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Polysaccharides in Fungi. III.[1] A Neutral Heteroglycan from Alkaline Extract of *Tremella fuciformis* Berk[2]

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A neutral polysaccharide has been isolated from the alkaline extract of the fruit bodies of *Tremella fuciformis* Berk, which grow in China. It was composed of xylose, mannose, galactose and glucose in molar ratios of 2: 4: 5: 35. Its molecular weight was estimated at 8000, and it showed a positive specific rotation ($[\alpha]_D^{20}$ +9° in H_2O). The results of periodate oxidation, Smith-type degradation, partial acid hydrolysis and methylation studies suggested that the heteroglycan consisted of main units of 1→6 linked glucopyranosyl residues possessing branch points at position 3 in part and small amounts of 1→3 linked glucopyranosyl residues, to which minor units of mannopyranosyl residues having branches at position 2 and 3, and galactofuranosyl (or galactopyranosyl) residues possessing branches at position 2 and 6 (or 3 and 6) attached. Also, the structure was proved to contain moieties of 1→4 linked glucopyranosyl, 1→2 and 1→6 linked galactfuranosyl, and non-reducing terminal glucopyranosyl and xylopyranosyl residues.

Keywords——*Tremella fuciformis*; polysaccharide; neutral heteroglycan; molecular weight; Smith degradation; partial acid hydrolysis; methylation analysis

In the previous paper,[1] we have studied the acidic polysaccharides (AC and BC) obtained from the hot aqueous extract of the fruit bodies of *Tremella fuciformis* Berk (Tremellaceae) which grow in China. However, neutral polysaccharides could not be found in the aqueous extract. We have now isolated a neutral heteroglycan from alkaline extract of the fruit bodies of this fungus. The present paper concerns the purification and characterization of the neutral polysaccharide.

The dried fruit bodies were washed with hot ethanol, and then the residue was treated with 0.5 N sodium hydroxide.

The alkaline extracts were neutralized with hydrochloric acid, and dialyzed against water. The non-dialyzable solution was concentrated and fractionated by the addition of ethanol. The resulting precipitate was dissolved in water, and treated with cetyltrimethylammonium bromide (CTAB) to remove acidic polysaccharides as water-insoluble salt. After centrifugation of the salt, the supernatant fraction was dialyzed for the removal of CTAB, precipitated with ethanol, and treated with Amberlite CG-120 (H^+). The solution thus obtained was chromatographed on DEAE-Sephadex (phosphate form) to remove acidic polysaccharides completely. The neutral fraction was dialyzed, and lyophilized to yield a crude polysaccharide. The crude product had a trace of insoluble material in 0.1 M sodium chloride. Therefore, the insoluble material was removed by centrifugation and filtration. The soluble fraction was purified by gel chromatography on Sepharose 6B, dialyzed against water, and lyophilized to yield the polysaccharide (CCS) as a colorless powder (yield; 0.1%), $[\alpha]_D^{20}$ +9° (c=1, H_2O).

CCS was homogeneous on gel chromatography with Sephadex G-100, and gave a single spot on glass-fiber paper electrophoresis using a borate buffer of pH 9.2. Neither nitrogen, phosphorus nor sulfur could be detected by elemental analysis.

1) Part II: S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.* (Tokyo), **25**, 338 (1977).
2) This work was presented at the 96th and 97th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1976, and Tokyo, April 1977, respectively.
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The calibration curve as shown in Fig. 1 was made by gel chromatography on Sephadex G-100 with standard dextrans. Molecular weight ($\overline{M}w$) of CCS thus estimated was about 8000. The degree of polymerization ($\overline{DP}$) of CCS was determined to be 46 by a modified method of Akai[4] if the reduced terminal group was considered to be linked through position 6 of a glucose residue.

