-7 was less specific as compared with serum 262. Thus, the former was inhibited quite well by p-nitrophenyl-β-D-glucuronide and sodium glucuronate, and only slightly by p-nitrophenyl-β-D-xyloside and D-xylose. In contrast, serum 262 was inhibited by p-nitrophenyl-β-D-glucuronide as well as p-nitrophenyl-β-D-xyloside. These facts suggest that the serum 261 have a large proportion of antibody with a specificity directed against structural groupings involving glucuronic acid residues, while the serum 262 have a large proportion of antibody with a specificity directed against structural grouping involving xylose residues, in addition to the same antibody as that of the serum 261.

The difference in the specificities of the two sera were also evident from the precipitation studies. Serum 262 precipitated much better with the cell wall and extracellular polysaccharides of strain T-19 than with corresponding polysaccharides from strain T-7 and the capsular polysaccharide of C. neoformans. Serum 261, on the other hand, precipitated quite well with the cell wall polysaccharide of strain T-7 and the capsular polysaccharide of C. neoformans, as well as with the cell wall and extracellular

polysaccharides of strain T-19. In agreement with these observations, the cell wall and extracellular polysaccharides of strain T-19 have shown to have some (1→2)-linked D-xylose side chains, while the corresponding polysaccharides of strain T-7 and the capsular polysaccharide of C. neoformans have shown to have no side chain of D-xylose.

Specific differences were also reflected in the Ouchterlony gel diffusion patterns. With the cell wall polysaccharide of strain T-19, serum 262 produced a line that spurred over the line produced with the cell wall polysaccharide of strain T-7. Serum 261 produced lines with the cell wall polysaccharide of both strain T-7 and T-19, which showed complete fusion.

However, the precise specific difference awaits further inhibition studies using oligosaccharides from the cell wall or extracellular polysaccharides of T. fuciformis.

The reactivity of the serum 261 with the capsular polysaccharide of C. neoformans confirms the conclusion, by use of chemical methods, concerning the possible taxomic relationship between Cryptococcus and Tremella. The capsular polysaccharide of C. neoformans was shown in the present study to react strongly with the serum 261 and poorly with the serum 262. In this respect, it resembles the cell wall polysaccharide of strain T-7 rather than that of strain T-19. These data reinforce the findings of the methylation analysis that the capsular polysaccharide and the cell wall polysaccharide of strain T-7 have no side chain of D-xylose.

It is interesting that the extracellular polysaccharide of strain T-7 reacted with the serum 261 poorly, although its structure estimated by methylation analysis is similar to that of the cell wall polysaccharide of strain T-7 and the capsular polysaccharide of C. neoformans. The similar phenomenon was reported by Helms et al. (48). They have carried out a

comparative immunochemical study on the acidic polysaccharides of C. laurentii and T. mesenterica using antisera against type II pneumococcal capsular polysaccharide, of which specificity is for glucuronyl determinants. They found that, while the native polysaccharide from Cryptococcus cross-reacted, the corresponding polysaccharide from Tremella did not. Fraser and co-workers (49) have carried out further studies concerning the cross-reaction of the acidic polysaccharide from Tremella mesenterica with a type II anti-pneumococcal serum, and they proposed that the failure of the Tremella polysaccharide to cross-react with type II antiserum is due to steric interactions. The critical factor involved in the steric hindrance can be attributed to some strategically placed xylose units located in the vicinity of the glucuronic acid determinants. Whether the same steric interactions contribute to the poor activity of the extracellular polysaccharide of strain T-7 with the serum 261 is not clear from the present data available.

Summary

Immunochemical studies, including quantitative precipitin and inhibition assay, and double diffusion in agar, confirmed the very close phylogenic relationship between Tremella, Auricularia, and Cryptococcus, which has been suggested by the structural studies of their acidic heteropolysaccharides.

It was also found that the two strains of Tremella fuciformis (T-7 and T-19) are serologically different from each other. This may be caused from the presence or absence of (1→2)-linked D-xylose side chains in the cell wall polysaccharides.

Chapter 6.

Purification and Characterization of β-D-Mannosidase and β-N-Acetyl-D-Hexosaminidase of *Tremella fuciformis*.

Introduction

Among various glycosidases occurring in nature, β-N-acetyl-D-hexosaminidase and β-D-mannosidase have recently been recognized as very useful enzymes for structural elucidations of the carbohydrate chains of many biologically important glycoproteins containing a particular sequence of sugar residues such as β-linked mannosyl-N-acetyl-glucosaminyl-N-acetyl-glucosamine. β-N-Acetyl-D-hexosaminidases are known to be widely distributed in plants, animal tissues and certain microorganisms, such as Jack bean (50), pinto bean (51), human placentae (52), *Aspergillus niger* (53) and *Bacillus subtilis* (54). Several reports concerning the isolation and characterization of β-N-acetyl-D-hexosaminidase have recently appeared. Although β-D-mannosidase have been found in snail (55), hen oviduct (56), pineapple bromelain (57) and also liver of *Turbo cornutus* (58), these sources usually contain other types of glycosidases, particularly, α-D-mannosidase.

In the course of studies on polysaccharides in the fruit body of *Tremella fuciformis*, known as an edible mushroom, and also from its yeast-like cells of the haploid strain (see, Chapter 2), the author became aware that the cell extract of the cultured *T. fuciformis* contained only two particular glycosidases, *i.e.*, β-D-mannosidase and β-N-acetyl-D-hexosaminidase, and no appreciable activities of other glycosidases were detected. Since these two enzymes were thought to be useful tools for investigation of the carbohydrate chains of glycoproteins, *e.g.*, the lectin

of Phaseolus lunatus (59), an attempt has been made to isolate these two enzymes in highly pure state. This chapter deals with the purification of a β-D-mannosidase and a β-N-acetyl-D-hexosaminidase, and their properties, such as substrate specificities and physical properties. When the present study was completed, the isolation of a β-D-mannosidase from a mushroom Polyporus sulfures, which is free of α-D-mannosidase, was reported by Wan et al. (60).

Materials and Methods

Materials—— Most of p-nitrophenyl glycosides used in this study were available commercially. p-Nitrophenyl-β-D-mannoside was also provided by Dr. K. Kawaguchi (61). The β-(1→4)-manno-oligosaccharides were prepared from the enzymic digestion of the mannan of "Dioscorea batatas Decne, forma Tsukune" (62). The chito-oligosaccharides and O-β-D-mannopyranosyl-(1→4)-2-amino-2-deoxy-D-glucopyranose were generous gifts of Prof. Goldstein of the University of Michigan. The haploid cells of Tremella fuciformis Berk (strain T-7) was provided by Dr. K. Tubaki, Tsukuba University.

