Polysaccharides in Fungi. VII.[1] Acidic Heteroglycans from the Fruit Bodies of *Auricularia auricula-judae* Quel[2]

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Two kinds of acidic polysaccharides, MEA ($[\alpha]_D^{23}$ +31°) and MHA ($[\alpha]_D^{23}$ +33°), have been isolated from the hot aqueous 70% ethanol and hot water extracts of the fruit bodies of *Auricularia auricula-judae* Quel, which grow in China. Both polysaccharides were homogeneous as judged by gel filtration, electrophoresis and ultracentrifugal analysis. They were composed of D-glucuronic acid, D-xylose and D-mannose in molar ratios of 1.0: 0.5: 2.8 (MEA) and 1.0: 0.6: 1.0 (MHA), together with a small amount of D-glucose, and contained O-acetyl groups. The molecular weights were estimated to be 37×10^4 for MEA and 30×10^4 for MHA.

The results of methylation, Smith degradation and partial acid hydrolysis studies suggested that MEA and MHA were constructed with α-1→3 linked D-mannopyranose residues as a backbone, some of which were substituted at position 2 with terminal β-D-glucopyranosyluronic acid residues, and at position 2 and position 6 with single residues or short chains of β-D-xylopyranose. Further, MEA and MHA appear to contain small amounts of 1→6 linked D-mannopyranose (glucose) and branching D-mannopyranose residues at positions 4 and 6. MEA and MHA differ significantly in the amounts of O-acetyl groups, 1→4 linked D-xylopyranose residues and the branching D-mannopyranose residues at positions 4 and 6.

Keywords——*Auricularia auricula-judae*; acidic polysaccharide; molecular weight; reduction with sodium borodeuteride; methylation analysis; Smith degradation; acidic oligosaccharide; structural features of acidic heteroglycans; chemotaxonomy

The fruit bodies of *Auricularia auricula-judae* Quel (Japanese name: kikurage) (Auriculariaceae) have been used as a food and as a drug. Previous studies in this series[1] have dealt with the structural analysis and biological activities of polysaccharides prepared from the fruit bodies of *Tremella fuciformis* Berk (Tremellaceae). In the course of these studies our attention was called to polysaccharides in the fruit bodies of *A. auricula-judae*, which belongs to the same subclass (Heterobasidae) as *T. fuciformis*. Recently, an acidic polysaccharide obtained from the fruit bodies of *A. auricula-judae* was reported by Misaki *et al.*,[3] though the homogeneity and physical properties of their polysaccharide, and the presence of O-acetyl groups in the molecule were not discussed. We have now isolated native pure acidic polysaccharides (MEA and MHA) from the aqueous ethanol and hot water extracts of the fruit bodies of this fungus. The present paper deals with the purification, characterization and structural analysis of the two acidic polysaccharides, MEA and MHA.

The crushed dried fruit bodies were washed with hot methanol, and the residue was extracted with hot 70% aqueous ethanol as described in our previous paper.[4] Compounds of low molecular weight and proteins in the extract were removed by dialysis, Pronase treatment, and the Sevag procedure.[5] The crude polysaccharide fraction (ME) thus obtained was fractionated by treatment with cetyltrimethylammonium bromide (CTAB) to yield the acidic polysaccharide (MEA) as colorless flakes in about 4% yield. The aqueous ethanol-insoluble fraction was further extracted with hot water. The extract was purified in the manner described above to afford the acidic polysaccharide (MHA), as colorless flakes in about 16% yield. The overall process is outlined in Chart 1.

the fruit bodies of *Auricularia auricula-judae* QUEL

| extracted with hot MeOH

extract   residue

| extracted with hot 70% EtOH
| (20 h, 3 times)

extract   residue

| digested with Pronase | extracted with water at 93—98°C
| dialyzed | (4 h, 4 times)
| deproteinized by the Sevag procedure
| extract
crude polysaccharide (ME)
| yield: *ca.* 5%   crude polysaccharide (MH)
| ⋮   yield: *ca.* 20%
| treated with CTAB

