[Chem. Pharm. Bull. 22(9)2058—2063(1974)]

UDC 547.458.02.05 : 581.192

Chemical Structure of the Water-soluble Glucan from the Cell Wall of *Cladosporium herbarum*.[1] Studies on Fungal Polysaccharide. XV[2]

Toshio Miyazaki[3a] and Yasuta Naoi[3b]

Tokyo College of Pharmacy[3a] and Tokyo Metropolitan Research Laboratory of Public Health[3b]

(Received February 12, 1974)

Main water-soluble polysaccharide, PS-1, $[\alpha]_D^{25}$ +166° (c=1, H_2O), from the cell wall of *Cladosporium herbarum* is a glucan. Results of periodate oxidation, Smith degradation, and methylation studies showed that the glucan consists of linear structure possessing a few branches. It contains (1→3) and (1→4) D-glucopyranosyl-linkages. Result of proton magnetic resonance studies showed the glycosidic linkage in the glucan to be α-configuration.

As a part of studies on fungal polysaccharides, we have examined the relationship between genus specificity and chemical structure of polysaccharides in the cell wall of fungi.[4]

In 1970, Lloyd[5] proved the presence of phosphorylated galactomannan-peptide complex in the intracellular polysaccharide of *Cladosporium werneckii*. On the other hand, our previous paper[6] reported the chemical structure of extracellular phosphate-free galactomannan in *Cladosporium herbarum*. This paper describes characterisation and chemical structure of a water-soluble glucan from the cell wall of *C. herbarum*, a kind of black yeast which belonging to imperfect fungi.

The purified cell wall was prepared from the filamentous cells mechanically with the French press, and followed by repeated washing and fractional centrifugation. The cell wall sedimentes were microscopically free from cytoplasmic materials.

Four consecutive extractions were carried out on the purified cell wall according to the following procedures. The cell wall defatted with ether–EtOH (1:1) was extracted with hot water. After centrifugation, the supernatant was concentrated and then dialyzed against running water. Non-dialyzable solution gave crude polysaccharide in an approximate yield of 32.8% of the cell wall.

The residue was stirred with 1N KOH at a room temperature. After centrifugation, the supernatant was neutralized and then dialyzed against running water. Non-dialyzable material was obtained in approximate yield of 2.3% of the cell wall. The residue was extracted with 1N KOH at 100°. After centrifugation, the supernatant was treated as described above. Non-dialyzable material was obtained in approximate yield of 0.5% of the cell wall. The residue was purified as described in the experimental part. The hot water extracted material was treated with Pronase E (Kakenkagaku Co. Ltd. Tokyo) and then by the Sevag method, followed by repeated diethylaminoethyl (DEAE)-cellulose column chromatography using $NaHCO_3$ and $Na_2B_4O_7$ for the elution.

1) A part of this work was presented at the 90th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, July 1970.
2) Part XIV: T. Miyazaki, M. Yamada, and T. Ohno, *Chem. Pharm. Bull.* (Tokyo), **22**, 1666 (1974).
3) Location: *a) Kitashinjuku 3-chome, Shinjuku-ku, Tokyo, 160, Japan; b) 24-1, 3-chome, Hyakunin-cho 3-chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) T. Miyazaki and T. Irino, *Chem. Pharm. Bull.* (Tokyo), **18**, 1930 (1970).
5) K.O. Lloyd, *Biochemistry*, **9**, 17, 3446 (1970).
6) T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1360 (1974).

The main fraction eluted with distilled water was further purified by zone electrophoresis using glass powder and borate buffer. The purified main polysaccharide (PS-1) thus obtained showed $[\alpha]_D^{25}$ +166° (c=1, H_2O), gave a single spot on paper electrophoresis using borate buffer (0.026M, pH 10.0) and did not give blue color with iodine. PS-1 contained 91% of total hexose (by phenol–sulfuric acid[7]). Neither of nitrogen and phosphorus was detected by elemental analysis and Fiske–Subbarow method.[8]

The component sugar of PS-1 was identified as D-glucose by paper chromatography of the acid hydrolyzate.

On periodate oxidation of PS-1, consumption of periodate per anhydro component sugar unit was 0.91 mole, the value of formic acid liberated from the unit was 0.04 mole, and that of formaldehyde was 0.09 mole after 24 hr.

