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An Extracellular Glucan of *Pythium debaryanum*.[1] (Studies on Fungal Polysaccharides. VII[2])

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Extracellular major polysaccharide of *P. debaryanum* is a glucan, $[\alpha]_D^{21}$ −28° (H_2O). Results of periodate oxidation, Smith-degradation, and methylation studies showed that the glucan has a highly branched structure possessing mainly (1→3)-linkage and a small amount of (1→6)-linkage.

Some species belonging to the genus *Pythium* are well known as phytopathogenic fungi, and in particular, *P. debaryanum* causes damping-off or root-rot of seedlings.[4] The genus *Pythium* (Pythiaceae, Oomycetes) is also an interesting group chemotaxonomically, because the major component of the mycelial cell wall of this genus is not chitin, which is the most commonly encountered component of fungal mycelial cell walls, but contains cellulose and non-cellulosic glucan.[5] Structural elucidation of extracellular polysaccharide of this fungus has not been carried out. It would be of interest to compare the chemical structure between intracellular and extracellular polysaccharides.

Crude polysaccharide isolated from the culture liquid was treated with pronase and by the Sevag method, followed by repeated DEAE-cellulose column chromatography using sodium hydrogencarbonate and then sodium borate for the elution. The major fraction was further purified by zone electrophoresis using glass powder and borate buffer.

The purified major polysaccharide thus obtained showed $[\alpha]_D^{21}$ −28° (c=1.02, water), and contained 0.85% of nitrogen, 0.17% of phosphorus, and 91.1% of total hexose (by phenol–sulfuric acid). Acid hydrolysis of the polysaccharide gave only D-glucose. There was no coloration with iodine. The absorbance at 890 cm^{-1} in the infrared (IR) spectrum of the polysaccharide suggests the presence of a β-glycosidic linkage.

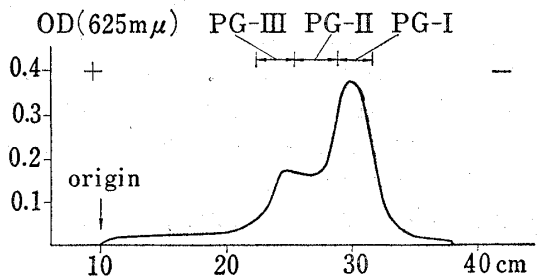

Fig. 1. Zone Electrophoresis of P-I

On periodate oxidation, the glucan consumed 0.66 mole of periodate per anhydroglucose unit, liberating 0.3 mole of formic acid and 0.06 mole of formaldehyde. When Smith–type degradation was carried out, the glucan afforded about 75% unoxidised glucose together with a small amount of glycerol derived from the oxidised glucose portion.

The glucan was methylated by the methods of Hakomori[6] and then of Purdie,[7] and the product was analyzed by gas-liquid chromatography. As shown in Fig. 2, methyl 2,3,4,6-tetra-

1) This work was presented at the 89th and 90th Annual Meetings of the Pharmaceutical Society of Japan, Nagoya, April 1969, and Sapporo, July 1970.
2) Part VI: T. Miyazaki and T. Yadomae, *Chem. Pharm. Bull.* (Tokyo), **17**, 361 (1969).
3) Location: *600 Kashiwagi 4-chome, Shinjuku-ku, Tokyo, 160, Japan.*
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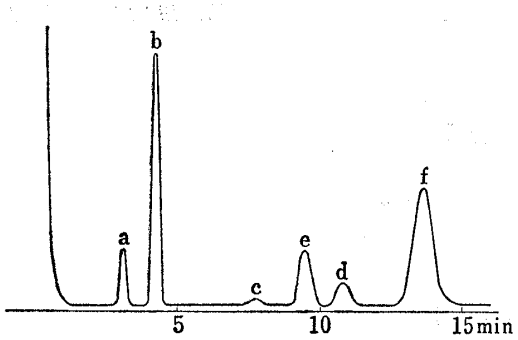

