[Chem. Pharm. Bull. 25(2) 338—344 (1977)]

UDC 547.458.02 : 581.192 : 582.28

Polysaccharides in Fungi. II.[1] Structural Analysis of Acidic Heteroglycans from *Tremella fuciformis* Berk[2]

Shigeo Ukai, Kazuo Hirose, Tadashi Kiho, and Chihiro Hara

Gifu College of Pharmacy[3]

(Received September 16, 1976)

The acidic heteroglycans (AC and BC) have been isolated from the fruit bodies of *Tremella fuciformis* Berk, which grow in China. The structures of the AC- and BC polysaccharides containing D-xylose, D-mannose, D-glucuronic acid and O-acetyl groups were investigated by Smith degradation and methylation analysis.

These results indicated that AC and BC were composed of α-1→3 linked D-mannopyranose backbone having highly branched points. D-Glucopyranosyluronic acid end groups, D-xylopyranose end groups and β-1→2 or β-1→3 linked D-xylopyranose side chains were linked to the position 2 of the mannopyranose main chain. Also, the results of Smith degradation suggest that O-acetyl groups are probably located at the glucuronic acid residues.

The results indicated that the structures of the polysaccharides, AC and BC, are essentially similar. However, significant difference between AC and BC has been observed in that the ratio of the xylose residues as end groups directly linked to the mannose backbone to the xylose side chains in BC is somewhat higher than that in AC, and the proportion of glucopyranose residues as end groups in BC is slightly larger than that in AC.

Keywords——*Tremella fuciformis*; polysaccharide; acidic heteroglycan; Smith degradation; methylation analysis; cross-reaction

In the previous papers,[1,4] we described the isolation, characterization and antitumor activity of the acidic heteroglycans (AJ and BJ; AC and BC) from the fruit bodies of *Tremella fuciformis* Berk (the edible mushroom) harvested in Japan and in China. The preceding paper[1] also showed that these polysaccharides were composed of xylose, mannose and glucuronic acid, and contained O-acetyl groups in their molecules.

In the present work, the structures of the acidic heteroglycans AC and BC obtained from Chinese fungus were investigated by Smith degradation and methylation analysis. Further, this paper deals with the cross reactivities of the polysaccharides with type II pneumococcal horse serum.

The native polysaccharides (AC and BC) and deacetylated polysaccharides (AC–D and BC–D) were subjected to periodate oxidations. The deacetylated polysaccharides (AC–D and BC–D) were obtained from the native polysaccharides by a treatment with sodium methoxide in methanol. The amounts of periodate consumption per anhydrohexose unit were 0.89, 0.90, 1.20, 1.10 moles for AC, BC, AC–D, BC–D, respectively. The native polysaccharides showed lower values than the deacetylated polysaccharides. The lower consumptions in AC and BC indicated that the presence of O-acetyl groups in their molecules increased the resistivity against the oxidation. The oxidation products were reduced with sodium borohydride, dialyzed, and lyophilized. Thus the polyalcohols (ACP, BCP, AC–DP and BC–DP) were obtained.

Smith degradation products[5] obtained by a mild hydrolysis of the polyalcohols with 0.5 N

1) Part I: S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1102 (1974).
2) Presented partly at the 95th Annual Meeting of the Pharmaceutical Society of Japan, Nishinomiya, April 1975.
3) Location: *6–1, Higashi-5-chome, Mitahora, Gifu, 502, Japan.*
4) S. Ukai, K. Hirose, and T. Kiho, *Chem. Pharm. Bull.* (Tokyo), **20**, 1347 (1972); S. Ukai, K. Hirose, T. Kiho, C. Hara, T. Irikura, T. Kanechika, and Y. Hasegawa, *ibid.*, **20**, 2293 (1972).
5) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).