The periodate oxidized PS-1 was treated by the Smith procedure. Paper chromatographic analysis of the hydrolyzate revealed the presence of erythritol, glycerol, and unoxidized component sugar. The molar ratio showed 1.7: 3.6: 1.0 (glucose, erythritol, glycerol) when these were estimated by the methods of Dubois, *et al.*,[7] (for hexose) and of Lambert–Neish[9] for polyalcohols.

After the main polysaccharide PS-1 was methylated by the methods of Hakomori[10] and then of Purdie,[11] methanolysis and hydrolysis were carried out. The resulting *O*-methyl monosaccharides were examined by paper chromatography, thin-layer chromatography, paper electrophoresis, and gas–liquid chromatography.

In the paper and thin-layer chromatographic analysis of the hydrolyzate of the methylated glucan, a large amount of tri-*O*-methyl-glucose and a small amount of tetra- and di-*O*-methyl-glucose were detected. Molar ratio was approximately 66: 1: 1. Di-*O*-methyl-monosaccharide fraction was identified as 3,6-di-*O*-methyl-monosaccharide (MG value; 0.54) by the paper electrophoresis using 1% borate. Tri-*O*-methyl-monosaccharide fraction was suggested as 2,4,6-tri-*O*-methyl- and 2,3,6-tri-*O*-methyl-monosaccharide by the paper electrophoresis using 1% borate.

The methylated PS-1 formed alditol acetates, and then the product was analyzed by gas–liquid chromatography. As shown in Fig. 1, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-glucitol were detected.

The methylated PS-1 was converted into methylglycoside by methanolysis. The product was further analyzed by gas–liquid chromatography under a different condition. As shown in Fig. 2, methyl-2,4,6-tri-*O*-methyl- and methyl-2,3,6-tri-*O*-methyl-D-glucosides were detected. From the results of gas–liquid chromatography, fractional components of methanolyzate were detected in the molar ratio of 1: 22: 44: 1 (2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl, 2,3,6-tri-*O*-methyl, 3,6-di-*O*-methyl).

Recently, Casu, *et al.*,[12] and one of the authors (T.M.)[2] examined glycosidic linkage in methylated polysaccharides by proton magnetic resonance (PMR) spectrum. PMR spectrum (Fig. 3) of the methylated PS-1 in $CDCl_3$ showed a doublet at 5.53 ppm for anomeric protons, H-1, of the α-D-(1—4)-linked residues in the glucan. In the infrared (IR) spectrum of PS-1, absorption maximum at 844 cm^{-1} suggests a CH bending vibration in the α-glycosidic linkage.

From these results, it is certain that the glucan has a linear structure possessing a few branches, and the main core contains α-1,4- and α-1,3-D-glucopyranosyl residues in a ratio

7) M. Dubois, K.A. Gillles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
8) C.H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **81**, 629 (1929).
9) M. Lambert and A.C. Neish, *Can. J. Res.*, **28B**, 83 (1956).
10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
11) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).
12) B. Casu, M. Regginani, G.G. Gallo, and A. Vigevani, *Tetrahedron*, **24**, 803 (1968).
13) S. Hirase, S. Nakai, and S. Otsuka, Proc. Japan. Cancer Assoc. 29th Annu. Meet., 1970, p. 227.

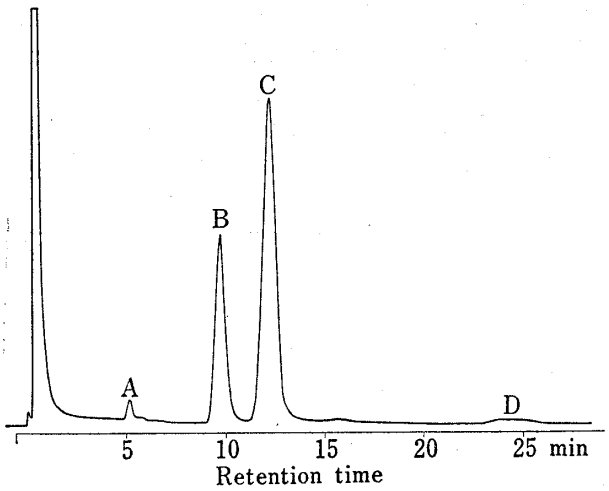

Fig. 1. Gas Chromatograms of Acetyl Alditol of the Methylated PS-1

A: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol
B: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol
C: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol
D: 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl glucitol
conditions: 5% of ECNSS-M on Chromosorb W; (60—80 mesh) 200×0.3 cm: 180°; N_2 50 ml/min