Fig. 2. Gas Chromatogram of Methanolysate of the Methylated Glucan

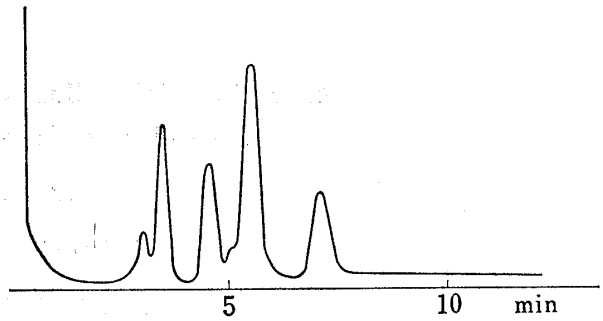

Fig. 3. Gas Chromatogram of Trimethylsilyl Derivatives of Methanolysate from the Methylated Glucan

TABLE I. Relative Retention Time of Methyl Glycosides

PG-1 Component	Authentic compds. with similar mobility O-Methyl-glucosides	
a : 0.99	2, 3, 4, 6-tetra-	β: 1.00
b : 1.38		α: 1.40
c : 2.53	2, 3, 4-tri-	β: 2.51
d : 3.48		α: 3.49
e : 3.12	2, 4, 6-tri-	β: 3.13
f : 4.45		α: 4.47

O-methyl-, methyl 2,3,4-tri-O-methyl-, and methyl 2,4,6-tri-O-methyl-D-glucosides were detected in the molar ratio of 10:3:18.

Di-O-methyl-D-glucose fraction was further analyzed by gas chromatography under a different condition, and the molar ratio was found to be almost equal to that of tetra-O-methyl-D-glucose fraction (Fig. 3) . The product from acid hydrolysis of the methylated glucan with formic acid and sulfuric acid was examined by paper chromatography and paper electrophoresis, and di-O-methyl-D-glucose was identified as 2,4-di-O-methyl-D-glucose.

From these results, it is concluded that the glucan has a highly branched structure possessing the following sugar units, and their glycosidic linkage may be in β-configuration.

$$\text{Glc}\underset{1}{-},\quad -\text{Glc}\underset{3}{-}\underset{1}{},\quad \text{Glc}\underset{1}{\overset{6}{-}},\quad -\text{Glc}\underset{3}{\overset{6}{-}}\underset{1}{}$$

A mannan was also isolated from the culture liquid as a minor polysaccharide. Detailed elucidation of the mannan and cell wall glucan will be made in near future.

Experimental

Isolation of Extracellular Crude Polysaccharide——The organism used in this study, *Pythium debaryanum* IFO-5919, was kindly supplied by the Institute of Fermentation, Osaka.

Incubation was carried out at 25° for 4 weeks in an Erlenmyer flask of 300 ml capacity containing 100 ml of Sabouraud medium which was prepared from 2% glucose and 1% dialyzable peptone. After the mycelium was removed by filtration, the culture fluid (10 liter) was dialyzed in a Visking Cellophane tubing against running water for 2 days to remove excess medium composition and low-molecular products.

The internal solution was concentrated to a small vloume under a reduced pressure below 35°. A precipitate formed on addition of EtOH to the concentrate was collected by centrifugation, washed with EtOH, acetone, and ether, and dried *in vacuo*.

Digestion of the Crude Polysaccharide with Pronase——About 30 g of the crude polysaccharide was dissolved in 600 ml of distilled water and adjusted to pH 7.5 with $NaHCO_3$. To the solution was added

pronase (1.5 g) to 5% of the content of the crude polysaccharide, incubated for 2 days at 35°, and then the mixture was dialyzed in the Visking Cellophane tubing against running water for 2 days. The solution in the tube was concentrated to 150 ml and shaken vigorously for 0.5 hr with 30 ml of $CHCl_3$–BuOH (4:1). The mixture was centrifuged and aqueous phase was collected. The same procedure was repeated until a gelatinous substances was no longer formed in the mixture. After centrifugation, the supernatant was concentrated to a small volume under a reduced pressure at below 35°, and 4 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*. The same procedure was repeated 3 times. Yield, 0.1 g from 1 liter of cultivated fluid.