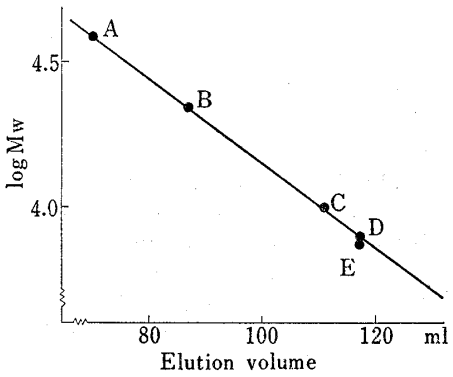

Fig. 1. Plot of Elution Volume against log $\overline{M}w$ for Dextran Fractions on Sephadex G-100 with 0.1 M NaCl

A; Dextran T-40, B; Dextran T-20, C; Dextran T-10, D; CCS, E; Dextran (7600).

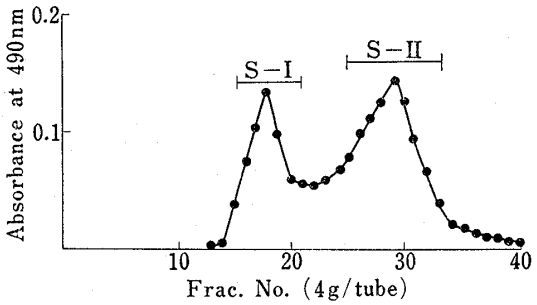

Fig. 2. Chromatogram on Sephadex G-25 of Controlled Smith Degradation Products of CCS

The component sugars of CCS were identified to be xylose, mannose, galactose and glucose by paper-partition chromatography (PPC) and gas-liquid chromatography (GLC) of the hydrolysate with sulfuric acid. The molar ratios of these sugars were estimated to be 2: 4: 5: 35, respectively, based upon GLC analysis of the alditol acetate derivatives.

On periodate oxidation of CCS, 1.43 mol of the amount of periodate per anhydrohexose unit was consumed.

Smith degradation products[5] of CCS were analysed by GLC as acetyl- and trimethylsilyl derivatives to detect ethylene glycol, glycerol, erythritol, threitol, arabinose, mannose, galactose and glucose in the molar ratio of 0.9: 6.5: 0.7: 0.7: 0.4: 1.0: 0.6: 1.7, respectively.

The controlled Smith degradation products of CCS were subjected to a column chromatography on Sephadex G-25. As shown in Fig. 2, two major fractions were eluted. The sugar component of each fraction was determined by GLC as trifluoroacetate derivatives of the hydrolysate. S–I fraction consisted of oligomers containing mannose, galactose, and small amounts of glucose and arabinose as sugar component. On the other hand, S–II fraction was composed of low molecular products containing glucose, mannose and a trace of arabinose. The appearance of arabinose indicated the presence of 1→2 and/or 1→3 linked galactofuranose. Those component sugars in S–II were degraded during the Smith degradation of both fractions, whereas mannose, galactose or glucose in S–I was not degraded. These results suggested that CCS contained an oxidation-resistant core consisting of mannose, galactose and glucose as main sugar.

CCS was partially hydrolyzed with sulfuric acid under four different conditions as follows; (a): 0.05 N, 100°, 2 hr. (b): 0.25 N, 100°, 3 hr. (c): 0.25 N, 100°, 2 hr. (d): 2 N, 100°, 6 hr. The outline of the treatment is shown in Chart 1. The fragments released were separated by gel chromatography on Sephadex G-25. The low molecular fraction (F–I–2) released by treatment (a) of CCS consisted of xylose, mannose, galactose and glucose in an approximate molar ratio of 1.0: 0.3: 0.8: 0.9. The low molecular fraction (F–II–2) released by treatment (b) of F–I–1

4) H. Akai, Y. Yokobayashi, A. Misaki, and T. Harada, *Biochim. Biophys. Acta*, **237**, 422 (1971).
5) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).

consisted of xylose, mannose, galactose and glucose in an approximate molar ratio of 1.0: 1.4: 1.2: 9.3. The fragments (F–III) released by treatment (c) of F–II–1 contained a large quantity of glucose and a small quantity of isomaltose and of higher oligosaccharides, which released only glucose by treatment (d). These results suggested the presence of acid-labile xylosyl residues and glucosyl-glucose units.