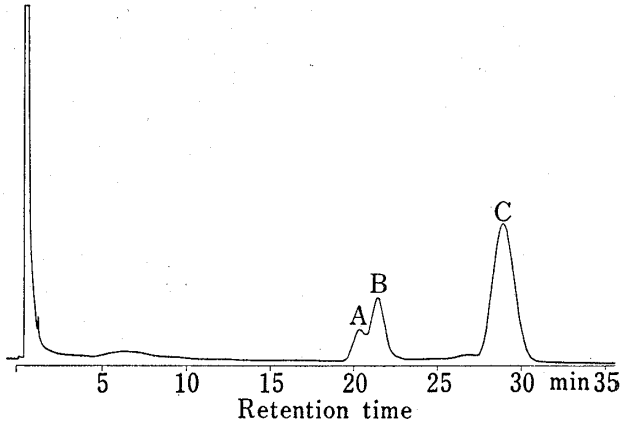

Fig. 2. Gas Chromatogram of Methyl Glycosides from Methylated PS-1

A: methyl 2,4,6-tri-*O*-methyl-glucoside (β)
B: methyl 2,3,6-tri-*O*-methyl-glucoside (β)
C: methyl 2,4,6-tri-*O*-methyl-(α) and methyl 2,3,6-tri-*O*-methyl-glucoside (α)
conditions: 15% poly butane-1,4-diol Succinate on Celite 545 (60—80 mesh); 200×0.3 cm; 175°; N_2 50 ml/min

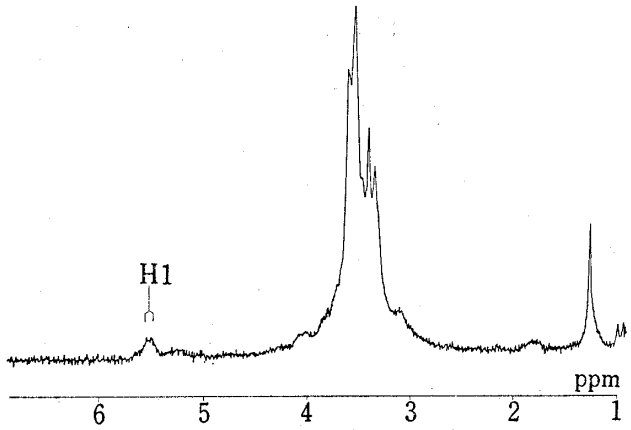

Fig. 3. PMR (60 MHz) Spectrum in $CDCl_3$ of Methylated PS-1

of 2:1. Similar structure is known in the case of polysaccharide fraction from the mycelium of *Coriolus versicolor*.[13]

The alkali-soluble but water-insoluble fractions of the minor components (PS-2 and PS-3) of the cell wall showed the presence of glucose and galactosamine, and glucose and glucosamine, respectively. Characterization of these materials will be discussed in a later comunication.

Paper chromatographic analysis of the acid hydrolyzate of alkali-extracted cell wall residue showed the presence of a large amount of glucosamine and a small amount of glucose.

Heteroglycan such as phosphorylated galactomannan-peptide complex, isolated from the cell wall of *C. werneckii* (in yeast form) by Lloyd, was not detected in spite of a similar medium used. However, it is not clear whether the difference is due to culture condition or to species.

