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Studies on Fungal Polysaccharides. VIII.[1] Extracellular Heteroglycans of *Rhizopus nigricans*[2]

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Main extracellular polysaccharide of *R. nigricans*, $[\alpha]_D+68.1°$, is a highly heterogenous glycan composed of fucose, mannose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine in a relative ratio of 1:22:4:5:3.8:3.8. The results of periodate oxidation, Smith degradation, and partial acid hydrolysis indicate that the polysaccharide contains (1→2) and (1→4)-linked hexopyranose residues as the main linkages.

The minor polysaccharide, $[\alpha]_D-45.6°$, is an acidic heteroglycan consisting of fucose, galactose, and glucuronic acid, and its chemical structure is discussed.

Extracellular polysaccharides of the fungi belonging to Mucorales have not been examined in detail. Relationship between taxonomy and chemical structure of extra- or intra-cellular polysaccharides is an interesting problem in connection with diagnosis of Mucormycosis.

Previously, Martin, *et al.*[4] reported that *Rhizopus oryzae, R. tamari*, and *R. tokiensis* produced a substance containing glucuronic acid and fucose, in addition to, galactose, glucose, and mannose. As the first step in our investigation, extracellular polysaccharides of *R. nigricans* IAM6070, a currently fermentative fungus, were examined.

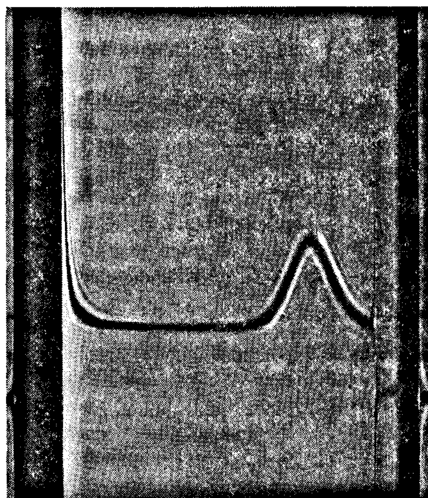

Fig. 1. Ultracentrifugal Pattern of PI

1% aqueous solution, $\mu=1.0$, 144 min
Spinco Model-E Ultracentrifuge (59780 rpm)

The crude polysaccharide isolated from the culture liquid was treated with pronase and by the Sevag method, followed by DEAE-cellulose column chromatography using sodium hydrogen carbonate for the elution and then rechromatography in a borate-form. The main fraction (PI), $[\alpha]_D+68.1°$ ($c=0.66$, H_2O), gave a single spot on a paper electrophoresis using borate buffer (0.026 M, pH 10.0), contained 1.4% of nitrogen, 74.9% of total hexose (by the procedure of Dubois, *et al.*[5]), 18.0% of total hexosamine (by the method of Blix,[6] as glucosamine), and no phosphorus. PI was found to be pure by the ultracentrigufal analysis (Fig. 1). The infrared (IR) spectrum of PI showed absorption bands due to α-configuration at 810, 877, and 970 cm^{-1} with acetamido group at 1640 and 1550 cm^{-1}.

The component sugars of PI were identified as D-mannose, D-galactose, D-glucose, L-fucose, D-glucosamine, and D-galactosamine by paper chro-

1) Part VII: T. Miyazaki and M. Yamada, *Chem. Pharm. Bull.* (Tolyo), **19**, 813 (1971).
2) A part of the work was presented at the 87th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 1967.
3) Location: *20-1, Kitashinjuku 3-chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) S.M. Martin and G.A. Adams, *Can. J. Microbiol.*, **2**, 715 (1956).
5) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
6) G. Blix, *Acta Chem. Scand.*, **2**, 467 (1948).

matography of the acid hydrolysate, and the molar ratio was estimated approximately as 22:5:4:1:3.8:3.8 by the methods of Dubois, *et al.*[5] (for hexoses), Dische[7] (for methylpentose), Blix[6] (for total hexosamine), and Ludowieg[8] (for galactosamine). Estimation of the reducing power of the material by the Park–Johnson method[9] revealed the ratio of the reducing end group to the monosaccharide unit of 1:0.007, calculated as fucose.