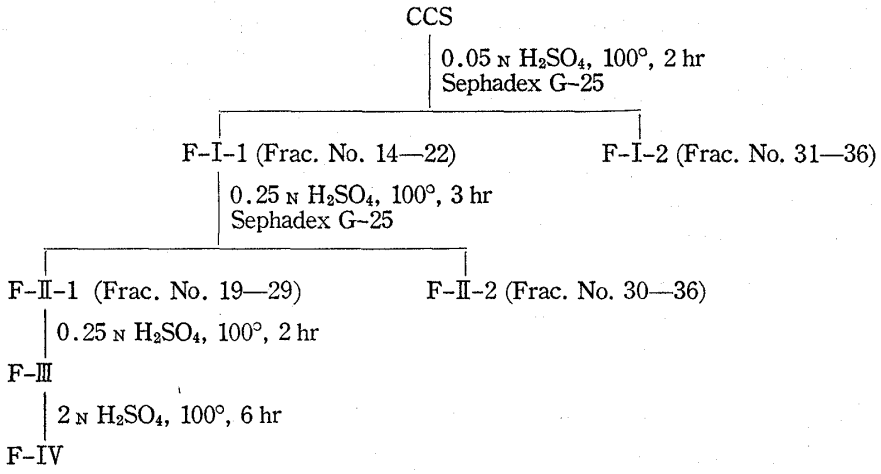

Chart 1. Partial Acid Hydrolysis of the Polysaccharide

After methylation of CCS by the methods of Hakomori[6] and Purdie,[7] methanolysis and hydrolysis of the product were carried out. The resulting partially O-methylated monosaccharides were converted into alditol acetates, and identified by GLC and gas-liquid chromatography-mass spectrometry (GLC–MS). The retention times were consistent with those of authentic samples (derivatives from O-methylated sugar except for O-methylated galactose) or the values in the literature.[8a] The results are shown in Table I.

Table I. Relative Retention Times and Composition of Methylated Products

Peak number	Component (alditol acetate derivative)	Relative retention time	Molar ratio
I	2,3,4-Tri-O-methyl-1,5-di-O-acetyl xylitol	0.68	2.0
II	2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl glucitol	1.00	7.0
III	2,4,6-Tri-O-methyl-1,3,5-tri-O-acetyl glucitol 3,5,6-Tri-O-methyl-1,2,4-tri-O-acetyl galactitol	1.91	3.1
IV	2,3,4-Tri-O-methyl-1,5,6-tri-O-acetyl glucitol[a] 2,3,6-Tri-O-methyl-1,4,5-tri-O-acetyl glucitol[b]	2.40	27.6
V	2,3,5-Tri-O-methyl-1,4,6-tri-O-acetyl galactitol 4,6-Di-O-methyl-1,2,3,5-tetra-O-acetyl mannitol	3.16	0.7 1.1
VI	2,4-Di-O-methyl-1,3,5,6-tetra-O-acetyl glucitol	4.86	4.5
VII	3,5(or 2,4)-Di-O-methyl-1,2,4,6(or 1,3,5,6)-tetra-O-acetyl galactitol	6.08	1.1

a) A large amount.
b) A small amount.

The mass spectra analyzed by GLC–MS were compatible with those in the literature[8b] in peak I, II, VI and VII. Peak III on the mass spectrum (*m/e*: 43, 45, 87, 101, 117, 129, 161) was identified as 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl glucitol. In addition, the presence

6) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
7) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).
8) *a*) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967); *b*) *Idem*, *Carbohyd. Res.*, **5**, 433 (1967).