CTAB-complex   CTAB-complex

| dissolved in 1 M MgCl₂   ⋮
| precipitated with EtOH   ⋮
| dialyzed and lyophilized   acidic polysaccharide
acidic polysaccharide   MHA   yield: *ca.* 16%
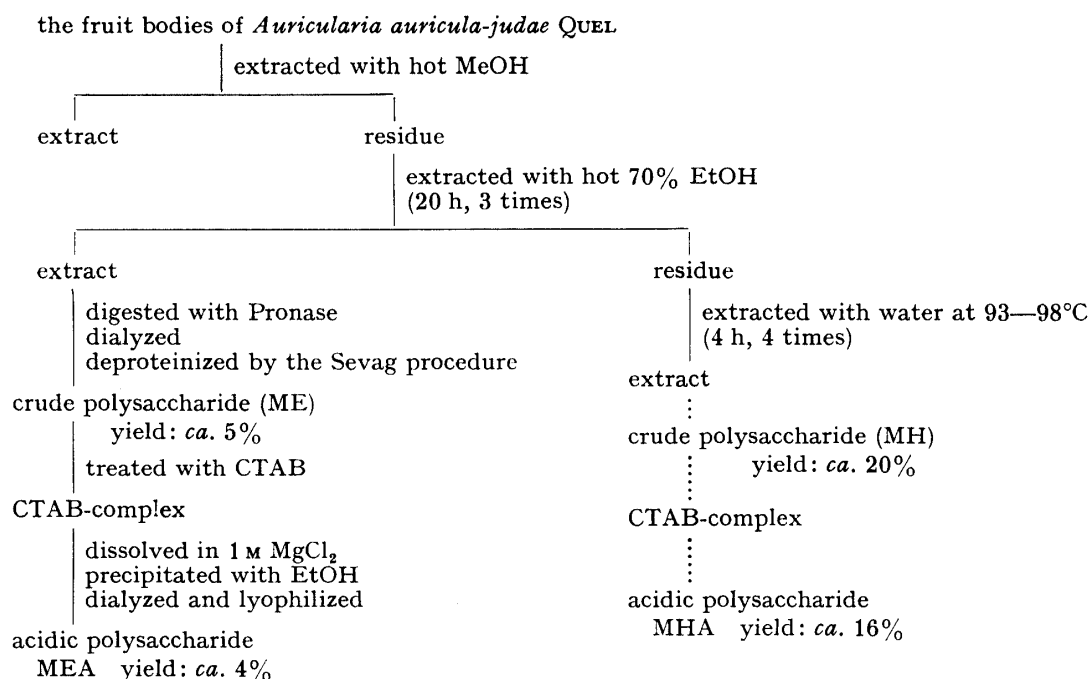MEA   yield: *ca.* 4%

Chart 1.   Isolation and Purification of the Polysaccharides

The dotted line in the case of MHA indicates that the procedure is the
same as that for MEA.

Each polysaccharide (MEA and MHA) gave a single peak on column chromatography with Sepharose 4B and with diethylaminoethyl (DEAE)-Sephadex A-25 (phosphate form) (Fig. 1).

Each polymer was found to be homogeneous by Tiselius-type electrophoresis and ultracentrifugal analysis (Fig. 2).   No nitrogen, phosphorus or sulfur was detected by elementary analyses.
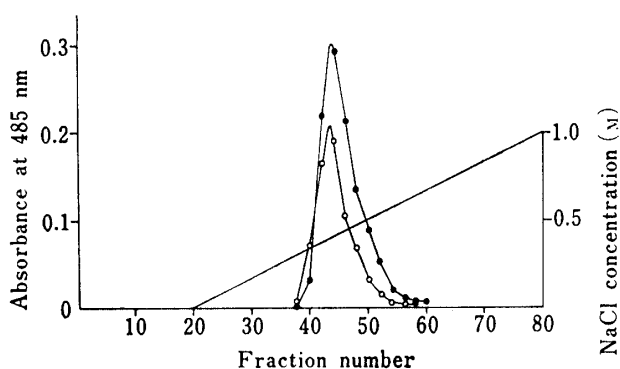

Fig. 1.   Chromatograms of MEA and MHA on a
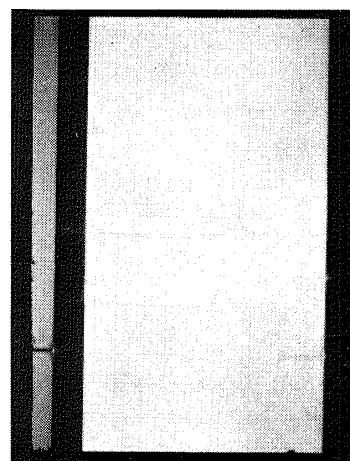Column of DEAE-Sephadex
—●—: MEA, —○—: MHA.

Fig. 2.   Ultracentrifugal Patterns of
MEA (upper) and MHA (lower)

The physical properties of MEA and MHA are shown in Table I.   These polysaccharides showed positive specific rotation values.   The values of sedimentation coefficient, intrinsic viscosity and partial specific volume of MEA were somewhat higher than those of MHA. The molecular weights of MEA and MHA were estimated to be approximately 37×10^4 and 30×10^4 by means of the equations used by Eyring[6] and Pancake,[7] respectively.   These

values are obviously smaller than those of the acidic polysaccharides (59×10^4 and 72×10^4) obtained from the fruit bodies of *T. fuciformis*.[8]

The infrared (IR) spectrum of each polysaccharide showed characteristic absorption bands at 1730 and 1250 cm^{-1}. Because two bands disappeared on treatment of these polymers with sodium hydroxide, it was assumed that *O*-acyl groups exist in these molecules. The acidic compound arising from the acyl groups was identified as acetic acid by gas–liquid chromatography (GLC), and characterization of the ester obtained by reaction with *p*-bromophenacyl bromide. The total acetyl contents of these polysaccharides were determined colorimetrically by the ferric hydroxamate method.[9] The acetyl content of MEA was larger than that of MHA, as shown in Table I.