Enzyme assays—— During the purification steps of β-D-mannosidase and β-N-acetyl-D-hexosaminidase, their enzyme activities were routinely assayed at 37°, using p-nitrophenyl-β-D-mannoside and p-nitrophenyl-β-N-acetyl-D-glucosaminide, respectively, as substrates. An enzyme solution (100 μl) was added to 400 μl of 1.25 mM p-nitrophenyl glycosides dissolved in 2.5 mM acetate buffer, pH 5.0. After incubation for 20 min or 30 min, 1.25 ml of 0.2 M sodium carbonate solution was added to stop the reaction, and liberated p-nitrophenol was determined spectrophotometrically at 420 nm. One unit was defined as the amount of the enzyme that releases 1 μmol of

p-nitrophenol per min, and specific activity was expressed as the number of units per mg protein. Protein content was determined by the method of Lowry et al. (63), using bovine serum albumin as a standard.

Analytical methods—— When the manno-oligosaccharides were used as substrates for β-D-mannosidase, D-mannose in the enzyme digest was quantitatively determined by the method of Perk-Johnson (64). N-Acetyl-D-glucosamine liberated from chito-oligosaccharides was determined colorimetrically by the method of Reissing et al. (65). Polyacrylamide disc gel electrophoresis was performed in 6% polyacrylamide gels, pH 9.3, as the method described by Davis (66). The enzyme preparation in 10% sucrose was applied on the top of the polymeric gels and the electrophoresis was conducted for 3 h. A 1% solution of Amido Black in 7% acetic acid was used for detection of the protein bands. Molecular weights of β-D-mannosidase and β-N-acetyl-D-hexosaminidase were determined according to the method of Andrews (67). A column of Sephadex G-100 (1.5 x 84 cm), which was equilibrated with 0.05 M acetate buffer, pH 5.5, was used. Calibration was made with proteins of known molecular weights, purchased from Mann Research Laboratories.

Purification of glycosidases—— Each enzyme preparation obtained at various stages of purification was concentrated by means of ultrafiltration using a Diaflo XM-100 membrane, which excludes materials under 100,000 molecular weight, under nitrogen pressure of 2 kg/cm^2. As will be described later, β-D-mannosidase appeared not to be stable, but it was found to be protected if an appropriate amount of dithiothreitol (DTT) is present. Therefore, purification steps of β-D-mannosidase were carried out with the buffer containing 1 mM DTT.

Results

Unless otherwise stated, all purification procedures of the enzymes were carried out at 0°—4°.

Preparation of the crude enzyme—— Tremella fuciformis, strain T-7, was grown at 30° for 4 days in a 30 liters jar fermentor contained 25 liters of the medium containing glucose (2%), yeast extract (0.5%), KH_2PO_4 (0.05%) and K_2HPO_4 (0.05%). The harvested cells were collected by centrifugation, washed twice with saline, and disintegrated three times with Sorvall-Ribi cell fractionator (RF-1) at 40,000 psi, or sonicated (20 KHz, for 30 min) in 0.05 M acetate buffer, pH 5.5. The cell-wall were removed by centrifugation at 12,600 x g for 30 min, and the supernatant was used as the crude extract for purification of the enzymes.

Ammonium sulfate fractionation of the crude enzyme—— To the cell extract (1.25 liters obtained from 50 liters culture), ammonium sulfate was added gradually to bring about 25% saturation. The resulting precipitate was collected by centrifugation (Fraction 1). The second precipitation was obtained at 50% saturation of ammonium sulfate (Fraction 2 2). The precipitated protein was collected by centrifugation. Fractions 1 and 2 were dissolved, respectively, in 100 ml of 0.05 M acetate buffer, pH 5.5. The third precipitation was carried out about 100% saturation in the same way described above (Fraction 3). Each enzyme solution so obtained was dialyzed against the same 0.05 M acetate buffer for 48 h at 4°. Since Fraction 2 contained only β-D-mannosidase, it was used for further purification of β-D-mannosidase, and Fraction 3, which contained most of β-N-acetyl-D-hexosaminidase and β-D-mannosidase, was used for the purification of β-N-acetyl-D-hexosaminidase.

Purification of β-D-Mannosidase

Step 1; Column chromatography on Sephadex G-200—— Fraction 2, precipitated by ammonium sulfate (25%—50%), was concentrated to 25 ml, and applied to a column of Sephadex G-200 (3.0 x 90 cm) equilibrated with 0.05 M acetate buffer (pH 5.5), containing 1 mM DTT, and eluted with the same buffer as shown in Fig. VI-1. Fractions of 3.0 ml were collected. The enzyme fractions containing the enzyme activities were pooled , and concentrated by membrane-ultrafiltration.

Step 2; Column chromatography on DEAE-cellulose—— The above concentrated β-D-mannosidase solution was applied to a DEAE-cellulose column (3.5 x 10 cm) equilibrated with 0.05 M acetate buffer, pH 5.5, containing 1 mM DTT. Linear gradient elution was carried out with 0.05 M acetate buffer, pH 5.5, containing 1 mM DTT, and the same buffer containing 1 M NaCl as shown in Fig. VI-2. The fractions which contained β-D-mannosidase were pooled and concentrated.

Step 3; Column chromatography on Hydroxylapatite—— The enzyme solution obtained by step 2 was dialyzed against 0.05 M potassium phosphate buffer, pH 5.5, containing 1 mM DTT for 24 h, and applied to a hydroxylapatite column (3.5 x 10 cm), which was equilibrated with the same buffer. Linear gradient elution was made with 0.05 M and 0.5 M potassium phosphate buffer. Fractions containing pure β-D-mannosidase were pooled and concentrated. The purification process of β-D-mannosidase is summarized in Table VI-I.

Purification of β-N-acetyl-D-hexosaminidase

Step 1; Column chromatography on Hydroxylapatite—— Fraction 3, which contained β-N-acetyl-D-hexosaminidase and β-D-mannosidase, was dialyzed against 0.05 M potassium phosphate buffer, pH 5.7, for 48 h, and the

non-dialyzable fraction was applied to a hydroxylapatite column (3.5 x 7 cm), elution was made with a continuous gradient of 0.05 M potassium phosphate buffer, pH 5.7, and 0.5 M of the same buffer containing 1% NaCl. Fractions of each 10 ml were collected. Fig. VI-3 shows the elution profile of the enzymes from the column. The column chromatography on hydroxylapatite gave a complete separation of β-D-mannosidase and β-N-acetyl-D-hexosaminidase. Each enzyme fraction was pooled and concentrated.

Step 2; Column chromatography on DEAE-cellulose——The partially purified β-N-acetyl-D-hexosaminidase was applied to a DEAE-cellulose column (3.5 x 9.0 cm). After washing the column with 0.05 M acetate buffer, pH 5.5, the column was eluted with a linear salt gradient from 0 to 1 M NaCl in the acetate buffer, pH 5.5. Fractions of each 5 ml were collected and assayed for protein and enzyme activity. The β-N-acetyl-D-hexosaminidase was not absorbed on the column and emerged before the gradient elution. Fractions containing the enzyme activity were pooled and concentrated by membrane-ultrafiltration to a small volume.

The purification of β-N-acetyl-D-hexosaminidase is summarized in Table VI-II.

The purification scheme described above yielded preparations of β-D-mannosidase and β-N-acetyl-D-hexosaminidase, respectively. Each preparation of β-D-mannosidase and β-N-acetyl-D-hexosaminidase was free from the other enzyme activity, and gave a single protein band, when examined by polyacrylamide gel electrophoresis at pH 9.3. This may be the first example that the β-D-mannosidase was highly purified to give a single protein band on disc gel electrophoresis.