of weak fragments (*m/e*: 89, 189)[9] and the occurrence of arabinose in the Smith degradation products led us to estimate the coexistence of 3,5,6-tri-O-methyl-1,2,4-tri-O-acetyl galactitol (*m/e*: 43, 45, 87, 89, 101, 117, 129, 161, 189). Methanolysis analysis as described below, and a large amount of glycerol and a small amount of erythritol in the Smith degradation products supported the idea that peak IV consisted of a large amount of 2,3,4-tri-O-methyl-1,5,6-tri-O-acetyl glucitol (*m/e*: 43, 87, 99, 101, 117, 129, 161, 189) and a small amount of 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl glucitol (characteristic fragments: 45, 113 and 233). The mass spectrum of peak V (*m/e*: 43, 45, 87, 101, 117, 129, 161, 261) revealed characteristic fragments for 4,6-di-O-methyl-1,2,3,5-tetra-O-acetyl mannitol except for *m/e* 117. The above result and the occurrence of threitol in the Smith degradation product indicated the presence of 2,3,5-tri-O-methyl-1,4,6-tri-O-acetyl galactitol. The ratio of mannose to threitol in the Smith degradation products suggested that 4,6-di-O-methyl mannose and 2,3,5-tri-O-methyl galactose were in the approximate ratio of 1.1 : 0.7.

GLC on methanolysis analysis using a column of NPGS revealed the presence of 2,3,4,6-tetra-O-methyl glucose and a large amount of 2,3,4-tri-O-methyl glucose. However, other O-methyl sugars were not identified due to the weakness of the peaks and the overlap with each other.

From these results, the neutral polysaccharide (CCS) from *T. fuciformis* seems to be composed of main units of 1→6 linked glucopyranosyl residues possessing branch points at position 3 in part and small amounts of 1→3 linked glucopyranosyl residues, to which minor units of mannopyranosyl residues having branches at position 2 and 3, and galactofuranosyl (or galactopyranosyl) residues possessing branches at position 2 and 6 (or 3 and 6) attached. Also, the structure was proved to contain moieties of 1→4 linked glucopyranosyl, 1→2, 1→6 linked galactofuranosyl, and non-reducing terminal glucopyranosyl and xylopyranosyl residues. The molecular weight (8000) of CCS was very small, when compared with that (AC: 590000, BC: 720000) of the acidic polysaccharides from hot water extract.[10] It is of interest that the neutral polysaccharide, which could not be found in the aqueous extract, was obtained by the alkaline extraction from the fruit bodies of this fungus.

Fraser, *et al.*,[11] reported that the glucan isolated from the culture medium of *T. mesenterica* NRRL-Y 6158 was a linear molecule composed of approximately 200 α-D-glucopyranose units linked 1→6 and 1→4 in the ratio 2:1. However, the polysaccharide (CCS) of *T. fuciformis* differs from the above glucan in the ratio of 1→6 to 1→4 linked glucose, in the existence of the other component sugars, and in the physical properties. An interesting information will be provided in the chemotaxonomy by comparison of our polysaccharide (CCS) with neutral polysaccharides obtained from T. mesenterica.[11,12]

Experimental

IR spectra were recorded on a Japan Spectroscopic Co., Model IRA-I spectrometer. Specific rotations were measured by the use of a JASCO Model DIP-4 automatic polarimeter. GLC was carried out by the use of a JEOL Model JGC-1100 gas chromatograph equipped with hydrogen flame ionization detector.

Isolation and Purification——The dried fruit bodies (200 g) harvested in China[10] were crushed and then washed with hot EtOH. The material was extracted with 0.5 N NaOH (6 l) at room temperature, centrifuged at 5000 rpm for 20 min. This process was repeated 3 times. The combined extracts were neutralized with a dilute HCl, filtered and then dialyzed against running water for 2 days. The internal solution was concentrated to a small volume under reduced pressure and to which was added 3 volumes of EtOH, and then centrifuged. The precipitate was dissolved in a small amount of water and 3% CTAB was added until no further precipitation occurred. After the mixture was allowed to stand at 37° for 24 hr, the supernatant was separated by centrifugation. The treatment of CTAB was carried out again. The combined supernatant

9) T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **23**, 157 (1975).
10) S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1102 (1974).
11) C.G. Fraser and H.J. Jennings, *Can. J. Chem.*, **49**, 1804 (1971).
12) D.S. Cameron and I.E.P. Taylor, *Biochim. Biophys. Acta*, **444**, 212 (1976).