Experimental

Preparation of Cell Wall Material——*Cladosporium herbarum* NIHS 4000[14] was grown in Sabouraud medium (dialyzable polypeptone 1%, glucose 4%) at 25° in an incubator for 40 days. The culture flasks were shaken once a day to obtain the filamentous form. The mycelium was separated from the medium by filtration through a Nylon cloth, washed thoroughly with distilled water, and the cell wall was prepared mechanically with the French press. This procedure was repeated 3 times at a pressure of 400 kg/cm² and it was usually sufficient to achieve total cell rupture. After this procedure, the cell wall was separated

14) The strain was supplied from the National Institute of Hygienic Sciences, Tokyo.

immediately from cytoplasmic debris and intracellular soluble materials by repeated centrifugation at 3000 rpm for 10 min to prevent enzymic degradation of the cell wall. The supernatant was discarded and the residue was resuspended in an aqueous solution of 0.1% sodium dodecylsulfate and was stirred for 24 hr vigorously. This treatment was repeated 7 times. The sedimented cell wall preparation was washed with distilled water until free of sodium dodecylsulfate and dried over P_2O_5 *in vacuo*. A charcoal grayish fine powder obtained in yield of 4.8 g per liter. Microscopic examination of this material showed that it was free from cytoplasmic contamination (tested with 0.02% Methylene Blue staining).

Extraction from the Cell Wall——1) Extraction with Acetone and EtOH: Ether (1: 1): The finely powdered cell wall (20.0 g) was extracted with acetone and then with EtOH–ether (1: 1, v/v) for 3 days at a room temperature. After the mixture was centrifuged, the supernatants were combined and evaporated to dryness. A very small amount of residue was obtained.

2) Extraction with Distilled Water at 100°: The dried fat-free cell wall was stirred with distilled water (300 ml) for 2 hr at 100° and this treatment was repeated until negative to the anthrone reagent. After centrifugation, the supernatant was dialyzed against running water for 2 days. The internal solution was centrifuged, the supernatant was concentrated to a small volume, and 10 volumes of EtOH were added. The resulting precipitate was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*. Yield of the water-soluble crude polysaccharide was 6.56 g (corresponding to 32.8% of the cell wall).

3) Extraction with 1N KOH at a Room Temperature: The residue left after extraction with hot water was extracted with 1N KOH (300 ml) for 2 hr at a room temperature and centrifuged. This treatment was repeated until almost negative to the anthrone reagent. The supernatant was neutralized with 1M AcOH, dialyzed against running water for 4 days, and then concentrated. Water-insoluble material appeared on dialysis. To the concentrate, 10 volumes of EtOH were added, and the precipitate that appeared was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*. Yield of the water-soluble crude polysaccharide, 0.46 g (corresponding to 2.3% of the cell wall) and water-insoluble material (PS-2), 1.13 g (corresponding to 5.7% of the cell wall).

4) Extraction with 1N KOH at 100°: The residue from the alkali treatment was suspended in 300 ml of 1N KOH, kept at 100° for 2 hr, and then treated as described above. Yield of water-soluble material, 0.1 g (corresponding to 0.5% of the cell wall) and water-insoluble material, 2.82 g (PS-3) (corresponding to 11.2% of the cell wall).

After the above four steps of extraction, the residue was washed with distilled water until free of KOH and dried with acetone and ether. Yield of the residue, 1.15 g.