Table I. Relative Retention Times[a] of Trimethylsilyl Derivatives of the Low Molecular Smith Degradation Products

	Condition A (5% SE-30)	AC	BC	AC-D	BC-D
Ethylene glycol	0.34	s	s	s	s
Glycolaldehyde (oxime)	0.48, 0.49	s	s	s	s
Glycerol	0.81	w	w	w	w
Glyceraldehyde (oxime)	0.89, 0.93	vw	t	vw	t
Glyceric acid	0.90	—	—	w	w
Erythrono-γ-lactone	1.02	t	t	—	—
Erythritol	1.16	t	—	t	—

s: strong, w: weak, vw: very weak, t: trace, —: not detected
a) relative to the trimethylsilyl derivative of trimethylolpropane

Table II. Component Sugars of Smith Degradation Products

	AC and BC	AC-D and BC-D
1st Smith degradation products		
mannose	+[a]	+
glucuronic acid	+[a]	—
xylose	trace	trace
2nd Smith degradation products		
mannose	+	+
glucuronic acid	—	—
xylose	—	—

a) Mannose and glucuronic acid in the molar ratios were 1:0.077 for AC, 1:0.071 for BC respectively.

hydrochloric acid were converted into the oxime derivatives[6] for aldehyde products and trimethylsilyl derivatives, then analyzed by gas–liquid chromatography (GLC). The low molecular degradation products from AC and AC–D were a large quantity of ethylene glycol and glycolaldehyde, a small amount of glycerol, a slight amount of glyceraldehyde and a trace of erythritol, whereas those from BC and BC–D were a large quantity of ethylene glycol and glycolaldehyde, a small amount of glycerol, a trace of glyceraldehyde as given in Table I.

Glyceric acid was not clearly identified by the above method. Therefore, Smith degradation products prepared by a hydrolysis of the polyalcohols with 0.5 N hydrochloric acid were separated into neutral and acidic fractions by ion-exchange chromatography, followed by analyzing those by GLC. The results showed that acidic fractions thus obtained from AC–D and BC–D contained glyceric acid, whereas those from AC and BC contained, instead, a trace of erythrono-γ-lactone.

The oxidation-resistant component sugars of the first- and second Smith degradation products were determined by paper partition chromatography (PPC) and GLC as shown in Table II. The first Smith degradation products were obtained by a complete hydrolysis of the polyalcohols (ACP, BCP, AC–DP and BC–DP) with 1 N sulfuric acid, and the second Smith degradation products were prepared by a successive periodate oxidation of the controlled Smith degradation products (ACS, BCS, AC–DS and BC–DS).

The presence of the residual mannose after periodate oxidation suggested that both AC and BC mostly consisted of 1→3 linked mannopyranosyl backbone with branching points. The source of ethylene glycol should be non-reducing terminal xylopyranose residues. The presence of xylose might be due to oxidation-resistant xylose residues such as 1→3 linked pyranose units. The appearance of glyceric acid and a slight amount of glyceraldehyde demon-

6) H. Yamaguchi, T. Ikenaka, and Y. Matsushima, *J. Biochem.* (Tokyo), **68**, 253 (1970).