was dialyzed against running water for 2 days, and the internal solution was concentrated. To the solution was added 3 volumes of EtOH. The resulting precipitate was dissolved in water, and stirred with Amberlite CG-120 (H^+) resin for 30 min and then filtered. The filtrate was purified by a column chromatography on DEAE-Sephadex A-25 (phosphate form) as described in a previous paper.[10] The neutral fractions were collected, dialyzed and lyophilized. The material thus obtained was dispersed in a small amount of 0.1 M NaCl to yield a trace of insoluble material, which was removed by centrifugation and filtration with Toyo Roshi membrane filter TM-1. The filtrate was applied to a column (2.6×100 cm) of Sepharose 6B (Pharmacia Co.), and eluted with 0.1 M NaCl at flow rate of 10 ml per hr. The main fraction (380—520 ml) corresponding to carbohydrate peak by the method of phenol–sulfuric acid[13] at 490 nm were collected, dialyzed, and lyophilized to give CCS as a colorless powder (yield: 0.1%).

Properties of CCS——Zone electrophoresis with Whatman GF 81 glass-fiber paper using alkaline borate buffer of pH 9.2 (0.05 M borax) showed a single spot (detected with α-naphtol–sulfuric acid reagent[14]). CCS had $[\alpha]_D^{20}$ +9° (c=1, H_2O), C, 40.93%; H, 6.25%; N, nil; ash, nil.

Determination of Molecular Weight——Molecular weight ($\overline{M}w$) estimation was made by gel filtration on a column (1.5×100 cm) of Sephadex G-100 (Pharmacia Co.) equilibrated with 0.1 M NaCl. The calibration curve was made with dextran T-40 ($\overline{M}w$, 22300), and T-10 ($\overline{M}w$, 10400), purchased from Pharmacia Co., and dextran ($\overline{M}w$, 7600) kindly supplied from Research Laboratories of Meito Sangyo Co..

Determination of the Degree of Polymerization——The average degree of polymerization ($\overline{DP}$) was estimated by a modified Akai method.[4] $\overline{DP}$ was calculated from the result of the determination of formaldehyde produced by periodate oxidation of the reduced CCS with $NaBH_4$.

Component Sugar of CCS——CCS (5 mg) was hydrolyzed with 2 N H_2SO_4 (2 ml) in a sealed tube at 100° for 8 hr, followed by neutralization with $BaCO_3$, centrifugation, and filtration. The filtrate was passed through a column of Amberlite CG-120 (H^+), and concentrated under reduced pressure. The hydrolysate was applied to PPC using Toyo Roshi No. 51 filter paper by double ascending method with the solvent system; n-BuOH: pyridine: H_2O (6: 4: 3). Sugars were detected by spraying a solution of alkaline $AgNO_3$[15] and p-anisidine hydrochloride.[16] R_{GLC} values of xylose, mannose, glucose and galactose were 1.17, 1.10, 1.00 and 0.90, respectively. A part of the hydrolysate was reduced with $NaBH_4$ to the corresponding alditols, trifluoroacetylated with trifluoroacetic anhydride, and the mixture were applied to GLC. GLC of the trifluoroacetate derivatives was carried out under condition A, a glass column (0.3 cm×2 m) packed with XF-1105 on Chromosorb P (80 to 100 mesh) at 140° with a flow of 38 ml per min of N_2.[17] Retention times of trifluoroacetates of xylitol, mannitol, glucitol, and galactitol were 12.6, 18.3, 24.9 and 27.1 min, respectively. On the other hand, the alditols were acetylated with Ac_2O–pyridine (1: 1) at 100° for 2 hr. GLC of the alditol acetate derivatives was carried out under condition B, a glass column (0.3 cm×2 m) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 190° with a flow of 43 ml per min of N_2.[18] Retention times of acetates of xylitol, mannitol, galactitol and glucitol were 20.2, 37.2, 43.4, 49.0 min, respectively. The results revealed that CCS was composed of xylose, mannose, galactose and a large amount of glucose. The approximate molar ratio of Xyl: Man: Gal: Glc were 2: 4: 5: 35, determined by GLC analysis of alditol acetates derivatives.