Protease Digestion and Separation of Hot Water Extracted Polysaccharide——The extract (6.56 g) was dissolved in 100 ml of H_2O and adjusted to pH 7.8 with $NaHCO_3$. To the solution was added Pronase (300 mg), the mixture was kept standing at 37° for 4 days, and then dialyzed against distilled water for 3 days. The solution remaining in the Visking cellulose tubing was concentrated to a small volume under a reduced pressure and 10 volumes of EtOH containing 0.1% AcOK was added to this concentrate. The precipitate formed was collected by centrifugation, washed with EtOH, acetone and ether, and dried *in vacuo*. Yield, 4.17 g (20.8% of the cell wall). The polysaccharide was separated by a DEAE-cellulose column (OH^-) using H_2O, $NaHCO_3$, and NaOH as eluants. A solution of the crude polysaccharide (1.8 g) in H_2O (20 ml) was applied to the column (4.5 × 50 cm) and stepwise elution was made with H_2O, 0.01M, 0.05M, 0.1M $NaHCO_3$, and then with 1N NaOH. The flow rate was 100 ml/hr and 10 ml fractions were collected. An aliquot of each fraction (0.5 ml) was mixed with 1.5 ml of H_2O and 4 ml of 0.2% anthrone reagent, and the optical density was read at 625 nm. Each fraction was dialyzed in a Visking cellulose tubing against distilled water for 2 days. Internal solution of the tube was concentrated to a small volume *in vacuo* and 10 volumes of EtOH was added to the concentrate. Each precipitate was collected by centrifugation, washed with EtOH, acetone and ether, and dried *in vacuo*. Yield was as follows: H_2O eluate (fract. I), 0.9 g (50%); 0.01M $NaHCO_3$ eluate, 0.27 g (15%); 0.05M $NaHCO_3$ eluate, 0.21 g (11.1%); 0.1M $NaHCO_3$ eluate, 0.13 g (7.2%); 0.1N NaOH eluate, nil. Fraction I (0.9 g) in H_2O (10 ml) was further chromatographed over a column (3 × 50 cm) of DEAE-cellulose. Stepwise elution with H_2O, 0.01M, 0.05M, and 0.1M $Na_2B_4O_7$, and finally with 0.1N NaOH was carried out in the same way as for the crude polysaccharide. Yield was as follows: H_2O eluate (CF-1), 0.88 g (97.8%); 0.01M $Na_2B_4O_7$ eluate, 13 mg (1.4%); 0.05M and 0.1M $Na_2B_4O_7$ eluates, nil; 0.1N NaOH eluate, nil. Total (0.61 g) of each fraction of $NaHCO_3$ eluates was dissolved in H_2O (50 ml) and further chromatographed over a column (3 × 45 cm) of DEAE-cellulose. Stepwise elution with H_2O, 0.01M, 0.05M, and 0.1M $Na_2B_4O_7$, and finally with 0.1N NaOH was carried out in the same way as for fraction I. Yield was as follows: H_2O eluate (CF-2), 0.31 g (50.8%); 0.01M, $Na_2B_4O_7$ eluate (CF-3), 0.17 g (27.7%); 0.05M $Na_2B_4O_7$ eluate, trace; 0.1M $Na_2B_4O_7$ eluate, nil; 0.1N NaOH eluate, nil.

Both H_2O fraction CF-1 (0.88 g) and CF-2 (0.31 g) were dissolved in 50 ml of distilled water. After centrifugation, the supernatant was concentrated to a small volume under a reduced pressure at below 35°, and 10 volumes of EtOH containing 0.1% AcOK was added to this concentrate. The precipitate was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*. Yield: 1.10 g (P-1; 61.1% of the crude polysaccharide).

Purification of P-1 by Zone Electrophoresis——Zone electrophoresis of P-1 (100 mg) was carried out using glass powder as a supporting medium (1 × 10 × 40 cm), for 5 hr in 1% $Na_2B_4O_7$ under applied current

of 35 mA. After migration, the zone was cut into 40 segments, each segment was quantitatively extracted with a constant volume (10 ml) of distilled water and the sugar content was determined with the anthrone reagent. The migration pattern showed a single peak. Yield of the polysaccharide (PS-1) obtained from the peak was 99.4%. From this result the polysaccharide (PS-1) is homogeneous.

Component Sugar of Main Polysaccharide PS-1——PS-1 (20 mg) in 3 ml of 0.5N H_2SO_4 sealed in a tube was heated in a boiling water bath for 6 hr. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolyzate was concentrated and applied to the Whatman No. 1 filter paper for detection of component sugars. Paper chromatography was carried out by the ascending method, using AcOEt–pyridine–H_2O (10: 4: 3) (solvent system A). Sugars were detected on the paper chromatogram by spraying a solution of *p*-anisidine hydrochloride[15] and alkaline $AgNO_3$.[16] PS-1 showed the presence of only glucose.

Properties of PS-1——PS-1 consisted of 91.0% of total hexose (C_6H_5OH–H_2SO_4), and no phosphorus.[8] It showed no coloration with I_2, and showed $[\alpha]_D^{25}$ +166° (c=1.0, H_2O). It was electrophoretically pure (detected with the periodate-Schiff reagent.[17]) IR ν_{max}^{KBr} cm^{-1}: 844 (α-glycosidic linkage).

Periodate Oxidation of PS-1——PS-1 (20 mg) was oxidized with 50 ml of 0.018M $NaIO_4$ at room temperature in the dark. A blank solution containing no glycan was processed similarly. The consumption of $NaIO_4$ and the formation of HCOOH and HCHO were determined with an aliquot of this solution by the procedures of Malaprade,[18] Whistler,[19] and of O'Dea and Gibbons,[20] respectively.