Table III. Relative Retention Times and Compositions of Methylated Products

	Condition			Molar ratio	
	B[a]	C[b]	D[b]	AC	BC
2,3,4-Tri-O-methyl-1,5-di-O-acetyl-D-xylitol	0.68				
2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl-D-glucitol	1.00				
2,4-Di-O-methyl-1,3,5-tri-O-acetyl-D-xylitol	1.36				
3,4-(or 2,3)-Di-O-methyl-1,2(or 4),5-tri-O-acetyl-D-xylitol	1.45				
2,4,6-Tri-O-methyl-1,3,5-tri-O-acetyl-D-mannitol	2.06				
2,3,4-Tri-O-methyl-1,5,6-tri-O-acetyl-D-glucitol	2.35[c]				
4,6-Di-O-methyl-1,2,3,5-tetra-O-acetyl-D-mannitol	3.19				
Methyl 2,3,4-tri-O-methyl-D-xyloside		0.49 0.61	0.44 0.56	1.0	1.1
Methyl 2,3,4,6-tetra-O-methyl-D-glucoside		1.00 1.39	1.00 1.45	trace	0.1
Methyl 3,4-di-O-methyl-D-xyloside		1.30 1.59	1.12 1.34	0.2	0.1
Methyl 2,3-di-O-methyl-D-xyloside		1.44 1.69	1.24 1.35 1.49	trace	—
Methyl 2,4-di-O-methyl-D-xyloside		1.47 1.90	1.26 1.68	0.3	0.3
Methyl 2,3,4-tri-O-methyl-D-glucuronide methyl ester		2.31 2.94	2.39 3.14	1.0	1.0
Methyl 2,4,6-tri-O-methyl-D-mannoside		3.47	3.55	1.6	1.4
Methyl 4,6-di-O-methyl-D-mannoside		10.58	10.05	2.3	2.3

a) 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol=1.00 (B: 3% ECNSS-M)
b) methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside=1.00 (C: 15% BDS; D: 5% NPGS)
c) The peak was detected only after carboxyl-reduction of methylated polysaccharides.

strated the presence of non-reducing terminal glucopyranosyluronic acid residues and a slight amount of 1→2 linked sugar residues in the materials. The detection of a trace of erythrono-γ-lactone from AC and BC might be arised from O-acetylated glucuronic acid residues.

Partial acid hydrolysates obtained from AC and BC by treatment with 0.05 N sulfuric acid were subjected to dialysis. The dialyzable fractions contained xylose and glucuronic acid as liberated sugars. On the other hand, the non-dialyzable fractions (ACH and BCH; yield: 68 and 63%) were composed of mannose, glucuronic acid and xylose residues (molar ratios in ACH and BCH; 4.1: 1.0: 1.0 and 3.7: 1.0: 1.1). The amounts of xylose in ACH and BCH were, however, less than those in the native polysaccharides. The specific rotations of ACH ($[\alpha]_D^{28}$ +32° in H_2O, c=1) and BCH ($[\alpha]_D^{28}$ +28° in H_2O, c=1) were higher than those of AC ($[\alpha]_D^{20}$ +10° in H_2O, c=1) and BC ($[\alpha]_D^{20}$ +12° in H_2O, c=1). Consequently, it may be assumed that xylose in AC and BC exists as β-D-linked sugar side chains. Infrared (IR) spectra of ACH and BCH indicated that O-acetyl groups still remained to a considerable extent in ACH, but little in BCH.

After AC and BC were methylated by the method of Hakomori,[7] methanolysis and hydrolysis were carried out, and the resulting O-methylated monosaccharides were identified by comparison with authentic samples on GLC. The fully methylated polysaccharides and their carboxyl-reduced derivatives, prepared by reduction of the former with lithium aluminum hydride because of the occurrence of glucuronic acid, were hydrolyzed and converted into the corresponding alditol acetates, which were analyzed by GLC and gas–liquid chromatography–mass spectrometry (GLC–MS).[8] The mass spectra were compatible with those in the literature.[8b] The 2,3,4-tri-O-methyl-D-glucose, obtained only after carboxyl-reduction, was obvi-

7) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
8) *a*) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967); *b*) *Idem*, *Carbohyd. Res.*, **5**, 433 (1967); *idem*, *Angew. Chem.*, **82**, 643 (1970).

Table IV. Immunological Behaviors of the Polysaccharides and their Smith Degradation Products from *Tremella*, *Cryptococcus*

Polysaccharides	Cross reaction for anti-Pn II
Tremella fuciformis	
Native (AC and BC)	positive
Deacetyl (AC-D and BC-D)	positive
Controlled Smith degradation products (ACS and BCS)	positive
Tremella mesenterica (Y-6158 and Y-6151)[9]	
Native	negative
Deacetyl	negative
Smith degradation product of the native	positive
Cryptococcus laurentii[9]	
Native	positive
Deacetyl	positive
Smith degradation product of the native	negative

ously derived from the terminal glucuronic acid residue.