Periodate Oxidation of CCS——CCS (10 mg) was oxidized with 0.02 M $NaIO_4$ (20 ml) at 5° in the dark. The periodate consumption was estimated by an arsenite method.[19] The mol of $NaIO_4$ consumed per anhydrohexose unit were as follows: 0.50 (1 hr), 0.88 (3 hr), 1.23 (6 hr), 1.29 (10 hr), 1.34 (24 hr), 1.41 (48 hr), 1.43 (72 hr), 1.43 (96 hr).

Smith Degradation of CCS and Analyses of the Products——After oxidation of CCS (20 mg) for 6 days as described above, the reaction mixture neutralized with $BaCO_3$ was reduced with $NaBH_4$ (50 mg) at 5° for 12 hr, and then excess of $NaBH_4$ was decomposed by an acidification with 1 M AcOH to the final pH 5. The solution was purified by use of a column (1.5×30 cm) chromatography on Sephadex G-10 (Pharmacia Co.) (eluted with H_2O), corresponding fractions were concentrated, and lyophilized to the polyalcohol (10 mg).

A part of the polyalcohol (3 mg) was completely hydrolyzed with 1 N H_2SO_4 (2 ml) at 100° for 10 hr. The hydrolysate neutralized with $BaCO_3$ was reduced with $NaBH_4$ by the usual manner, and acetylated with Ac_2O–pyridine (1: 1) at 100° for 2 hr, and then subjected to GLC. GLC was carried out under condition B except for column temperature 187°. Retention times of acetates of glycerol, erythritol, threitol, arabinitol, mannitol, galactitol and glucitol were 1.6, 5.5, 6.7, 17.4, 43.8, 50.8, 58.2 min, respectively. Their molar ratios were approximately 6.5: 0.7: 0.7: 0.4: 1.0: 0.6: 1.7 (glycerol: erythritol: threitol: arabinose: mannose: galactose: glucose).

A part of the polyalcohol (1 mg) was hydrolyzed with 0.5 N HCl (1 ml) at 35° for 12 hr in a sealed tube. The hydrolysate was concentrated to dryness, and dissolved in pyridine containing trimethylolpropane as an

13) J.E. Hodge and B.T. Hofreiter, *Meth. Carbohyd. Chem.*, **1**, 388 (1962).
14) H. Jacin and A.R. Mishkin, *J. Chromatogr.*, **18**, 170 (1965).
15) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature*, **166**, 444 (1950).
16) L. Hough and J.K.N. Jones, *Meth. Carbohyd. Chem.*, **1**, 28 (1962).
17) T. Imanari, Y. Arakawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1967 (1969).
18) J.S. Sawardeker, J.H. Sloneker, and A. Jeanes, *Anal. Chem.*, **37**, 1602 (1965).
19) P.F. Fleury and J. Lange, *J. Pharm. Chem.*, **17**, 107 (1933); *idem*, *ibid.*, **17**, 196 (1933).

internal standard and trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane as described in the previous paper,[1] then applied to GLC. GLC was carried out on a glass column (0.3 cm × 2 m) packed with 5% SE-30 on Chromosorb W (AW) (60 to 80 mesh); programmed column temperature increasing 4° per min from 70° to 200°; carrier gas, N_2 (43 ml). Relative retention times of trimethylsilyl derivatives of ethylene glycol and glycerol to internal standard were 0.31 and 0.79. The molar ratios of ethylene glycol and glycerol were identified as approximate 1.0: 7.2.