The number of moles of $NaIO_4$ consumed per anhydrohexose unit of polysaccharide was as follows: 0.25 (1 hr), 0.69 (3 hr), 0.87 (6 hr), 0.88 (12 hr), 0.91 (24 hr), 0.92 (48 hr), 0.92 (72 hr), 0.93 (96 hr). The value of HCOOH: 0.02 (1 hr), 0.04 (3 hr), 0.04 (6 hr), 0.04 (12 hr), 0.04 (24 hr), 0.04 (48 hr), 0.04 (72 hr); the value for HCHO was 0.09 (2 hr), 0.09 (12 hr), 0.09 (24 hr), 0.09 (48 hr), 0.08 (72 hr), 0.07 (96 hr).

Smith-type Degradation of Periodate Oxidized PS-1——PS-1 (20 mg) was oxidiezed with $NaIO_4$ as described above, followed by Smith degradation. To destroy the excess periodate, ethylene glycol (0.4 ml) was added after 72 hr and the solution was dialyzed against running water for 24 hr. The internal solution was concentrated to about 20 ml and $NaBH_4$ (*ca.* 100 mg) was added to the concentrate with continuous stirring overnight and then the excess $NaBH_4$ was decomposed by acidification with AcOH. The mixture was dialyzed against distilled water for 2 days, concentrated to a syrup, and hydrolyzed with 1N H_2SO_4 (3 ml) in a boiling water bath for 6 hr. The hydrolyzate was neutralized with $BaCO_3$, $BaSO_4$ formed was removed by filtration, and the filtrate was concentrated to a small volume *in vacuo*. Examination by paper chromatography using the solvent system A showed three spots corresponding to glucose, erythritol, and glycerol. In order to estimate the relative molar ratio of the main products, the syrup was spotted on a filter paper and multiple developing was carried out 4 times with the solvent system A. After air drying, the corresponding area on the paper chromatogram were quantitatively extracted with distilled water and the extract was filtrerd through a sintered glass filter. Hexose was determined by the method of Dubois, *et al.*,[9] and glycerol and erythritol by the method of O'Dea and Gibbons.[20]

Methylation of PS-1——NaH (1.5 g) was mixed with 15 ml of Me_2SO and the mixture was heated at 60° for 1 hr with stirring. To this solution was added PS-1 (50 mg) in 5 ml of Me_2SO with stirring. After 5 hr, 0.4 ml of MeI was added dropwise to the reaction mixture with stirring at room temperature and the mixture was stirred for 12 hr. All the procedures were carried out under nitrogen atmosphere. The methylated product thus obtained was methylated again under the above condition. The partially methylated PS-1 thus obtained was dissolved in MeI (5 ml) and then stirred in a water bath of 40° for 12 hr with occasional addition of Ag_2O (50 mg). Methylated sugar was extracted with $CHCl_3$ from this reaction mixture which showed no significant OH bond in 3500 cm^{-1} region in its IR spectrum.

Acetylation of the Methylated PS-1——The methylated PS-1 (50 mg) was heated with 90% HCOOH in a boiling water bath for 4 hr. HCOOH was distilled off and the residue was further hydrolyzed with 0.5N H_2SO_4 for 4 hr in a boiling water bath. The reaction mixture was neutralized with $BaCO_3$, passed through an Amberlite IR-120 (H^+) column, and the clear filtrate was concentrated to a syrup. The syrup was reduced in water (20 ml) with $NaBH_4$ (*ca.* 100 mg) for 15 hr. After treatment with Amberlite IR-120 and concentration, boric acid was removed by codistillation with MeOH and the product was treated with Ac_2O–pyridine, (1: 1, 10 ml) at 100° for 1 hr. The acetylation mixture was either injected into the column or was first diluted with water, concentrated to dryness, and dissolved in acetone.

Methanolysis of Methylated PS-1——Methylated PS-1 was converted into methyl glucosides by heating with 0.7N MeOH–HCl (10 ml) in a sealed tube for 6 hr in a boiling water bath. MeOH was evaporated and HCl was removed by evaporation in a vacuum desiccator over $CaCl_2$ and KOH pellets.