This method does not permit to distinguish between 2,3-di-O-methyl and 3,4-di-O-methyl pentose.[8a] In addition, di-O-methyl xylose and tetra-O-methyl hexose were not clearly identified. The methylated AC and BC were therefore converted into methylglycosides by methanolysis. The methanolysates were analyzed by GLC. These results were fully consistent with those of the alditol acetates of AC and BC (Table III).

The results of these methylation analyses suggested that AC and BC were composed of terminal glucuronic acid residues, mannose residues linked to position 3, branching mannose residues linked to positions 2 and 3, terminal xylose residues and terminal glucose residues. Distinct differences between AC and BC were observed in the amount of 3,4-di-O-methyl-D-xylose and in the ratio between di-O-methyl and tri-O-methyl D-mannose. These data also demonstrate that these sugars have pyranose rings. The structures of AC and BC deduced from these results well agreed with those from the Smith degradations.

The first controlled Smith degradation products (ACS, BCS, AC–DS and BC–DS) were obtained by a mild hydrolysis[10] of the polyalcohols (ACP, BCP, AC–DP and BC–DP) described above followed by dialysis and lyophilization. These products showed a high positive specific rotation as follows: ACS ($[\alpha]_D^{23}$ +96° in H_2O, c=1); BCS ($[\alpha]_D^{23}$ +76° in H_2O, c=1); AC–DS ($[\alpha]_D^{23}$ +113° in 1 N NaOH, c=1); BC–DS ($[\alpha]_D^{23}$ +103° in 1 N NaOH, c=1).

The methylation analyses of AC-DS and BC-DS indicate that they consist of mostly 1→3 linked mannopyranose and of a trace of terminal xylose. The result was supported by the fact that mannose still existed as a resistant component sugar after the second Smith degradation (the Smith degradations of AC-DS and BC-DS) (Table II). The values of optical rotations in AC-DS and BC-DS and the presence of IR absorption at 820 cm^{-1} suggest that they have α-mannosidic linkage. ACS and BCS consist of mannose, a trace of xylose in addition to a small amount of glucuronic acid. As shown in Table II, the component sugar of the second Smith degradation products from AC and BC was only mannose, and they contained neither xylose nor glucuronic acid. These results support that the backbone is composed of α-1→3 linked D-mannopyranose units.

From these results, both AC and BC seem to be constructed with α-1→3 linked D-mannopyranosyl backbone, which is substituted at position 2 with non-reducing terminal D-glucopyranosyluronic acid and β-D-xylopyranose, and with β-1→2 or β-1→3 linked D-xylopyranosyl

9) *a*) C.M. Helms, P.Z. Allen, and D.S. Feingold, *Immunochemistry*, **6**, 269 (1969); *b*) C.G. Fraser, H.J. Jennings, and P. Moyna, *Can. J. Biochem.*, **51**, 225 (1973).
10) E.C. Yurewcz, M.A. Ghalambor, and E.C. Heath, *J. Biol. Chem.*, **246**, 5596 (1971).

side chains. On the basis of Smith degradations in AC, BC, AC-D and BC-D, it could be posturated that O-acetyl groups are located at the glucuronic acid residues.

In a previous paper,[1] we have reported that differences between AC and BC were in a molecular weight, in O-acetyl content and in antitumor activity. The present structural analysis indicates following differences: the amount of 1→2 linked xylopyranose in AC is slightly larger than that in BC; non-reducing glucopyranose in BC is a significant amount; BC has relatively highly branched structure, compared with AC. These data indicate that BC is somewhat higher than AC in the ratio of the xylopyranose residues as end groups directly linked to the mannopyranosyl backbone to the xylopyranose side chains.