Properties of β-D-mannosidase and β-N-acetyl-D-hexosaminidase

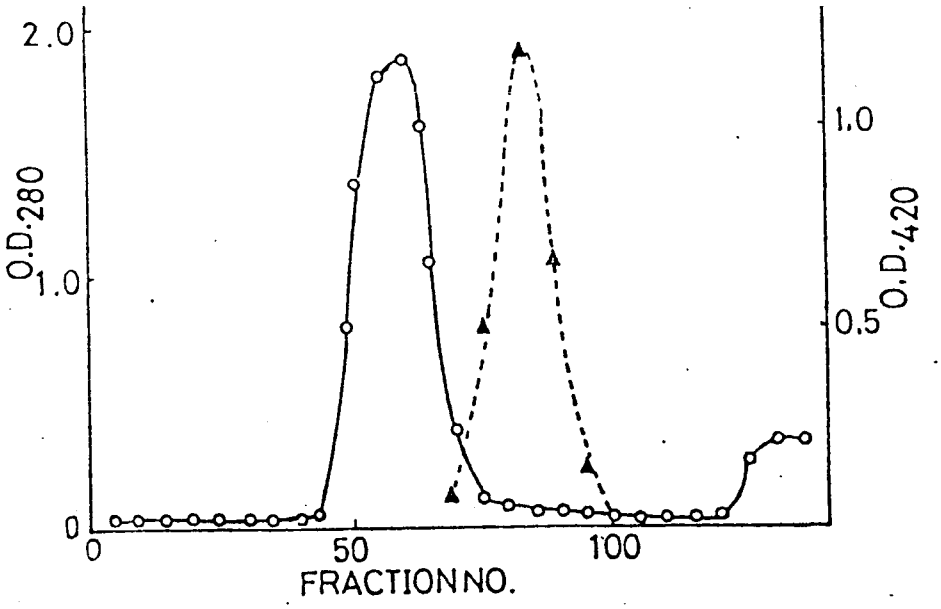


Fig. VI-1. Sephadex G-200 column chromatography of the second fraction of ammonium sulfate precipitation.

A 25 ml aliquot of the concentrated β-D-mannosidase fraction (33.8 mg of protein) was applied to a Sephadex G-200 column (3 x 90 cm) equilibrated with 0.05 M acetate buffer (pH 5.5) containing 1 mM dithiothreitol. The column was eluted with the same buffer and 3 ml fractions were collected and fractions 75-95 were pooled for further purification. 280 nm absorbance, -o-o- ; β-D-mannosidase, -▲-▲- .

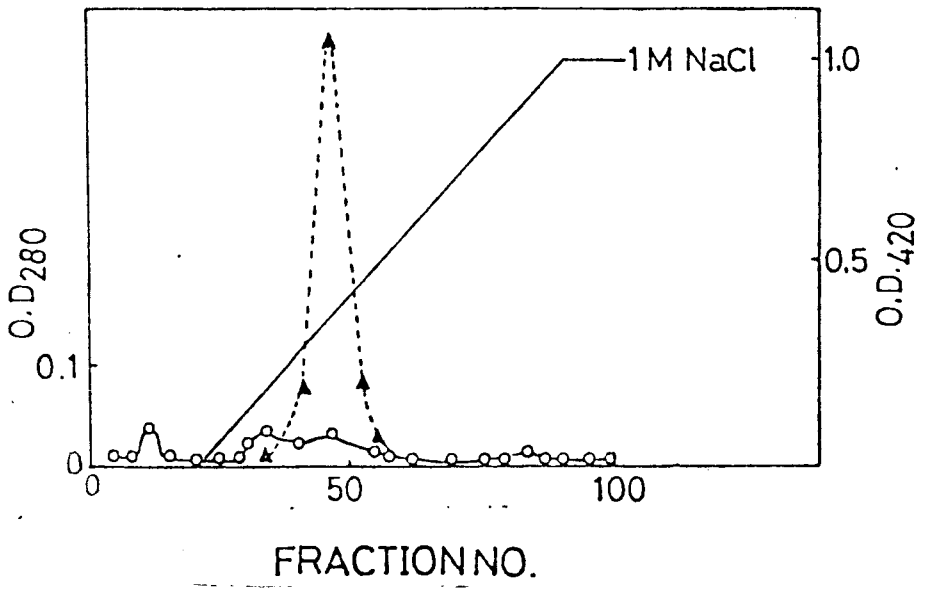


Fig. VI-2. DEAE-cellulose column chromatography of β-D-mannosidase from Sephadex G-200 column chromatography.

A 10 ml solution of β-D-mannosidase (4.3 mg of protein) was applied to a column (3.5 x 10 cm) of DEAE-cellulose equilibrated with 0.05 M acetate buffer, pH 5.5, containing 1 mM dithiothreitol. The column was eluted with a continuous linear salt gradient of 200 ml Of 0.05 M acetate buffer containing 1 mM dithiothreitol and 200 ml of 1 M NaCl in the same buffer. Fractions of 5 ml were collected. 280 nm absorbance, -o-o- ; β-D-mannosidase, -▲-▲- .

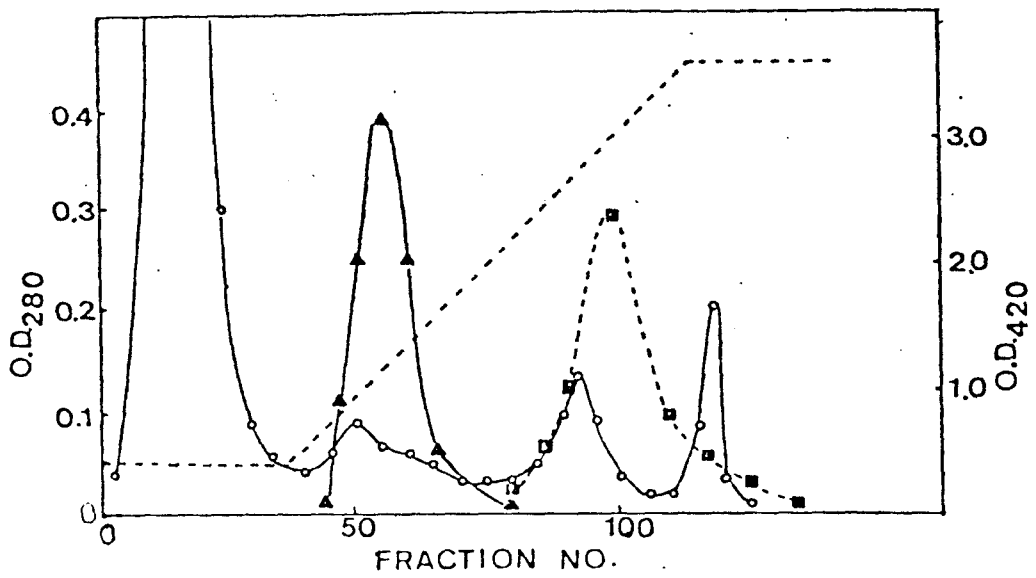


Fig. VI-3. Hydroxylapatite chromatography of Fraction 3 of ammonium sulfate precipitation.