On periodate oxidation of PI, consumption of periodate from anhydro component sugar unit was 1.03 moles, the values of formic acid and acetaldehyde liberated from the unit were 0.32 and 0.01 mole. Formaldehyde was not produced. The periodate-oxidized PI was treated by the Smith procedure.[10] Paper chromatographic analysis of the hydrolysate revealed the presence of a small amount of the componental sugars in addition to a large amount of glycerol and tetrahydric alcohol. The molar ratio, approximately in 1.0:0.7:0.5:0.9:2.5:21.7:6.2 (Man, Gal, Glc, Fuc, HexN, Gly, and tetrahydric alcohol), was estimated by the procedures of Dubois,[5] Blix,[6] Dische,[7] and O'dea & Gibbons[11] (for polyhydric alcohols).

Acid hydrolysis with 0.01 N sulfuric acid for 1 hr at 90° liberated only a trace of fucose. Further hydrolysis with 0.1 N sulfuric acid for 3 hr at 90° liberated fucose, mannose, glucose, galactose, and *N*-acetylhexosamine in an approximate ratio of 1:1:1:2:0.23. Non-dialyzable fraction from this treatment (component sugars: Man, Glc, Gal, and HexN, molar ratio: 4.1:0.8:1.0:1.3) was further hydrolyzed with 0.5 N sulfuric acid for 3 hr at 100°. As dialyzable fragments of the hydrolysate, mannose, glucose, galactose, hexosamine, N-acetylhexosamine (approx. molar ratio: 8:3:2:0.36:1.2), and few oligosaccharides were detected. The methanol-insoluble fraction from the dialyzable fragments, FI, was electrophoretically pure, $[\alpha]_D+14.3°$ (c=0.57, H_2O), and consisted of mannose, glucose, galactose, and N-acetylhexosamine (molar ratio: 8:1:2:0.5), and its IR spectrum showed the presence of an acetamido group. On the periodate oxidation, FI consumed about 1.30 moles of periodate and the values of formic acid and formaldehyde were 0.23 and 0.04 moles, respectively. Smith degradation of the fragment gave glycerol, tetrahydric alcohol, and no hexose. The molar ratio was 8:3 (Gly, tetrahydric alcohol). During this treatment, hexosamine portions were not examined.

From these results, it is concluded that (1) the main extracellular polysaccharide, PI, is a highly heterogenous glycan composed of fucose, mannose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine, (2) all of fucose and a small amount of other componental sugars are liberated by mild acid hydrolysis (0.1 N sulfuric acid, 90°, 3 hr), (3) highly heterogenous fragment, FI, $[\alpha]_D+14.3°$, which is composed of the same componental sugars without fucose and has a similar molar ratio to PI except hexosamine, was isolated by partial acid hydrolysis and, therefore, it suggests that each component sugar in PI would be intricate, (4) on the periodate oxidation, a large portion of mannose and about 2/3 of hexosamine are oxidized but not fucose, (5) as the main linkage in PI, (1→2) and (1→4)-linked hexopyranose residues would exist because a large quantity of glycerol and tetrahydric alcohol are formed by the Smith degradation, and (6) majority of fucose and a small portion of all other componental sugars would exist as (1→3)-linkage or branching point because they are not oxidized by periodate.

On the other hand, minor polysaccharide, PII, $[\alpha]_D-45.6°$ (c=0.77, H_2O), was obtained from the 0.5 M sodium hydrogen carbonate-eluted fraction and was electrophoretically pure. Componental sugars of PII were L-fucose, D-galactose, and D-glucuronic acid (molar ratio: 2.1:1.0:3.5). On periodate oxidation of PII, the consumption of periodate was about 1.0 mole and the values of formic acid and formaldehyde were 0.026 and 0.04 mole, respectively. Acet-

7) Z. Dische and L.B. Shettles, *J. Biol. Chem.*, **172**, 515 (1948).
8) J. Ludowieg and J.D. Benmaman, *Anal. Biochem.*, **19**, 80 (1967).
9) J.T. Park and M.J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).
10) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **73**, 5907, 5910 (1956).
11) J.F. O'dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).

aldehyde was not produced. The Smith degradation of PII gave a large amount of glycerol and unoxidized three componental sugars (molar ratio: Fuc, Gal, GlcA, Gly, 3:1:5:6.8).

Thus, the minor extracellular polysaccharide, PII, was an acidic heteroglycan which is composed of fucose, galactose, and glucuronic acid. From the result of the Smith degradation, it is considered that PII contains glycerol-producible linkage such as (1→2)-linked galactopyranose and (1→3)-linked sugar residues. Low value of formic acid production on periodate oxidation suggests that PII has a non-branching structure. Further structural elucidation will be discussed in a later communication.