A comparison of the structure of the polysaccharides (AC and BC) obtained from the fruit bodies of *T. fuciformis* with that of the extracellular acidic polysaccharide[11b] produced by *T. mesenterica* NRRL-6158 (Tremellaceae) indicates that distinct differences among them are in the molar ratio of component sugars and in O-acetyl content in addition to kinds of branching position of mannose, xylopyranosyl linkage and the length of xylopyranose side chains. Thus, contrary to the polysaccharide of *T. mesenterica*, both AC and BC possess short chains of xylopyranose, and the methylation analysis showed the absence of 3,6-di-O-methyl-D-mannose and the presence of 2,4-di-O-methyl-D-xylose.

It has been reported[11] that the structure of extracellular polysaccharides in *T. mesenterica* bore some resemblance to that in *Cryptococcus laurentii*. Moreover, the cross reactions of a number of polysaccharides containing glucuronic acid residues with antisera to type II pneumococcal capsular polysaccharide have been studied.[9,12] Therefore, the cross reactivities of the native- and deacetylated polysaccharides (AC, BC, AC-D and BC-D) and the first controlled Smith degradation products(ACS, BCS, AC-DS and BC-DS) were compared with the data[9] of those from *T. mesenterica* and *C. laurentii* (Table IV). In the polysaccharides of *T. mesenterica*, Fraser[9b] has proposed that critical factor in the failure of the acidic polysaccharides of *T. mesenterica* to cross reaction with type II antiserum was the strategic location of some xylose residues in the vicinity of the glucuronic acid residue. In the native- and deacetylated polysaccharides (AC, BC, AC-D and BC-D) of *T. fuciformis*, the xylose residues presumably do not inhibit the cross reaction. Thus, AC and BC widely differ from the polysac charides of *T. mesenterica* on the basis of the data of the cross reaction as well as the structural analysis.

These structural analyses give also interesting informations on relationship of antitumor activity between AC and BC, together with chemotaxonomy. Further structural analysis including the determination of the location of O-acetyl groups in AC and BC will be reported in a near future.

Experimental

IR spectra were recorded on a Japan Spectroscopic Co., Model DS-403G spectrometer. Specific rotations were measured by the use of a JASCO Model DIP-S automatic polarimeter. GLC was carried out by the use of a JEOL Model JGC-1100 gas chromatograph equipped with hydrogen flame ionization detector, and GLC-MS was performed by the use of a JEOL Model JMS-D100 mass spectrometer.

Deacetylation of the Acidic Polysaccharides (AC and BC)——Each sample (200 mg) was stirred with 0.1 M sodium methoxide in MeOH for 8 hr at room temperature. The products were filtered, washed with MeOH, dissolved in H_2O, dialyzed, and lyophilized. The IR spectra of the deacetylated products (AC-D and BC-D; yield: 188 mg and 180 mg) showed no absorption of acetyl groups.

Analytical Periodate Oxidations (AC, BC, AC-D and BC-D)——Each sample (50 mg) was oxidized with 0.05 M $NaIO_4$ (100 ml) at room temperature in the dark. The periodate consumptions were estimated by an arsenite method.[13] As shown in Fig. 1, the moles of $NaIO_4$ consumed per anhydrohexose unit were 0.89, 0.90, 1.20, 1.10 for AC, BC, AC-D, BC-D, respectively.

11) *a*) M.E. Slodki, L.J. Wickerham, and R.J. Bandoni, *Can. J. Microbiol.*, **12**, 489 (1966); M.E. Slodki, *ibid.*, **12**, 495 (1966); *b*) C.G. Fraser, H.J. Jennings, and P. Moyna, *Can. J. Biochem.*, **51**, 219 (1973).
12) M. Heidelberger, *Fortsch. Chem. Org. Naturstoffe*, **18**, 503 (1960); I. Cornell and L. Wofsy, *Immunochemistry*, **4**, 183 (1967).
13) P.F. Fleury and J. Lange, *J. Pharm. Chem.*, **17**, 107 (1933); *idem*, *ibid.*, **17**, 196 (1933).