After the dialysis against 0.05 M potassium phosphate buffer, pH 5.7, Fraction 3 was applied to a hydroxylapatite column (3.5 x 7 cm) and eluted with a continuous gradient of 0.05 M potassium phosphate buffer, pH 5.7 and 0.5 M of the same buffer containing 1% NaCl. Fractions of each 10 ml were collected. 280 nm absorbance,-o-o-; β-D-mannosidase,-▲-▲-; β-N-acetyl-D-hexosaminidase ,--■--■--; concentration of phosphate buffer,--------.

TABLE VI-I. Purification of β-D-Mannosidase of Tremella fuciformis.

Step and procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	3500	146	0.043	—
$(Am)_2SO_4$ precipitation	33.8	19.2	0.57	13
Sephadex G-200	4.3	6.0	1.4	3.7
DEAE-cellulose	1.1	5.5	5.0	3.7
Hydroxyl apatite	0.2	4.0	20.0	2.7

TABLE VI-II. Purification of β-N-Acetyl-D-Hexosaminidase of Tremella fuciformis.

Step and procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	3500	46	0.015	—
$(Am)_2SO_4$ precipitation	260	35.1	0.14	77
Pressure dialysis	130	30.3	0.23	66
Hydroxylapatite column	25	19.3	0.77	42
DEAE-cellulose column	4.5	13.8	3.04	30

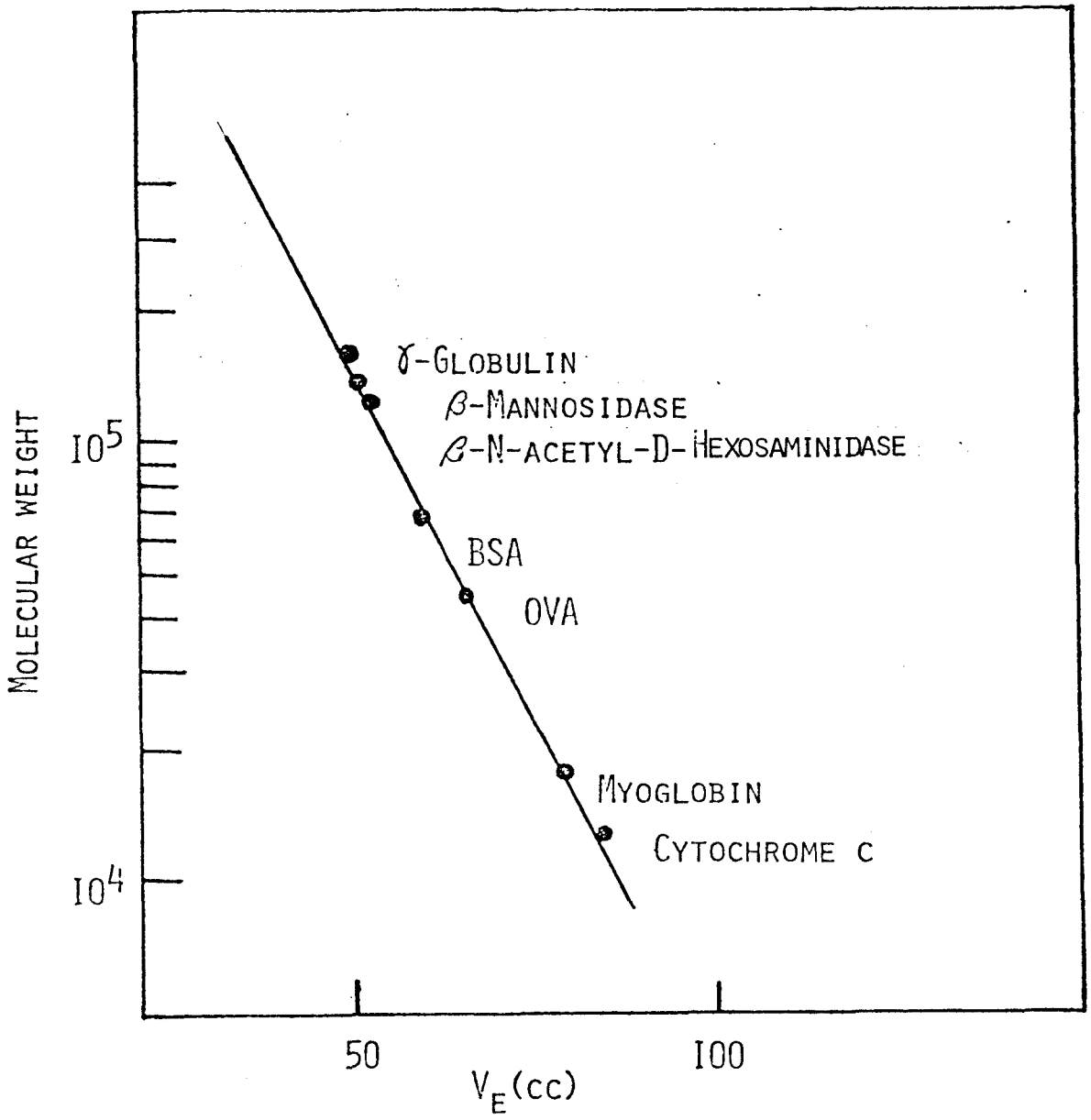


Fig. VI-4 Determination of the molecular weights of β-D-mannosidase and β-N-acetyl-D-hexosaminidase by Sephadex G-100 chromatography. The elution volumes of the protein standards were determined by measuring absorbance at 280 nm, whereas that for the β-D-mannosidase and β-N-acetyl-D-hexosaminidase was monitored by assaying for enzyme activity. Details are described in the text.

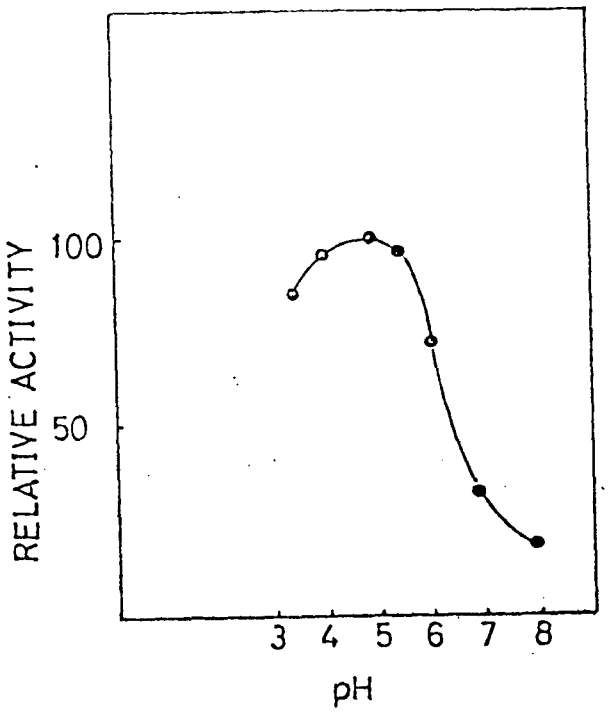


Fig. VI-5a. Effect of pH on activity of β-D-mannosidase.