TABLE I.   Some Physical and Chemical Properties of Acidic Heteroglycans

Polysaccharide	Specific rotation $[\alpha]_D^{23}$ ($c=1$, H_2O)	Intrinsic viscosity (dl/g)	Partial specific volume (ml/g)	Sedimentation coefficient s_{20}	Molecular weight $\times 10000$
MEA	$+31°$	5.65	0.50	5.8	37
MHA	$+33°$	4.75	0.48	5.6	30

Polysaccharide	Component sugars[a] Molar ratio			Acetyl content (%)	Elemental analysis			IR $_{max}^{KBr}$ cm^{-1}
	GlcA	Xyl	Man		C	H	N(%)	
MEA	1.0	0.5	2.8	15.0	37.7	5.2	nil	1730, 1250
MHA	1.0	0.6	3.0	11.7	40.3	5.4	nil	1730, 1250

a)   MEA and MHA each contained a small amount of glucose in addition to glucuronic acid (GlcA), xylose (Xyl) and mannose (Man).

The component sugars of MEA and MHA were identified as xylose, mannose, glucuronic acid and a small amount of glucose by paper partition chromatography (PPC) of the hydrolysates and GLC of the alditol acetates prepared from the hydrolysates. The molar ratios of these sugars (shown in Table I) were determined by colorimetric determination of the polysaccharide solutions and by GLC analyses of the hydrolysates for neutral sugars.

The native MHA and the fully *O*-acetylated MEA were methylated by the method of Hakomori,[10] and then the fully *O*-methylated polysaccharides were hydrolyzed with acid. Since the native MEA was only slightly soluble in dimethyl sulfoxide (DMSO), the *O*-acetylated MEA (readily soluble in DMSO) was used. The resulting partially *O*-methylated monosaccharides were analyzed by GLC and gas–liquid chromatography–mass spectrometry (GLC–MS) after conversion to the corresponding *O*-methyl-*O*-acetyl alditols. These alditol derivatives, except for those of *O*-methylated glucuronic acid and di-*O*-methylated xylose, were identified by comparing the retention times and the mass spectra with those of authentic samples or with the values in the literature.[11]

As 2,3-di-*O*-methyl- and 3,4-di-*O*-methyl-xylose give the same alditol acetates, sodium borodeuteride instead of sodium borohydride was used in the preparation of alditols, and partially methylated xylitol acetates deuterated at C-1 were analyzed by GLC–MS. The relative proportions of these sugars were determined by comparing the relative intensities of characteristic fragments (*m/e*: 117, 118, 189, 190) as shown in Chart 2 in the MS of the mixture.

Since some *O*-methylated derivatives of D-mannose and D-glucose cannot be separated under the present GLC conditions, definite differentiation of 2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl and 2,3,4-tri-*O*-methyl derivatives of D-mannose and D-glucose was not achieved. However, the presence of methyl 2,4,6-tri-*O*-methyl-D-mannoside was revealed by GLC analysis
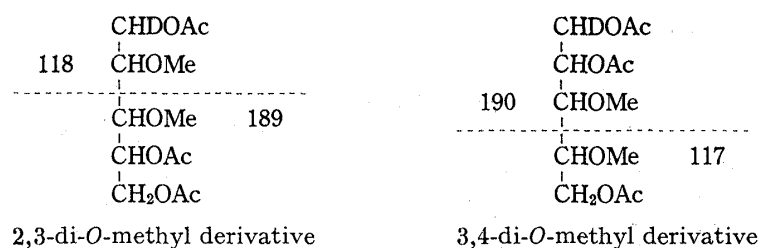
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                CHDOAc                              CHDOAc
        118     CHOMe                               CHOAc
        --------|--------------                 190 CHOMe
                CHOMe     189                        --------|--------------
                CHOAc                               CHOMe     117
                CH2OAc                              CH2OAc
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2,3-di-O-methyl derivative   3,4-di-O-methyl derivative

Chart 2.   Characteristic Fragments of Deuterated (C-1) Partially
Methylated Xylitol Acetates in MS

of the methanolysates of the fully O-methylated MEA and MHA. Identification of methyl (methyl 2,3,4-tri-O-methyl-D-glucopyranosid)uronate in the methanolysates and an increase in the intensity of the peak of the alditol acetates corresponding to 2,3,4-tri-O-methyl-D-glucose on GLC after carboxyl-reduction of the fully O-methylated polysaccharides indicate that glucuronic acid residues in MEA and MHA exist as non-reducing terminal residues. Table II shows the results of methylation analyses.