Controlled Smith Degradation——A part of the polyalcohol (3.8 mg) was mildly hydrolyzed with 0.1 N H_2SO_4 (2 ml) at room temperature for 24 hr. The solution was neutralized with $BaCO_3$, and filtered. The filtrate was deionized by a treatment with a small column of Amberlite CG-120 (H^+). After concentration to a small volume, the solution was applied to a column (1.5 × 100 cm) of Sephadex G-25 (Pharmacia Co.), and eluted with H_2O. Fractions of 4 g were collected and analyzed by phenol–sulfuric acid method.[13] As shown in Fig. 2, two major fractions were eluted. S-I and S-II fractions were pooled respectively, concentrated to small volumes, and then lyophilized. A part of both fractions was hydrolyzed with 2 N H_2SO_4 at 100° for 6 hr. The alditols of hydrolysates were trifluoroacetylated, and analyzed by GLC under condition A. A part of both fractions was further oxidized with $NaIO_4$ as described above. The final degradation products were analyzed by GLC as described above.

Partial Acid Hydrolysis of CCS——Partial acid hydrolysis of CCS (4 mg) using H_2SO_4 was examined under different conditions. The first fragments treated with 0.05 N H_2SO_4 (2 ml) at 100° for 2 hr were separated by gel filtration on a column (1.5 × 100 cm) of Sephadex G-25. The column was eluted with H_2O and each fraction was collected at 4 g, and analyzed phenol–sulfuric acid method.[13] The first high molecular fraction (F-I-1) was further hydrolyzed with 0.25 N H_2SO_4 (2 ml) at 100° for 3 hr, followed by gel filtration as described above. Each of low molecular fractions (F-I-2 and F-II-2) was examined by PPC as previously shown, and GLC of the alditol acetates under condition B. After the F-II-1 fraction obtained by the second treatment was hydrolyzed with 0.25 N H_2SO_4 (2 ml) at 100° for 2 hr, and examined by PPC as described above. R_{GLC} value of isomaltose was 0.56. A part of the above partial acid hydrolysate (F-III) was completely hydrolyzed with 2 N H_2SO_4, 100°, 6 hr and examined by GLC under condition B.

Methylation Analysis——Methylation of CCS (10 mg) was carried out by the method of Hakomori[6] as described in our previous paper.[1] The partially methylated CCS thus obtained was dissolved in MeI (3 ml) and then stirred at 45° for 12 hr with occasional addition of Ag_2O (30 mg). The reaction mixture was filtered to remove inorganic materials and the collected solid was fully washed with $CHCl_3$. The filtrate and combined washings were concentrated to dryness. After the twice procedure by the method of Purdie,[7] the product showed no free hydroxyl absorption in IR spectrum. A part of the fully methylated CCS was allowed to heat with 90% HCOOH (2 ml) at 100° for 1 hr in a sealed tube. On cooling, the mixture was concentrated, and then heated with 0.5 N H_2SO_4 (2 ml) at 100° for 15 hr. The hydrolysate neutralized with $BaCO_3$ was reduced with $NaBH_4$ and the resulting alditols were acetylated with Ac_2O–pyridine (1: 1) at 100° for 1 hr, and treated by the procedure described in our previous paper.[1] GLC of partially methylated alditol acetates were carried out under condition B except for column temperature 180°. Another portion of the fully methylated CCS was methanolysed with 5% MeOH–HCl in a sealed tube for 15 hr in a boiling water bath, and neutralized with Ag_2CO_3, and then filtered. The mixture of methyl glycosides was examined by GLC under the condition of a flow rate of 43 ml per min of N_2 on a glass column (0.3 cm × 2 m) packed with 5% neopentylglycol succinate polyester (NPGS) on Chromosorb G (AW) (60 to 80 mesh) at 152°. Methyl 2,3,4,6-tetra-O-methyl glucoside (t_R; 1.00, 1.44) and methyl 2,3,4-tri-O-methyl glucoside (t_R; 2.31, 3.29) were detected.

For GLC-MS spectrometry, alditol acetates derived from fully methylated CCS were dissolved in $CHCl_3$ and injected into a Shimadzu Model LKB-9000 gas-mass spectrometer using a glass column (0.4 cm × 1 m) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 188° with a flow of 30 ml per min of helium. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 60 μA and a temperature of the ion source of 290°.

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