The enzyme was incubated with p-nitrophenyl β-D-mannoside in acetate buffer of pH 3.0 to 5.5 and tris-malate buffer of pH 6.0 to 8.0.

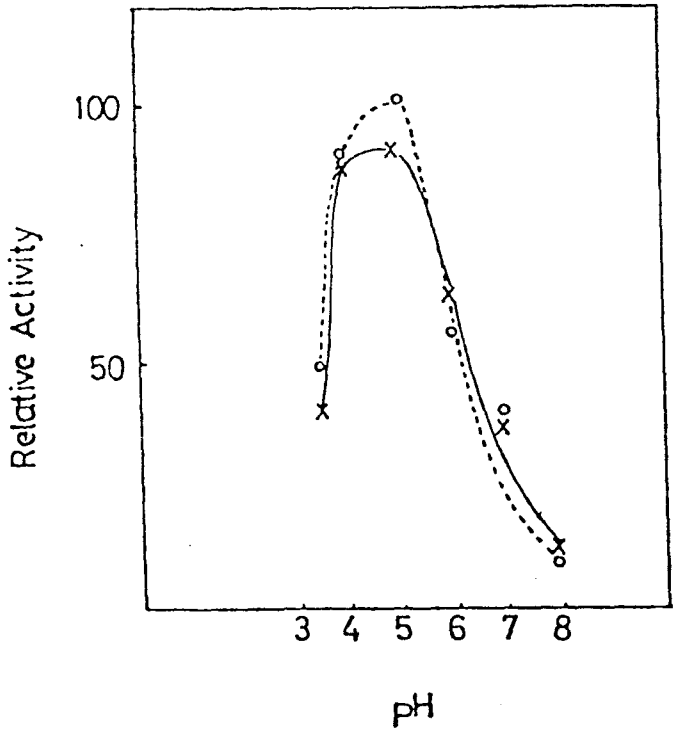


Fig. VI-5b. Effect of pH on activity of β-N-acetyl-D-hexosaminidase.

The enzyme was incubated with p-nitrophenyl β-N-acetyl-D-glucosaminide (–x—x–) and N,N'-diacetyl-chitobiose (--o-o--) in acetate buffer and tris-malate buffer.

Molecular weight—— The molecular weights of the two enzymes were estimated by comparing their elution volumes on a column of Sephadex G-100 with those of a series of proteins of known molecular weights on the same column, as shown in Fig. VI-4. The molecular weight of β-D-mannosidase was estimated to 140,000. In the same manner, the molecular weight of β-N-acetyl-D-hexosaminidase was 125,000.

pH Optima and stability of the glycosidases—— The effects of pH on the activities of the two enzymes were examined using p-nitrophenyl-β-D-mannoside for β-D-mannosidase, and p-nitrophenyl-β-N-acetyl-D-glucosaminide and N,N'-diacetyl-chitobiose for β-N-acetyl-D-hexosaminidase, respectively. The pH-activity curve of β-D-mannosidase shown in Fig. VI-5a indicated its pH optimum of 5.0. The β-N-acetyl-D-hexosaminidase showed its pH optimum of 5.0, with both p-nitrophenyl-β-D-glucosaminide and N,N'-diacetyl-chitobiose, as shown in Fig. VI-5b. The effect of pH on stability of the activity was examined by incubation of the enzyme with buffers of appropriate pH between 3 and 9 for 1 h at 37° followed by the assay of the residual activity at pH 5.0 by the method described in "Materials and Methods". The β-D-mannosidase was stable in the pH range between 5.0 and 5.5, its activity was lost rapidly below pH 3.5. The β-N-acetyl-D-hexosaminidase was found to be stable around pH 7.0.

Effect of substrate concentration—— By incubation of the β-D-mannosidase with p-nitrophenyl-β-D-mannoside at various concentration ranging from 1.25 mM to 20 mM in 0.05 M acetate buffer, pH 5.0, a Lineweaver-Burk plot was obtained. The plot of 1/V against 1/S showed a straight line relationship. The value of K_m, computed from the plot was 2.1 mM and that of V_{max} was 0.21 μmol per min per mg protein. A Lineweaver-Burk plot of β-N-acetyl-D-hexosaminidase was obtained by use of two analogous substrates,

i.e., p-nitrophenyl-β-N-acetyl-D-glucosaminide and p-nitrophenyl-β-N-acetyl-D-galactosaminide. The values for apparent K_m 0.31 mM and V_{max} 4.6 μmol per min per mg protein were computed from the graph with p-nitrophenyl-β-N-acetyl-D-glucosaminide, whereas the values of K_m 0.19 mM and V_{max} 0.6 μmol per min per mg protein were obtained with p-nitrophenyl-β-N-acetyl-D-galactosaminide under the same condition.

Effects of metal ions and chelating agents——In order to know the inhibitory or stimulatory effects of ions on the glycosidase activity, various metal ions were examined. As listed in Table VI-III a and b, mercury ion was found to be a potent inhibitor on both the glycosidase activities. Silver ion inhibited β-D-mannosidase activity completely at the concentration of 0.01 mM, but caused only 13% inhibition of β-N-acetyl-D-hexosaminidase activity at the same concentration. Calcium and magnesium ions and the chelating agents such as EDTA did not show any significant effect on both the enzyme activities. On the other hand, p-chloromercuribenzoate had no effect on β-N-acetyl-D-hexosaminidase activity, but did inhibit strongly the β-D-mannosidase activity. Surprisingly, the manno-γ-lactone did not exert significant inhibitory effect on β-D-mannosidase activity, even at a concentration of 20 mM. The galactono-γ-lactone and the gulono-γ-lactone did not show any inhibitory effects on β-D-mannosidase activity at concentrations of 10 mM and 5 mM, respectively. N-Acetyl-D-glucosamine was found to be an effective competitive inhibitor of β-N-acetyl-D-hexosaminidase.

Effects of the sulfhydryl reagents on β-D-mannosidase activity——During the purification steps, the β-D-mannosidase activity appeared to be gradually reduced. Therefore, the protective effects of sulfhydryl reagents, such as L-cystein, 2-mercaptoethanol and dithiothreitol, on the β-D-mannosidase activity were examined. A solution of β-D-mannosidase, which was inactivated

by an atomosphere of air for 4 days at 4°, pH 5.5, was incubated at 27° with 2 mM of L-cystein, 2-mercaptoethanol or 1 mM of dithiothreitol in 0.05 M acetate buffer, pH 5.5. At suitable time intervals, 1 ml aliquots of the incubation mixtures were taken, and the activity of the β-D-mannosidase in each tube was assayed by the standard method. As shown in Fig. VI-6, inactivated β-D-mannosidase was recovered its original activity by the addition of 1 mM of dithiothreitol, but no appreciable effect was observed with other sulfhydryl reagents, e.g., L-cystein and 2-mercaptoethanol.