TABLE II.   Relative Retention Times and Compositions of Methylated Products

Methylated sugar (as alditol acetate)	Relative retention time[a]	Molar ratios		Mode of linkage
		MEA	MHA	
2,3,4-Tri-O-Me-D-Xyl	0.73	1.0	1.0	Xyl 1→
2,3,4,6-Tetra-O-Me-D-Man(Glc)	1.00	Trace	0.1	Man (Glc) 1→
2,4-Di-O-Me-D-Xyl	1.33	0.1	0.06	→3 Xyl 1→
2,3-Di-O-Me-D-Xyl	1.49	0.1	Trace	→4 Xyl 1→
3,4-Di-O-Me-D-Xyl	1.49	0.07	0.1	→2 Xyl 1→
2,4,6-Tri-O-Me-D-Man	1.99	2.5	3.0	→3 Man 1→
2,3,4-Tri-O-Me-D-Man(Glc)	2.54	0.1	0.3	→6 Man (Glc) 1→
4,6-Di-O-Me-D-Man	3.12	1.9	1.7	$\begin{smallmatrix}\to 2\\\to 3\end{smallmatrix}$ Man 1→
2,3-Di-O-Me-D-Man	4.15	0.3	Trace	$\begin{smallmatrix}\to 4\\\to 6\end{smallmatrix}$ Man 1→
2,4-Di-O-Me-D-Man	5.00	1.4	1.0	$\begin{smallmatrix}\to 3\\\to 6\end{smallmatrix}$ Man 1→
2,3,4-Tri-O-Me-D-GlcA[b]				GlcA 1→

a)   Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.
b)   The methylated sugar was also identified by GLC analysis as its methyl glycoside methyl ester.

The native polysaccharides (MEA and MHA) and their de-O-acetylation products (MEA-D and MHA-D) obtained by alkali treatment of MEA and MHA, respectively, were subjected to periodate oxidation. The amounts of periodate consumption per anhydrohexose unit were 0.53, 0.49, 0.67, and 0.72 mol for MEA, MHA, MEA-D, and MHA-D, respectively. Reduction of the oxidation products with sodium borohydride followed by dialysis and lyophilization yielded the polyalcohols (MEAP, MHAP, MEA-DP, and MHA-DP). The component sugars in the products of Smith degradation[12] (the complete hydrolysate of the polyalcohol with acid) were mannose, glucuronic acid and a trace of xylose for MEA and MHA, while no glucuronic acid was evident in the component sugars of MEA-D and MHA-D.

The controlled Smith degradation products (MEA-DS and MHA-DS) were prepared by mild acid-hydrolysis of the corresponding polyalcohols (MEA-DP and MHA-DP).[13] The results of methylation analyses of MEA-DS and MHA-DS indicated that they consist mostly of 1→3 linked D-mannopyranose. The values of specific rotations (MEA-DS $[\alpha]_D^{23}$ +100° in 1 N NaOH, MHA-DS $[\alpha]_D^{23}$ +109° in 1 N NaOH) and the presence of IR absorption at 820 cm^{-1} suggest that they have α-D-mannosidic linkages.[4,14] These results demonstrate that the backbones of both MEA and MHA are composed of α-1→3 linked D-mannopyranose units.

From the partial acid-hydrolysates of each polysaccharide, the following oligosaccharides were isolated: an aldobiouronic acid, 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannopyranose; an aldotriouronic acid, *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-D-mannopyranose; an aldotetraouronic acid, *O*-(β-D-glucopyranosyluronic acid)-(1→2)-*O*-α-D-mannopyranosyl-(1→3)-*O*-α-D-mannopyranosyl-(1→3)-D-mannopyranose. The structures of these compounds were confirmed by methylation analyses and comparison with authentic samples prepared from the acidic polysaccharides of *T. fuciformis*.[15] The isolation of the acidic oligosaccharides showed that β-D-glucopyranosyluronic acid residues are linked to position 2 of the α-1→3 linked mannopyranosyl chain in MEA and MHA.

Mild acid-hydrolysis of MEA and MHA followed by dialysis yielded the degraded polysaccharides (MEA–H and MHA–H) as non-dialyzable fractions composed of D-mannose, D-glucuronic acid and a small amount of D-xylose residues (molar ratios: MEA–H, 2.4: 1.0: 0.2; MHA–H, 2.5: 1.0: 0.3). The amounts of xylose in MEA–H and MHA–H were less than those in the native polysaccharides. The specific rotations of MEA–H ($[\alpha]_D^{19}$ +46°) and MHA–H ($[\alpha]_D^{19}$ +48°) were higher than those of MEA ($[\alpha]_D^{23}$ +31°) and MHA ($[\alpha]_D^{23}$ +33°). Consequently, it may be assumed that xylose residues in MEA and MHA exist as β-D-linked sugar side chains.