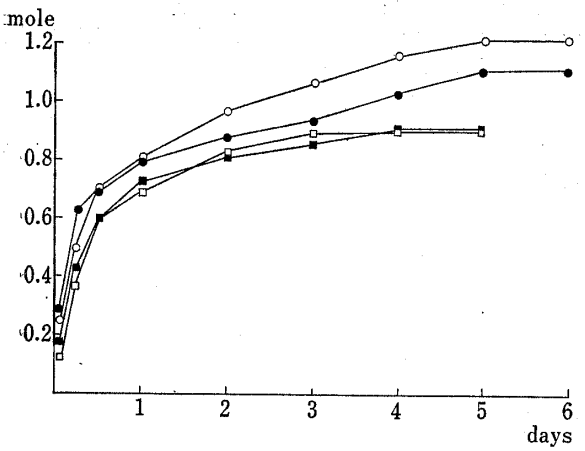

Fig. 1. Periodate Oxidations of Polysaccharides
—□—: AC, —■—: BC, —○—: AC-D, —●—: BC-D

Smith Degradations of the Acidic Polysaccharides (AC, BC, AC-D and BC-D) and Analyses of the Products——Each sample (200 mg) was oxidized with 0.05 M $NaIO_4$ (200 ml) for 5 days (AC and BC) or 6 days (AC-D and BC-D) respectively at room temperature in the dark. After additions of ethylene glycol, reaction mixtures were dialyzed against deionized water. Inner solution were concentrated to small volumes, reduced with $NaBH_4$ at room temperature for 12 hr. The excess of $NaBH_4$ was decomposed by an acidification with 1 M AcOH, dialyzed and lyophilized. The yields of the polyalcohols were as follows: ACP, 150 mg; BCP, 110 mg; AC-DP, 143 mg; BC-DP, 130 mg.

A part of the polyalcohols was mildly hydrolyzed with 0.5 N HCl at 35° for 12 hr in a sealed tube. The hydrolysate was treated with hydroxylamine hydrochloride at 80° for 30 min in a sealed tube. The reaction mixtures were dissolved in pyridine (0.1 ml) containing trimethylolpropane as an internal standard and trimethylsilylated with hexamethyldisilazane (0.05 ml) and trimethylchlorosilane (0.03 ml),[14] then applied to GLC. Condition A, a glass column (2 m×0.3 cm) packed with 5% SE-30 on Chromosorb W (AW) (60 to 80 mesh); programmed column temperature increasing 4° per min from 70° to 250°; carrier gas, N_2 (38 ml per min).

A part of the polyalcohols (ACP, BCP AC-DP, and BC-DP) was hydrolyzed with 0.5 N HCl at 100° for 4 hr. The hydrolysate neutralized with $BaCO_3$ was subjected to ion-exchange column chromatography on Amberlite CG-400 (acetate form) (0.8×6 cm). After elution with H_2O, the eluent with 1 N HCl gave the acidic fraction. The neutral and acidic fractions were trimethylsilylated and examined by GLC as described above. The polylalcohols were completely hydrolyzed with 1 N H_2SO_4 at 100° for 12 hr and component sugars were analyzed by GLC and PPC. The hydrolysate was reduced with $NaBH_4$ by the usual manner. The resulting alditols were treated with trifluoroacetic anhydride and the reaction mixtures were injected to GLC as described in our previous paper.[1] PPC of the hydrolysate was carried out by ascending method using Toyo-Roshi No. 51 filter paper. The solvent used was *n*-BuOH: pyridine: H_2O (6: 4: 3). Sugars were detected with *p*-anisidine hydrochloride[15] and alkaline $AgNO_3$[16] reagents. Areas corresponding to the spots on the chromatogram were quantitatively extracted with deionized water. Mannose was determined by the phenol–H_2SO_4 method,[17] and glucuronic acid by the method of Galambos.[18]