Substrate specificity——To characterize the substrate specificities of the two glycosidases, the hydrolysis rates of various oligosaccharides were measured. Table VI-IV shows the relative rates of hydrolyses of p-nitrophenyl-β-D-mannoside, methyl-β-D-mannoside and β-(1→4)-linked manno-oligosaccharides (DP, 2—4), when the β-D-mannosidase (0.03 unit) was incubated with 3 mM of each substrate at 37° for 1 h in 0.02 M acetate buffer, pH 5.0. It is apparent that p-nitrophenyl-β-D-mannoside and β-(1→4)-linked mannobiose were good substrates but the hydrolysis rate of β-manno-oligosaccharides tended to decrease with the increase in the degree of polymerization of the substrates. Methyl-β-D-mannoside was scarcely hydrolyzed, and the rate of hydrolysis was only 2% of that with p-nitrophenyl-β-D-mannoside. In the same condition, O-β-D-mannopyranosyl-(1→4)-2-amino-2-deoxy-D-glucopyranose (0.12 μmol) was slowly but completely hydrolyzed by the β-D-mannosidase (0.03 unit), and after 16 h N-acetyl-D-glucosamine was quantitatively liberated.

Table VI-V shows the relative rates of hydrolyses of p-nitrophenyl-β-N-acetyl-D-glucosaminide and N-acetyl-chito-oligosaccharides (DP, 2—4). The β-N-acetyl-D-hexosaminidase was incubated with 1 mM of each substrate at 37° for 30 min in 0.03 M acetate buffer, pH 5.0. The rate of hydrolysis

TABLE VI-III (a). Effect of various compounds on β-D-mannosidase activity

β-D-Mannosidase was preincubated at 37° with inhibitors (0.01 mM—20 mM) for 6 min, and then assayed the residual activity for p-nitrophenyl β-D-mannosidase by standard method. Enzyme, inhibitors and substrate were dissolved in 0.05 M acetate buffer, pH 5.5.

Compound	% Inhibition						
Concentration	0.01 mM	0.5 mM	1 mM	2 mM	5 mM	10 mM	20 mM
$HgCl_2$	65			100		100	
$CdCl_2$	26			53		100	
$AgNO_3$	100						
$MgSO_4$	19	11		3	(14)	(26)	
$Co(NO_3)_2$	24	11					
$Ca(NO_3)_2$	(5)	(22)		14	45		
$ZnSO_4$	0	(25)		14	19	30	
p-CMB	85	100		100		100	
EDTA					(11)		

( ) indicates % stimulation.

p-CMB, p-chloromercuribenzoate.

TABLE VI-III (b). Effect of various compounds on β-N-acetyl-D-hexosaminidase activity.

The residual activity was measured using p-nitrophenyl-β-N-acetyl-D-glucosaminide as a substrate. Other conditions were the same in Table VI-III (a).

Compound	% Inhibition				
Concentration	0.01 mM	0.5 mM	2 mM	5 mM	10 mM
$HgCl_2$	37		100		
$CdCl_2$	14		20		67
$ZnSO_4$	30		58		73
$Ca(NO_3)_2$			14		
$MgSO_4$			8		0
p-CMB		43			
EDTA				23	

p-CMB, p-chloromercuribenzoate.

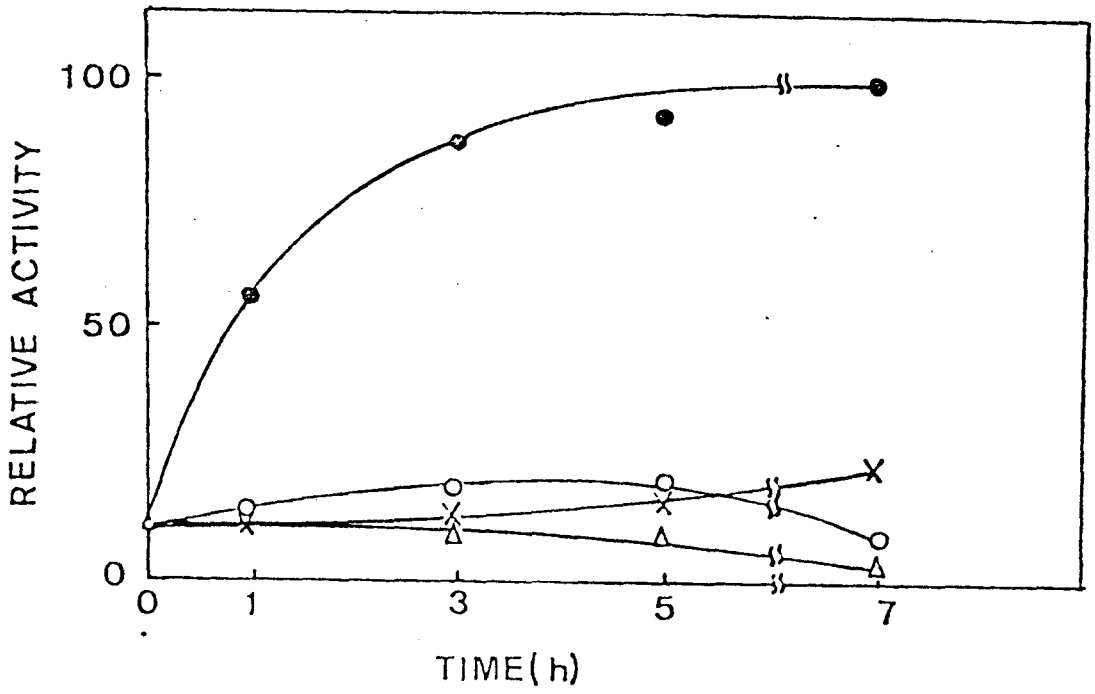


Fig. VI-6. Effects of the sulfhydryl reagents on β-D-mannosidase activity. The inactivated β-D-mannosidase was incubated at 27° with one of the sulfhydryl reagents in 0.05 M acetate buffer, pH 5.5, and at designated time intervals 1 ml aliquots of the incubation mixtures were taken, and the activity of the β-D-mannosidase was determined by the standard method. control, -Δ-Δ-; 2-mercaptoethanol, -×-×-; L-cystein, -o-o- ; dithiothreitol, -●-●-

TABLE VI-IV. Substrate specificity of *T. fuciformis* β-D-mannosidase
The β-D-mannosidase was incubated with 3 mM of each substrate at 37° for 1 h in 0.02 M acetate buffer, pH 5.0.

Substrate	Relative Rate
p-nitrophenyl-β-D-mannoside	100
methyl-β-D-mannoside	2
β-(1→4)-D-mannobiose	31
β-(1→4)-D-mannotriose	9
β-(1→4)-D-mannotetraose	4
β-(1→4)-D-mannosyl-N-acetyl-D-glucosamine	+

+ N-Acetyl-D-glucosamine (0.12 μmole) was released from the substrate (0.12 μmole) by 16 h incubation with 0.03 unit enzyme as determined colorimetrically.