From these results, both MEA and MHA seem to be composed of α-1→3 linked D-mannopyranosyl backbone, some of which is substituted at position 2 with non-reducing terminal β-D-glucopyranosyluronic acid, and at position 2 and position 6 with single residues or short side chains (1→2, 1→3, 1→4 linked) of β-D-xylopyranose. Furthermore, these polysaccharides appear to contain a small amount of 1→6 linked D-mannopyranose (and/or D-glucopyranose), terminal D-mannopyranose (and/or D-glucopyranose) and branching D-mannopyranose at positions 4 and 6. The results obtained in the present study also demonstrated that significant differences exist between MEA and MHA in molecular weights (MEA: 37×10⁴; MHA: 30× 10⁴), *O*-acetyl contents (MEA: 15.0%; MHA: 11.7%), and the amounts of 1→4 linked xylopyranose and branching mannopyranose at positions 4 and 6.

Misaki *et al.*[3] recently isolated an acidic polysaccharide ($[\alpha]_D$ −20°) from the fruit bodies of *A. auricula-judae*, and suggested the structure on the basis of methylation analyses. However, the paper did not describe the homogeneity, the presence of *O*-acetyl groups, or the physical properties of the polysaccharide. Furthermore, the specific rotation ($[\alpha]_D$ −20°) of their polysaccharide differs from those (MEA: $[\alpha]_D^{23}$ +31°; MHA: $[\alpha]_D^{23}$ +33°) of ours. The main structural differences between our polysaccharide and theirs were that their polysaccharide contained a large amount of non-terminal glucose, and did not contain non-terminal xylose, 1→6 linked hexose, or the branching mannose at positions 4 and 6. Except in these respects, their structure seems to be very similar to ours.

The acidic polysaccharides[15] isolated from the fruit bodies of *T. fuciformis*, belonging to the same Heterobasidiae, were shown to have a similar structural unit with terminal β-D-glucopyranosyluronic acid and β-D-xylopyranosyl side chains attached to position 2 of D-mannopyranose residues which are linked α-1→3 in a backbone. Such a structural unit was also reported in the extracellular polysaccharide of *T. mesenterica*[16] and in the capsular polysaccharides of *Cryptococcus neoformans*.[17,18] However, the essential difference among these polysaccharides is found in the structural detail of the xylopyranosyl side chains. The previous paper indicated that polysaccharides of *Tremella* and *Cryptococcus* species showed some chemotaxonomic relationship between the two species.[19] Recently, the perfect state of *C. neoformans* (Deuteromycetes) has been confirmed to be *Fillobasidiella neoformans* (Basidiomycetes)[20] by mating experiments, and the classification of the fungus has been discussed in detail by mycologists.[20a,21] This result is of interest in connection with the structural features of fungal polysaccharides.

The data presented in this work indicate that a certain chemotaxonomic relationship exists among *A. auricula-judae* and *T. fuciformis* (Heterobasidae), and also *F. neoformans* (Hemibasidae), which belongs to Basidiomycetes. The results of investigations including

determination of the location of O-acetyl groups in MEA and MHA will be reported in the near future.

Experimental

Solutions were concentrated below or at 40°C in a rotary evaporator under reduced pressure. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrometer. GLC was carried out on a JEOL JGC-1100 gas chromatograph equipped with a hydrogen flame ionization detector. GLC–MS was performed with a JEOL JMS-D 300 gas chromatograph and mass spectrometer.

Materials——The fruit bodies of *Auricularia auricula-judae* QUEL growing in Ssuchuan (China) were obtained as dry material. Pronase (45000 PUK/g) was purchased from Kaken Chemical Ind., Ltd. DEAE-Sephadex A-25 and Sepharose 4B were purchased from Pharmacia Fine Chemicals.

Isolation and Purification——The fruit bodies (50 g) were crushed and then washed with hot MeOH. The residue was extracted with 70% aqueous EtOH (1 l) for 20 h in a boiling water bath, and centrifuged at 5000 rpm for 20 min. This process was repeated 3 times. The combined extracts were concentrated to a small volume, and water (700 ml) was added. The solution was adjusted to pH 7.7 with 1 N NaOH. The mixture was treated with Pronase at 37°C until the pH dropped no further and was then dialyzed against distilled water for 3 d. The internal solution was further deproteinized by the Sevag procedure.[5] The aqueous phase was concentrated, and lyophilized to afford a crude polysaccharide (ME) as pale brown flakes (yield: 5%). The residue after 70% EtOH extraction was extracted with hot water (1.5 l) for 4 h, and centrifuged. This process was repeated 4 times. The combined extracts were deproteinized by Pronase treatment and by the Sevag procedure,[5] and dialyzed, then lyophilized to afford a crude polysaccharide (MH) as pale brown flakes (yield: 20%).