Partial Acid Hydrolysis (AC and BC)——The polysaccharides were hydrolyzed with 0.05 N H_2SO_4 at 100° for 6 hr. The hydrolysates were neutralized with $BaCO_3$, centrifuged, and filtered. Then, the filtrates were dialyzed against deionized water. The specific rotations and yields of the non-dialyzable fractions were as follows: ACH, $[\alpha]_D^{28}$ +32° (*c*=1, H_2O), 68%; BCH, $[\alpha]_D^{28}$ +28° (*c*=1, H_2O), 63%. The component sugars of dialyzable fraction were detected by PPC as described above, while those of non-dialyzable fraction were estimated by the methods of Dishe (for pentose[19a] and hexose[19]) and Galambos.[18]

Methylation of the Acidic Polysaccharide (AC and BC)——The polysaccharide (200 mg) was stirred with Me_2SO (30 ml) at 60° for 6 hr. After the solution was cooled to room temperature, the solution of methylsulfinyl carbanion freshly prepared from NaH (0.5 g) and Me_2SO (10 ml) was added, and the reaction mixture was stirred for 12 hr. On cooling to 20°, MeI (5 ml) was added and the mixture was stirred overnight at room temperature. All procedures carried out in nitrogen atmosphere. After dilution with small amount of H_2O, the solution was dialyzed against running water, and extracted with $CHCl_3$. The combined extracts were dried and evaporated to dryness. No significant free hydroxyl absorption was observed in the IR spectrum.

Hydrolysis and Analysis of the Methylated Products——The fully methylated polysaccharide was allowed to heat with 90% HCOOH at 100° for 3 hr in a sealed tube. On cooling, the mixture was concentrated, and then hydrolyzed with 0.5 N H_2SO_4 at 100° for 20 hr. The hydrolysate was neutralized with $BaCO_3$, filtered and concentrated. The methylated sugars were reduced with $NaBH_4$ overnight, then neutralized with Amberlite CG-120 (H^+). The filtrate was evaporated and boric acid was removed by a repeated addition and

14) C.C. Sweely, R. Bently, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
15) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, **1950**, 1702.
16) W.E. Trevelyan, D.P. Proctor, and J.S. Harrison, *Nature*, **166**, 444 (1950).
17) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
18) J.T. Galambos, *Anal. Chem.*, **19**, 119 (1967).
19) *a*) Z. Dishe, *J. Biol. Chem.*, **181**, 379 (1949); *b*) Z. Dishe, L.B. Shettles, and M. Osnos, *Arch. Biochem.*, **22**, 169 (1949).

evaporation of MeOH. The product was acetylated with Ac_2O–pyridine (1 : 1) at 100° for 1 hr. The acetylation mixture was injected into the column either directly or after working-up involving dilution with H_2O, concentration to dryness, and dissolving in $CHCl_3$. GLC was carried out under conditions B, a glass column (2 m × 0.3 cm) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 180° with a flow rate of 38 ml per min of N_2.

A part of the methylated polysaccharide was dissolved in tetrahydrofuran, and the solution was added into 1.25% suspension of $LiAlH_4$ in tetrahydrofuran. The mixture was stirred for 24 hr. The excess hydride was destroyed by dropwise addition of H_2O with stirring. The solution was filtered and the residue was extracted with $CHCl_3$. The filtrate and combined extracts were evaporated to dryness. The methylated carboxyl–reduced sugars were hydrolyzed with 90% HCOOH at 100° for 2 hr, and with 0.5 N H_2SO_4 at 100° for 15 hr. The neutralized sugars were converted into alditol acetates and analyzed by GLC and GLC–MS. GLC of the acetylation mixture was carried out under the condition B as described above. GLC–MS was also performed under the same condition.