TABLE VI-V. Substrate specificity of *T. fuciformis* β-N-acetyl-D-hexosaminidase
The β-N-acetyl-D-hexosaminidase was incubated with 1 mM of each substrate at 37° for 30 min in 0.03 M acetate buffer, pH 5.0

Substrate	Relative Rate
p-nitrophenyl-β-N-acetyl-D-glucosaminide	100
N,N'-diacetyl-chitobiose	40
N,N',N'-triacetyl-chitotriose	32
N,N',N',N'-tetraacetyl-chitotetraose	32

of N,N'-diacetyl-chitobiose was 40%, and that of N,N',N''-triacetyl-chitotriose and N,N',N'',N'''-tetraacetyl-chitotetraose was 32% of that with p-nitrophenyl-β-N-acetyl-D-glucosaminide. The β-N-acetyl-D-hexosaminidase was shown to also be active on a β-glucosaminyl-muraminyl-peptide isolated from bacterial cell wall peptideglycan, from which the terminal N-acetyl-D-glucosamine was liberated, but its hydrolysis rate appeared to be very small.

Discussion

Recent structural studies of glycoproteins and glycopeptides from various biological origins have shown successful applications of purified glycosidases for determination of the sequences and anomeric linkages of sugar residues in the carbohydrate chains. The isolation of β-D-mannosidase from several sources were reported (55-58), but most of these enzyme sources are known to contain both α- and β-D-mannosidase, in addition to other glycosidases, except Polyporus sulfureus (60) which contains only β-D-mannosidase.

The present study indicated that the extract of yeast-like cells of Tremella fuciformis, grown in a simple medium, contains only two types of glycosidase, i.e., β-D-mannosidase and β-N-acetyl-D-hexosaminidase. Each glycosidase was highly purified, approximately 500- and 200-fold, respectively, by relatively simple procedures. The crude enzyme in the second fraction of cell extract, precipitated with ammonium sulfate at 25%—50%, was used for the purification of the β-D-mannosidase, since this fraction was free of β-N-acetyl-D-hexosaminidase, though it contained approximately 15% of the total activities of the β-D-mannosidase. The purification was effected by three steps, involving column chromatographies using Sephadex G-200, DEAE-

cellulose and hydroxylapatite. The purified β-D-mannosidase showed a single protein band in the acrylamide gel electrophoresis, and had a molecular weight 140,000, about two times as large as that from Polyporus sulfureus (mol. wt. 64,000)(60), and larger than that from Hen oviduct (mol. wt. 100,000)(56). Its optimal pH 5.0, for hydrolysis of p-nitrophenyl-β-D-mannoside and β-linked D-manno-oligosaccharides may be similar to those of known mannosidases(55-58). The present β-D-mannosidase readily hydrolyzes p-nitrophenyl-β-D-mannoside, but cannot hydrolyze methyl-β-D-mannoside. With regards to β-(1→4)-linked-D-manno-oligosaccharides, the hydrolysis rate appears to be markedly reduced with increases in the degree of polymerization. Thus, β-mannotriose is hydrolyzed at a rate of 30% and, β-mannotetraose at a rate of 13%, respectively, relative to the mannobiose. It may be noteworthy that the enzyme is able to hydrolyze slowly but completely O-β-(1→4)-D-mannosyl-N-acetyl-D-glucosamine, which is a common building unit of the carbohydrate chains of most N-glycosidic linked glycoproteins. Unlike other mannosidases, such as α- and β-mannosidase from Hen oviduct (56), the mannosidase activity was not inhibited by manno-γ-lactone, known as an effective competitive inhibitor, even at a concentration of 20 mM, suggesting that the enzyme action of the present β-D-mannosidase involves somewhat a differnt mechanism, with those of known glycosidases. As regards stability of the β-D-mannosidase, it must be pointed out that the activity was gradually lowered during column chromatography on a Sephadex G-200, and also in the process of concentration by means of dialysis under an aerobic condition. The later could be prevented by substitution of air with nitrogen, suggesting that such an inactivation of the enzyme involves the modification of linkages between certain amino acid residues under the oxidative condition. This prompted the author to examine the effect of

sulfhydryl reagents, since some enzymes, e.g., threonine deaminase of E. coli (68) and metapyrocatechase of Pseudomonas sp. (69) were known to be inactivated under the oxidative condition, and the activities might be recovered by the addition of an appropriate concentration of sulfhydryl reagent, such as mercaptoethanol and dithiothreitol (DTT). The experiments revealed that the activity of β-D-mannosidase, dropped in the presence of oxygen, is recovered almost completely by the addition of 1 mM DTT (see, Fig. VI-6), but not with other sulfhydryl reagents, so far as tested. This fact, and also a strong inhibition of the enzyme activity with p-chloromercuribenzoate, suggest that the present β-D-mannosidase most probably contains sulfhydryl groups in or near the active sites of the enzyme molecule. The strong effect of DTT, as compared to other sulfhydryl reagents, on the reactivation of the enzyme may be attributed to its pronounced cyclization property, that is capable of reducing disulfide groups in the enzyme molecule (69, 70).

The β-N-acetyl-D-hexosaminidase was purified from the third fraction of the cell extract, precipitated with ammonium sulfate, at a concentration of 50%—100%, which contains most activities of β-N-acetyl-D-hexosaminidase and also some of β-D-mannosidase. The purification procedures involved two steps of column chromatographies using hydroxylapatite and then DEAE-cellulose, the former being useful for removal of β-D-mannosidase. The enzyme purified, approximately 200-fold, showed a single protein band in gel electrophoresis. Its molecular weight, 125,000, as estimated by gel exclusion chromatography, indicates that the molecular size may be similar to those obtained from Jack bean (mol. wt. 110,000)(50) and Pinto bean (mol. wt. 112,000 ± 1,000)(51), and appears to be larger than that of Bacillus subtilis(mol. wt. 75,000)(54). This enzyme readily hydrolyzes

p-nitrophenyl-β-N-acetyl-D-glucosaminide. It is also active to N,N'-diacetyl-chitobiose, and the higher N-acetyl-chito-oligosaccharides, with hydrolysis rates ranging 40%—30%, relative to that with p-nitrophenyl-β-N-acetyl-D-glucosaminide. This purified enzyme, like other β-N-acetyl-D-hexosaminidases, was found to be active to β-N-acetyl-D-galactosaminide, although it hydrolyzes p-nitrophenyl-β-D-glucosminide eight times faster than the the corresponding galactosaminide.

The biological roles of these two particular glycosidases are not clear, but the β-N-acetyl-D-hexosaminidase may be related to the morphological changes in Tremella fungi, which involves the degradation of chitin or glycoprotein in the cell wall (71,72). As regards the occurence of β-D-mannosidase in the cells, there may be a possibility that β-mannosidic linkages are present in either cell wall polysaccharides or glycoproteins, and be degraded during the life cycle, or alternatively, the constitutive occurence of β-D-mannosidase may be related to utilization of β-linked mannans or galactomannans in woods during growth of this particular fungus.