Each crude polysaccharide (ME and MH) was dissolved in water (1% solution), and 1% CTAB solution was added until no further precipitation occurred. The mixture was allowed to stand at 37°C for 1 d, then the precipitate was collected by centrifugation, and dissolved in 1 M MgCl$_2$. The solution was precipitated with EtOH. Each precipitate fraction obtained by the above repeated treatment was dialyzed and lyophilized to give an acidic polysaccharide as colorless flakes (yield: MEA 4%; MHA 16%).

Gel Chromatography——Each polysaccharide (*ca.* 2 mg) was dissolved in 0.1 M NaCl (1 ml), and applied to a column (15 × 90 cm) of Sepharose 4B. The column was eluted with 0.1 M NaCl at a flow rate of 8 ml per h. Fractions of 3 g were collected, and analyzed by the phenol–H$_2$SO$_4$ method at 485 nm.[22]

Ion-Exchange Chromatography on DEAE-Sephadex A-25——Each polysaccharide (*ca.* 2 mg) in 0.1 N phosphate buffer (2 ml) at pH 6.1 was applied to DEAE-Sephadex A-25 which had previously been equilibrated with the buffer. The column (1.5 × 40 cm) was eluted with the buffer (60 ml), and then with an NaCl gradient (180 ml) (0 to 1 M). Fractions of 3 g were collected, and analyzed by the phenol–H$_2$SO$_4$ method at 485 nm.[22]

Tiselius-type Electrophoresis——Electrophoresis of 0.4% polysaccharide was carried out with a Hitachi HID-1 boundary electrophoresis apparatus in 0.05 M sodium tetraborate buffer at pH 9.3 for 80 min. Electrophoretic mobility: MEA, 1.20×10^{-4} cm^2/V·s; MHA, 1.27×10^{-4} cm^2/V·s.

Physical Analyses——The physical measurements were carried out in 0.3 M NaCl–0.001 M sodium phosphate buffer (pH 6.9) at 20° C as described in a previous paper.[8]

The viscosities were measured with an Ubbelohde viscometer at four concentrations in the range of 0.04 to 0.10%. The partial specific volumes were calculated from density measurements made with a 5 ml density bottle. The sedimentation velocities were determined at 51000 rpm in a Hitachi UCA-1 analytical ultracentrifuge with a schlieren optical system. The sedimentation coefficient (s_{20}) was obtained at three concentrations (0.1, 0.15 and 0.2%) for each polysaccharide.

The molecular weight (M) was calculated by means of the equation, $M^{2/3} = N[\eta]^{1/3}\eta_0 s/2.5 \times 10^6 (1 - \bar{v}\rho)$ used by Eyring[6] and Pancake.[7] Here N is Avogadro's number, η the intrinsic viscosity, η_0 the solvent viscosity, s the sedimentation coefficient, $\bar{v}$ the partial specific volume, and ρ the solvent density.

O-Acetyl Determination——Each polysaccharide (500 mg) was hydrolyzed with 1 N NaOH (100 ml) for 8 h at room temperature. The hydrolysate was treated by addition of EtOH (3 volumes), and centrifuged. The supernatant was reacted with p-bromophenacyl bromide to yield the p-bromophenacyl ester according to the procedure described earlier.[8] The ester (mp 85°C) was identified as the p-bromophenacyl acetate by a mixed melting point test and comparison of the IR spectrum with that of an authentic sample.

A part of the supernatant was treated with Amberlite CG-120 (H$^+$) resin, and applied to GLC. GLC was carried out under the following conditions: a glass column (0.3 cm × 2 m) packed with 2% phosphoric acid-Porapack Q (80 to 100 mesh) at 205°C at a flow rate of 50 ml per min of N$_2$. The retention time of each sample was identical with that of acetic acid (t_R: 4.8 min). Total acetyl contents of these polysaccharides were determined by the ferric hydroxamate method[9] using penta-O-acetyl-β-D-glucose as a standard.

Component Sugars——Each polysaccharide (3 mg) was hydrolyzed with 2 N H$_2$SO$_4$ in a sealed tube at 100 °C for 8 h. After neutralization with BaCO$_3$ and filtration, the filtrate was passed through a column of

Amberlite CG-120 (H+), and concentrated to a syrup. The hydrolysates were subjected to PPC using Toyo Roshi No. 51 filter paper by the double ascending method with n-BuOH: pyridine: H_2O (6: 4: 3). Sugars were detected with alkaline $AgNO_3$[23] and p-anisidine hydrochloride[24] reagents. R_{glc}: D-xylose 1.17, D-mannose 1.10, D-glucose 1.00, D-glucuronic acid 0.28.