Methanolysis and Analysis of the Methylated Products——A part of the fully methylated polysaccharide was treated with 5% methanolic HCl in a sealed tube for 20 hr in a boiling water bath. After neutralization with Ag_2CO_3 and filtration, the mixture of methyl glycosides was examined by GLC. The following conditions (C and D) were used. Condition C, a glass column (2 m × 0.3 cm) packed with 15% 1,4-butandiol succinate polyester (BDS) on Chromosorb W (AW) (60 to 80 mesh) at 175° with a flow rate of 38 ml per min of N_2; D, a glass column (2 m × 0.3 cm) packed with 5% neopentylglycol succinate polyester (NPGS) on Chromosorb G (AW) (60 to 80 mesh) at 155° with a flow rate of 38 ml per min of N_2.

Controlled Smith Degradation Products——The polyalcohols (ACP, BCP, AC-DP and BC-DP) prepared as described above were dissolved in 0.1 M AcOH (adjusted to pH 2 with dil. HCl). The solutions were heated at 100° for 35 min, and dialyzed against deionized water for 12 hr. As the inner solutions gave small amounts of insoluble material, the solutions were centrifuged, and supernatants were lyophilized (ACS, BCS, AC-DS, and BC-DS). The specific rotations and yields of products based on the polyalcohols were as follows: ACS, $[\alpha]_D^{23}$ +96° (c=1, H_2O), 71%; BCS, $[\alpha]_D^{23}$ +76° (c=1, H_2O), 72%; AC-DS, $[\alpha]_D^{23}$ +113° (c=1, 1 N NaOH), 27%; BC-DS, $[\alpha]_D^{23}$ +103° (c=1, 1 N NaOH), 38%.

2nd Smith Degradations (Smith Degradations of the First Controlled Smith Degradation Products)——The samples (ACS, BCS, AC-DS and BC-DS) were degraded by $NaIO_4$ as described above. The final degradation products were analyzed by PPC and GLC in the same manner as described above. The oxidation–resistant sugar in ACS, BCS, AC-DS, and BC-DS was only mannose.

Methylation Analysis of the Controlled Smith Degradation Products (AC-DS and BC-DS)——Methylation of the samples (10 mg) were performed with NaH and MeI in Me_2SO according to the method of Hakomori[7] as described above. The methylated sugars were hydrolyzed with 90% HCOOH at 100° for 2 hr, and 0.5 N H_2SO_4 at 100° for 15 hr. Partially methylated alditol acetates were analyzed by GLC under the condition B. The result revealed that the products of AC-DS and BC-DS contained a large amount of 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl-D-mannitol, a trace of 2,3,4-tri-O-methyl-1,5-di-O-acetyl-D-xylitol and 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-mannitol and 4,6-di-O-methyl-1,2,3,5-tetra-O-acetyl-D-mannitol.

Cross Reaction of the Polysaccharides (AC, BC, AC-D, BC-D, ACS, BCS, AC-DS and BC-DS)——Immunological analysis was carried out according to the Heidelberger method[20] by the use of each polysaccharide (0.05 mg) and type II horse antipneumococcal serum H 513 (0.5—1 ml). The reaction mixture was allowed to stand at 0° for 6 to 8 days. The resulting precipitation was qualitatively analyzed. The results (scale: − to ++++) of the reaction were as follows: AC, ++±; BC, ++±; AC-D, +++; BC-D, ++±; ACS, ++±; BCS, ++±; AC-DS, −; BC-DS, −.

Acknowledgement The authors are grateful to Prof. M. Heidelberger, Department of Pathology, New York University, for immunological analysis of the polysaccharides from *Tremella fuciformis* Berk.

20) M. Heidelberger and F.E. Kendall, *J. Exper. Med.*, **55**, 555 (1932); *idem*, *ibid.*, **61**, 559 (1935).