Fractionation of the Crude Polysaccharide——The crude polysaccharide (1 g) was applied to a DEAE-cellulose column (3.5×50 cm). Stepwise elution was carried out with H_2O, 0.1 M $NaHCO_3$, and 0.1 M NaOH, at the flow rate of 100 ml/hr. Each fraction (0.1 ml) was added with 2.0 ml of H_2O and 4 ml of 0.2% anthrone reagent, and the optical density was read at 625 mμ on colorimeter. Each fraction was dialyzed in a Visking Cellophane tubing against distilled water for 3 days. The internal solution of the tubes was concentrated to a small volume *in vacuo* and 10 volumes of EtOH was added to the concentrate. The precipitate formed was collected by centrifugation, washed with EtOH, acetone, and ethere, and then dried *in vacuo*. Yield was as follows: H_2O eluate (fract. 1), 480 mg (48%); 0.1 M $NaHCO_3$ eluate (fract. 2), 50 mg (5%); 0.1 M NaOH eluate (fract. 3), 4 mg (0.4%).

Fract. 1 (480 mg) in H_2O (3 ml) was further submitted to a column (2.2×45 cm) of DEAE-cellulose. Stepwise elution with H_2O, 0.01 M, 0.05 M, and 0.1 M $Na_2B_4O_7$, and fiinally with 0.01 M NaOH was carried out in the same way as that of the crude polysaccharide. Yields were as follows: H_2O eluate (P-1) 173 mg (36%); 0.01 M $Na_2B_4O_7$ eluate (P-2), 86 mg (18%); 0.05 M $Na_2B_4O_7$ eluate (P-3), trace; 0.1 M $Na_2B_4O_7$ eluate (P-4), trace; 0.01 M NaOH eluate, nil.

Purification of P-1 by Zone Electrophoresis——Zone electrophoresis of P-1 (50 mg) was carried out using glass powder as a suporting medium (1×7×40 cm), for 8.5 hr in 0.8% $Na_2B_4O_7$ under as 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 suger constant was determined with anthrone reagent. The result is shown in Fig. 1. Yield of the main peak (PG-1) was about 60% and the minor peaks were named PG-2 and PG-3.

Component Sugar of Each Fraction——Each fraction (*ca.* 5-10 mg) in 2ml of 2N H_2SO_4 sealed in a tube was heated in a boiling water bath for 7 hr. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolysate was concentrated and applied to Toyo Roshi No. 50 filter paper for detection of componental 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[8] and alkaline $AgNO_3$.[9] PG-1 showed the presence of glucose alone, but PG-2 and PG-3 had glucose and mannose in different ratios.

Properties of PG-1——PG-1 consisted of 99.1% of total hexose (PhOH–H_2SO_4), and contained 0.17% of phosphorus[10] and 0.85% of nitrogen. It showed no coloration with I_2, and showed $[\alpha]_D^{21}$ −28.0° (c=1.02, H_2O). IR ν_{max}^{KBr} cm^{-1}: 890 (β-glycosidic linkage).

Periodate Oxidation of PG-1——PG-1 (15 mg) was oxidized in 25 ml of 0.017 M $NaIO_4$ at room temperature in the dark. A blank solution containing no glucan was processed similarly. An aliquot of 3 ml was taken at different periods for the determination of $NaIO_4$ consumption and formation of HCOOH and HCHO by the procedures of Maraprade,[11] Whilstler,[12] and of O'Dea and Gibbons,[13] respectively.

The number of moles of $NaIO_4$ consumed per anhydroglucose unit of polysaccharide was as follows: 0.33 (1 hr), 0.54 (3 hr), 0.64 (6 hr, 12 hr), 0.69 (24 hr), 0.71 (48 hr), 0.72 (72 hr). The value of HCOOH: 0.13 (1 hr), 0.23 (3 hr), 0.29 (6 hr), 0.31 (24 hr), 0.30 (48 hr), 0.32 (72 hr); value for HCHO was 0.06 (1 hr), 0.06 (6 hr), 0.06 (24 hr), 0.05 (72 hr).