Summary

β-D-Mannosidase (EC 3.2.1.25) and β-N-acetyl-D-hexosaminidase (EC 3.2.1.30) were purified approximately 500- and 200-fold, respectively, from the cell extract of Tremella fuciformis. Both glycosidases showed single protein bands in disc gel electrophoresis and the molecular weights of β-D-mannosidase and β-N-acetyl-D-hexosaminidase were about 140,000 and 125,000, respectively, as estimated by Sephadex gel exclusion chromatography. The substrate specificities and kinetics of the two enzymes were tested with p-nitrophenyl glycosides and their related oligosaccharides. The β-D-mannosidase hydrolyzed

p-nitrophenyl-β-D-mannoside, with K_m 2.1 mM and V_{max} 0.21 μmol per min per mg protein. 4-O-β-D-Mannosyl-D-mannose was readily hydrolyzed, but β-(1→4)-linked mannotriose and mannotetraose were hydrolyzed much slower. Unlike other known β-D-mannosidases, its activity was not inhibited by D-manno-γ-lactone, but strongly inhibited by p-chloromercuribezoate. The β-D-mannosidase tended to be inactivated in the presence of oxygen but reactivated with dithiothreitol. The β-N-acetyl-D-hexosaminidase hydrolyzed p-nitrophenyl-β-N-acetyl-D-glucosaminide, with K_m 0.31 mM and V_{max} 4.6 μmol per min per mg protein. It was active on N,N'-diacetyl-chitobiose and their higher saccharides (DP, up to 4) and liberated N-acetyl-D-glucosamine. The glycosidase preparation was also slightly active on p-nitrophenyl-β-N-acetyl-D-galactosaminide (K_m 0.19 mM, and V_{max} 0.6 μmol per min per mg protein). Both β-D-mannosidase and β-N-acetyl-D-hexosaminidase had an optimum pH 5.0. Inhibition by various metal ions to both glycosidases and their stabilities were tested.

Chapter 7.

Conclusion

Fungal polysaccharides have long been studied by numerous groups of workers in relation to biological functions, taxonomy and potential utilizations. Generally speaking, previous investigations of the fungal polysaccharides, however, have been restricted to the classes Phycomycetes and Ascomycetes. Therefore, the author attempted to clarify the chemical nature of the polysaccharides of the higher fungi of Basidiomycetes, particulary of the sub-class Heterobasidiae.

The present thesis is mainly concerned with the structural studies of the polysaccharides of Tremella fuciformis and Auricularia auricula-judae, by use of chemical and immunochemical methods, in relation to the chemical taxonomy, and the isolation and characterization of two glycosidases, β-D-mannosidase and β-N-acetyl-D-hexosaminidase of the haploid strain of Tremella fuciformis. These experimental results are summarized as follows.

Chapter 2. The haploid cells of Tremella fuciformis produced an acidic heteropolysaccharide into the culture medium abundantly. The acidic extracellular polysaccharide was consisted of D-xylose, L-fucose, D-mannose and D-glucuronic acid, and partly O-acetylated. The polysaccharide was found to be built up with (1→3)-linked mannose backbone to which β-linked D-glucuronic acid , L-fucose and D-mannose residues, and single or short side chains of (1→2)-linked D-xylose units are attached by (1→2)-linkages. This structural feature is very similar to that of the acidic polysaccharide of Cryptococcus laurentii and the capsular polysaccharide of C. neoformans. These structural similarity in the extracellular polysaccharides of Tremella and Cryptococcus indicates the very close

phylogenic relationship between these groups proposed by Slodki and associates.

Chapter 3. The cell wall fraction prepared from the yeast-like cells of Tremella fuciformis was found to be mainly consisted of glucurono-xylo-mannan and glucurono-xylo-manno-glucan. The glucurono-xylo-mannan was isolated by hot-water extraction from the cell wall, and showed the immunological activity to the antiserum prepared against the cells of T. fuciformis, indicating to be originated from the outer layer of the cell wall. The structure of glucurono-xylo-mannan from strain T-7 was suggested to be consisted 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 units, and D-mannose and D-glucuronic acid residues. This structural feature resembles that of the extracellular polysaccharide of T. fuciformis strain T-7. The structural resemblance between the cell wall polysaccharide and the extracellular polysaccharide was also found in the cells of T. fuciformis strain T-19. The glucurono-xylo-manno-glucan was isolated as alkali- and dimethyl sulfoxide insoluble material. This polysaccharide was comprised two polysaccharide moieties, glucurono-xylo-mannan and β-D-glucan. The structure of the former resembled that of the cell surface acidic polysaccharide. The glucan moiety was shown to be a branched structure consisting of β-(1→3)- and β-(1→6)-linkages. The structural feature of the glucan moiety may be somewhat similar to that of yeast (Saccharomyces cerevisiae) cell wall glucan. The relation of the water soluble glucurono-xylo-mannan and the alkali-insoluble glucurono-xylo-manno-glucan in the cell wall of T. fuciformis may be comparable to that of α-mannan and β-glucan of the cell wall of Saccharomyces cerevisiae.

Chapter 4. Two kinds of β-D-glucans and an acidic heteropolysaccharide

were isolated from the fruit body of Auricularia auricula-judae. The acidic heteropolysaccharide isolated from the hot water extract was found to be consisted of a backbone of (1→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. The isolation of the acidic heteropolysaccharide, of which structure is similar to that of the polysaccharide of A. auricula-judae, from the fruit body of T. fuciformis was already reported. Therefore, it may be concluded that the acidic heteropolysaccharides of such structures as described above are common in the polysaccharides of the fruit bodies of the species which belong to Heterobasidiae. A water soluble glucan consisted of a backbone chain of β-(1→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 alkali-insoluble residue, was also β-(1→3)-glucan with single branches at the C-6 positions, but it had an extremely highly branched structure. There have been several reports concerning antitumor activities of β-1,3-, 1,6-glucans, such as scleroglucan and lentinan. In relationship between the structure and activity of this type of glucans, it is interesting that the water-soluble glucan exhibited potent activity, while the alkali-insoluble glucan appered to be less active.

Chapter 5. Immunochemical studies using the antiserum against the haploid cells of T. fuciformis confirmed the structural similarities between the glucurono-xylo-mannans from Tremella, Auricularia, and Cryptococcus, estimated by use of chemical methods. The capsular polysaccharide of C. neoformans strongly cross-reacted with the antiserum against the cells of T. fuciformis strain T-7, indicating the very close taxonomic relationship between Tremella and Cryptococcus.

Chapter 6. β-D-Mannosidase (EC 3.2.1.25) and β-N-acetyl-D-hexosaminidase (EC 3.2.1.30) were purified approximately 500- and 200-fold, respectively, from the cell extract of T. fuciformis. Both glycosidases showed single protein bands in disc gel electrophoresis. The studies on the substrate specificities of these two enzymes showed that these glycosidases are useful for investigations of the carbohydrate chains of glycoproteins.

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4) Production and Characterization of the Extracellular Heteropolysaccharide of Tremella fuciformis.
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5) Structural and Immunochemical Relationships of the Cell Wall Polysaccharides of Tremella fuciformis and Cryptococcus neoformans.
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