An aqueous solution of each hydrolysate (1 mg in 2 ml) was treated with $NaBH_4$ (2 mg) for 3 h at room temperature, and, after removal of boric acid,[4] the reduction product was acetylated with Ac_2O and pyridine (1: 1, 0.5 ml) at 100°C for 1 h. The resultant O-acetyl alditols were then subjected to GLC under the following conditions: condition A, a glass column (0.3 cm × 2 m) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 182°C at a flow rate of N_2 (2.0 kg/cm²). The retention times of acetates of xylitol, mannitol and glucitol were 22.6, 42.8 and 59.2 min. The molar ratios of glucose to mannose were 0.06 in MEA and 0.07 in MHA.

The component sugars were estimated for each polysaccharide solution by the phloroglucinol–HCl method[25] for xylose, the anthrone method[26] for mannose, and the method of Galambos[27] for glucuronic acid.

Methylation Analysis——Since MEA (20 mg) was slightly soluble in DMSO, it was acetylated with a mixture of pyridine (0.4 ml)–Ac_2O (0.3 ml) in formamide (0.8 ml). MHA (20 mg) and the fully acetylated MEA were methylated by the method of Hakomori[10] as described in our previous paper.[19] The fully O-methylated polysaccharides showed no hydroxyl absorption band in the IR spectra.

A part of each fully O-methylated polysaccharide was dissolved in tetrahydrofuran (THF) (10 ml), and the solution was treated with 1% suspension of $LiAlH_4$ in THF (8 ml). The mixture was stirred for 24 h at room temperature, and treated in the usual manner to afford the O-methylated carboxyl-reduced polysaccharide, which showed no carbonyl absorption band in the IR spectrum.

Portions of the fully O-methylated polysaccharide and the O-methylated carboxyl-reduced polysaccharide were successively treated with 90% HCOOH (2 ml) at 100°C for 2 h and 0.5 N H_2SO_4 (2 ml) at 100°C for 20 h. After being neutralized with $BaCO_3$, the solutions were treated with $NaBH_4$ and $NaBD_4$ to provide the corresponding alditols, acetylated with a mixture of Ac_2O–pyridine as described above, then subjected to GLC and GLC–MS. GLC was carried out under condition A using 3% ECNSS-M on Gaschrom Q at 180°C. GLC–MS was carried out on the same column (0.2 cm × 1 m) at 180°C at a flow rate of helium of 0.8 kg/cm². The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 230°C. Table II shows the retention times and molar ratios of methylated sugars as alditol acetates relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. On GLC–MS of alditol acetates obtained by reduction with $NaBH_4$, prominent fragments in the mass spectrum (MS) of the peak corresponding to 2,3- or 3,4-di-O-methyl xylitol acetates were m/e: 43, 87, 88, 101, 117, 118, 129, 130, 189, 190.

Another portion of the fully O-methylated polysaccharide was treated with 5% methanolic HCl in a sealed tube for 20 h in a boiling water bath. After neutralization with Ag_2CO_3 and filtration, the mixture of methyl glycosides was analyzed by GLC. GLC was carried out under condition B: a glass column (0.3 cm × 2 m) packed with 5% neopentylglycol succinate polyester (NPGS) on Chromosorb G (AW) (60 to 80 mesh) at 154°C with a flow rate of N_2 of 2.2 kg/cm². Relative retention times with respect to methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside: methyl 2,3,4-tri-O-methyl-D-xyloside 0.43, 0.56; methyl 3,4-di-O-methyl-D-xyloside 1.12, 1.32; methyl (methyl 2,3,4-tri-O-methyl-D-glucopyranosid)uronate 2.36, 3.20; methyl 2,4,6-tri-O-methyl-D-mannoside 3.54; methyl 2,4-di-O-methyl-D-mannoside 7.80; methyl 4,6-di-O-methyl-D-mannoside 10.42.

Periodate Oxidation——The de-O-acetylated polysaccharides (MEA-D and MHA-D) were prepared by treatment of the native polysaccharides (MEA and MHA) with 0.1 M MeONa as described in a previous paper.[19] Each sample (MEA, MHA, MEA-D and MHA-D) (10 mg) was oxidized with 0.01 M $NaIO_4$ (20 ml) at 4°C for 5 d in the dark. After various times, the periodate consumptions were estimated by an arsenite method.[28] The amounts (mol) of periodate consumed per mol of anhydrohexose unit were 0.53, 0.49, 0.67 and 0.72 for MEA, MHA, MEA-D and MHA-D, respectively.