Smith-type Degration of Periodate Oxidised PG-1——After oxidation of PG-1 as described above, excess $NaIO_4$ was decomposed by the addition of 0.5 ml of ethylene glycol. The reaction mixture was dialyzed in a Visking Cellophane tubing against distilled water with stirring for 15 hr, the internal solution was concentrated to a small volume *in vacuo*, and the resulting polyaldehyde was reduced by stirring with 10 mg of $NaBH_4$ at room temperature for 17 hr. Excess of $NaBH_4$ was decomposed with AcOH. The reaction mixture was dialyzed in a Visking Cellophane tubing against distilled water, the internal solution was evaporated to dryness, and the residue was heated with 0.1 N H_2SO_4 (2 ml) in a boiling water bath for 4 hr. The hydrolysate was neutralized with $BaCO_3$, $BaSO_4$ formed was removed by filtration, and the filtrate was

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concentrated to a small volume *in vacuo* to be examined by paper chromatography using the solvent A. Two spots corresponding to standard glycerol and glucose were detected as the main products on the paper chromatogram. 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 solvent system A. After air drying, the corresponding area on the paper chromatogram were quantitatively extracted volume of distilled water and the extract was filtrered through a sintered glass filter.

Glucose was determined by the method of Dubois, *et al.*,[14] and glycerol and erythritol by the method of O'Dea and Gibbons.[13]

Methylation of PG-1 A solution (5 ml) of $MeSOCH_2Na^+$ in Me_2SO was added to the solution containing 15 mg of PG-1 (5 ml). The reaction mixture was incubated with continuous stirring for 15 hr at room temperature and then 1 ml of MeI was added. Stirring was continued for 1 hr and a clear solution was obtained. This solution was dialyzed for 43 hr against distilled water and then lyophilized. The same procedure was repeated. The partially methylated PG-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 the 3500 cm^{-1} region in its IR spectrum.

Gas-Liquid Partition Chromatography of Methanolysate of Methylated PG-1 and Trimethylsilyl Derivatives of the Methanolysate——Methylated PG-1 was converted into methyl glucosides by heating with 1N MeOH–HCl (2 ml) in a sealed tube for 10 hr in a boiling water bath. Methanol was evaporated and HCl was removed by evaporation in a vacuum desicator over $CaCl_2$.

The resulting methyl glucosides were dissolved in a minimum amount of MeOH and the solution was used for gas–liquid partition chromatography. After removal of methanol and HCl, the methanolysate was trimethylsilylated according to Sweeley, *et al.*[15] Gas-liquid partition chromatography of the methyl glucosides and trimethylsilylated derivatives of methyl glucosides was effected in a Simadzu DC-1C unit, equipped with a flame ionization detector, using a 175×0.5 cm glass column packed with 15% polybutane-1,4-diol succinate on Chromosorb W-80 (100 mesh); column temperature 165°; N_2 flow rate, 55 ml/min, and column temperature, 170°; N_2 flow rate, 50 min/ml.

Paper Chromatography and Paper Electrophoresis of Hydrolysate of Methylated PG-1——The methylated PG-1 was heated with 85% 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 5 hr in a boiling water bath. The reaction mixture was neutralized with $BaCO_3$, filtered, and the clear filtrate was cncentrated to a syrup.

Paper chromatography of the hydrolysate of methylated glucan was examined using AcOEt–AcOH–H_2O (9:2:2), and the three spots detected corresponded to tetra-O-methylglucose, tri-O-methylglucose, and di-O-methylgulcose. Di-O-methyl-D-glucose was identified by paper electrophoresis using 0.026 M borate buffer as 2,4-di-O-methyl-D-glucose (M_G; 0.0). Reference[16] di-O-methylglucose had M_G 0.0 (2,4-), 0.135 (2,3-), 0.28 (3,4-), 0.546 (3,6-), and 0.185 (4,6-).

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