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 (1958).
17) E. Köiwa and A. Grönwall, *Scand. J. Clin. Lab. Invest.*, **4**, 279 (1952).
18) L. Malaprade, *Bull. Soc. Chim. France*, **1**, 5, 833 (1934).
19) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
20) L.F. O'Dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).

Gas-liquid Partition Chromatography of Methanolyzate of the Methylated PS-1 and Acetyl-Alditol of the Methanolyzate——Gas–liquid chromatography of the methyl glucosides was carried out with a Shimadzu GC-5A unit, equipped with a flame ionization detector, using a 200×0.3 cm glass column packed with 15% polybutane-1,4-diol succinate on Celite 545 (60—80 mesh); column temperature 175°; N_2 flow rate, 50 ml/min.

Gas–liquid chromatography of acetyl alditol of the methanolyzate was carried out at the gas flow rate of 50 ml/min of N_2 on glass column (200×0.3 cm) containing 5% (w/w) ECNSS-M on Chromosorb W (aw-dmcs, 60—80 mesh), at 180°.

Paper Chromatography, Thin-layer Chromatography, and Paper Electrophoresis of Hydrolyzate of Methylated PS-1——The methylated PS-1 was heated with 90% HCOOH in a boiling water bath for 5 hr. HCOOH was distilled off and the residue was further hydrolyzed with 1N H_2SO_4 for 4 hr in a boiling water bath. The reaction mixture was neutralized with $BaCO_3$, filtered, and the clear filtrate was concentrated to a syrup. Paper chromatography of the hydrolyzate of methylated PS-1 was examined using AcOEt–AcOH–H_2O (9: 2: 2), and the three spots detected corresponded to tetra-*O*-methyl-, tri-*O*-methyl-, and di-*O*-methyl-monosaccharides. Thin–layer chromatography of the hydrolyzate of methylated PS-1 was examined using benzene–AcOH (1: 1) on a silica-gel plate, and the three spots detected corresponded to tetra-*O*-methyl-, tri-*O*-methyl-, and di-*O*-methyl-monosaccharides. Di-*O*-methyl monosaccharide was identified by paper electrophoresis using 1% borax solution as 3,6-di-*O*-methyl-D-glucose (MG value, 0.54). Reference di-*O*-methylglucose had MG values of 0.00 (2,4-), 0.135 (2,3), 0.28 (3,4), 0.564 (3,6), and 0.185 (4,6). MG value of the tri-*O*-methylmonosaccharide fraction showed 0.00. Therefore, this fraction was not 3,4,6-tri-*O*-methyl-glucose (MG value, 0.32).

PMR Studies on the Methylated PS-1——The PMR spectra were obtained with a Varian NV-14 spectrometer (60 MHz), ppm values were relative to tetramethylsilane. The PMR spectrum of the methylated PS-1 in $CDCl_3$ (*ca.* 50 mg/ml) exhibited signals at 5.55 ppm (H-1′), and distinct signals at 3.34, 3.40, 3.52, and 3.59 ppm for methoxyl groups.

Component Sugars of the Water-insoluble Fraction (PS-2 and PS-3) from the Alkali Extract——Each fraction (*ca.* 30—40 mg) in 5 ml of 2N HCl or 4N HCl sealed in a tube was heated in a boiling water bath for 10 hr. After neutralization (Ag_2CO_3) and filtration, a portion of the hydrolyzate was concentrated and applied to Whatman No. 1 filter paper for detection of component sugars. Paper chromatography was carried out by the ascending method, using solvent system A and AcOEt–pyridine–AcOH–H_2O (5: 5: 1: 3) (solvent system B). Sugars were detected on the paper chromatogram by spraying a solution of *p*-anisidine hydrochloride and alkaline $AgNO_3$. Component sugars of PS-2 showed the presence of glucose and galactosamine, and PS-3, glucose and glucosamine.

Examination of the Alkali-extracted Cell Wall Residue——The alkali-extracted residue was hydrolyzed with 2N HCl at 100° for 10 hr and then with 4N HCl for 10 hr in a boiling water bath. The paper chromatographic analysis of the hydrolyzates using the solvent system A and B revealed that it was consisting of a large amount of glucosamine and a small amount of glucose.