Smith Degradation——The solutions of the oxidized polysaccharides obtained as described above were added to ethylene glycol (0.2 ml), and then dialyzed against deionized water. The inner solutions were treated with $NaBH_4$ (10 mg) for 12 h at room temperature. The resulting solutions were acidified with 0.1 N AcOH to pH 5 and dialyzed, and the non-dialyzable fractions were hydrolyzed with 1 N H_2SO_4 at 100°C for 12 h. The component sugars of the hydrolysates were analyzed by GLC (as alditol acetates) and by PPC as described above.

Controlled Smith Degradation——Each de-O-acetylated polysaccharide (MEA-D 31 mg, MHA-D 49 mg) was oxidized with $NaIO_4$ and reduced with $NaBH_4$. The resulting polyalcohols (MEA-DP 24 mg, MHA-DP 37 mg) were mildly hydrolyzed with 0.1 M AcOH (adjusted to pH 2 with dil. HCl) at 100°C for 35 min, and then dialyzed for 12 h. The non-dialyzable fractions were lyophilized to yield the controlled Smith degradation products (MEA-DS 17 mg, MHA-DS 27 mg). The specific rotations of MEA-DS and MHA-DS were $[\alpha]_D^{23}$ +100° and +109° ($c=0.17$, 1 N NaOH), respectively. The IR spectra of these products showed absorption at 820 cm⁻¹ (α-D-configuration).

Each controlled Smith degradation product (3 mg) was methylated by the method of Hakomori.[10] The fully O-methylated products were hydrolyzed with 90% HCOOH (2 ml) at 100°C for 2 h, and then 0.5

N H_2SO_4 (2 ml) at 100°C for 15 h.   The partially O-methylated alditol acetates prepared in the usual manner were analyzed by GLC under condition A at 180°C.   The results indicated that the products of MEA-DS and MHA-DS contained a large amount of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-mannitol and traces of 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-D-mannitol and 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-mannitol.

Acidic Oligosaccharides——Preparation of oligosaccharides from MEA and MHA was performed as in the foregoing experiment.[15)]   Each polysaccharide (MEA and MHA) (300 mg) was successively treated with 1 N H_2SO_4 at 37°C for 24 h and at 100°C for 4 h.   The partial acid hydrolysates were fractionated on a column of Amberlite CG-400 (CH_3COO^-) with H_2O, followed by gradient elution with AcOH (0 to 1 M). The acidic fractions were further purified by PPC (Whatman 3MM paper; solvent system, n-BuOH: AcOH: $H_2O=2$: 1: 1).   Thus, three acidic oligosaccharides (F-1, F-2 and F-3) were isolated from MEA and MHA. The specific rotations of the oligosaccharides were as follows: F-1, $[\alpha]_D^{26}$ $+19°$ ($c=0.23$, H_2O); F-2, $[\alpha]_D^{26}$ $-3°$ ($c=0.25$, H_2O); F-3 $[\alpha]_D^{26}$ $-42°$ ($c=0.16$, H_2O).

The Rf values of F-1, F-2 and F-3 on PPC (Toyo Roshi No. 51; double ascending method; solvent system, n-BuOH: AcOH: $H_2O=2$: 1: 1) were consistent with those of the aldotetraouronic acid (R_{glc} 0.36), the aldotriouronic acid (R_{glc} 0.49), and the aldobiouronic acid (R_{glc} 0.68) which were isolated from the hydrolysate of the acidic polysaccharide of $T.$ $fuciformis$.[15)]   Oligosaccharides were methylated by the method of Hakomori.[10)]   Methanolysates of the fully O-methylated oligosaccharides on GLC under condition B gave peaks which had retention times identical with those of authentic methyl (methyl 2,3,4-tri-O-methyl-D-glucopyranosid)uronate (I) 26.4, 35.0 min, methyl 3,4,6-tri-O-methyl-D-mannoside (II) 30.9 min and methyl 2,4,6-tri-O-methyl-D-mannoside (III) 39.6 min.   The molar ratios of the products I, II and III were 1.0: 0.9: 2.2 in F-1, 1.0: 0.9: 1.0 in F-2 and 1.0: 1.0: 0 in F-3.

Degraded Polysaccharides——Each polysaccharide (MEA or MHA) (100 mg) was hydrolyzed with 0.1 N H_2SO_4 (100 ml) at 100° C for 6 h.   The hydrolysates were neutralized with $BaCO_3$, filtered, and then passed through a column of Amberlite CG-120 (H^+).   The eluates were dialyzed against deionized water for 2 d, and the inner solutions were lyophilized to afford the degraded polysaccharides (MEA-H 55 mg, MHA-H 42 mg). The specific rotations of MEA-H and MHA-H were $[\alpha]_D^{19}$ $+46°$ ($c=0.55$, H_2O) and $[\alpha]_D^{19}$ $+48°$ ($c=0.42$, H_2O). The component sugars were determined as described above.

References and Notes

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