Experimental

Isolation of the Extracelluar Polysaccharide——Large batches of *Rhizopus nigricans* IAM6070, were grown in Sabouraud medium (dialyzable polypeptone 1%, glucose 4%) at 25±2° in an incubator for 14 days. In order to obtain a filamentous form, the culture flasks were shanken once a day. The medium was separated from the mycelium by filtration through a Nylon cloth. The filtrate was dialyzed in a Visking Cellophan tubing against running water for 2 days. The internal solution was concentrated to a small volume and 3 volumes of EtOH was added to the concentrate. The precipitate was collected by centrifugation, washed with EtOH, acetone and ether and dried *in vacuo* (yield, 0.7 g/liter).

Protease Digestion of the Crude Polysaccharide——Crude material (10.0 g) was dissolved in 500 ml of H_2O, and adjusted to pH 7.8 with $NaHCO_3$. To the solution was added pronase (1.0 g), the mixture was kept standing at 37° for 4 days, and then dialyzed against distilled water for 2 days. The solution remaining in the Visking Cellophane tubing was concentrated to about 200 ml and shaken with 0.2 volume of $CHCl_3$–BuOH (4:1) for a 0.5 hr. The same procedure was repeated until gelationous substances no longer formed in the mixture. After centrifugation, the supernatant was concentrated to a small volume under a reduced pressure and 4 volumes of EtOH containing 0.1% AcOK was added to this concentrate. The greysh-brown precipitate was collected by centrifugation and further digested with pronase, followed by the method of Sevag as described above.

Separation and Purification by DEAE-Cellulose Column Chromatography——The separation of the polysaccharide was achieved on a DEAE-cellulose column using H_2O, $NaHCO_3$, and NaOH. A solution of the polysacchride (480 mg) in water (10 ml) was applied to the column (3×33 cm) and stepwise elution was effected with H_2O, and 0.02M, 0.05M, 0.1M, 0.2M, and 0.5M $NaHCO_3$, and then with 0.1M NaOH. The rate of flow through the column was 100 ml/hr and 10 ml fractions were collected by an automatic fraction collector. An aliquot of each fraction (0.5 ml) was mixed with 1.5 ml of H_2O followed by 4 ml of 0.2% anthrone reagent and the optical density was read at 625 mμ on a colorimeter. Each fraction was dialyzed in a Visking Cellophane tubing against distilled water for 2 days. Internal solution of the tubes was concentrated to a small volume *in vacuo* and 4 voumes of EtOH was added to these concentrates. Each precipitate was collected by centrifugation, washed with EtOH, acetone and ether and dried *in vacuo*. Yields were as follows: H_2O eluate, 169.0 mg (35.4%); 0.02M $NaHCO_3$ eluate, 15.2 mg (5.0%); 0.05M and 0.1M $NaHCO_3$ eluate, trace; 0.2M $NaHCO_3$ eluate, 48.0 mg (10.0%); 0.5M $NaHCO_3$ eluate, 61.5 mg (12.8%); and 0.1 M NaOH eluate, 10.3 mg (3.3%).

The water-eluted polysaccharide (164.4 mg) in H_2O (5 ml) was applied on the column (1.5×30 cm) of DEAE-cellulose (borate form). Stepwise elution was effected with H_2O, and 0.01 M, 0.02 M, 0.05 M, and 0.1 M $Na_2B_4O_7$ and then with 0.1 M NaOH. Each fraction was collected as descrived above (except the borate-eluted fractions which were acidified with AcOH to decompose borate complex and dialyzed against distilled water until free of borate ions). Yields were as follows: H_2O eluate, 126.0 mg (78.0%); 0.01 M $Na_2B_4O_7$ eluate, 6.8 mg (4.2%); 0.02 M $Na_2B_4O_7$ eluate, trace; 0.05 M $Na_2B_4O_7$ eluate, 6.8 mg (4.2%); 0.1 M $Na_2B_4O_7$ eluate, 6.9 mg (4.2%); and 0.1 M NaOH eluate, 3.0 mg (1.8%). The water-eluted polysaccharide (126.6 mg), corresponding to 27.3% of the crude polysaccharide, was dissolved in H_2O (1 ml) and centrifuged at 4000 rpm for 10 min. The supernatant was mixed with 4 volumes of EtOH. The white Precipitate (PI) thereby formed was centrifuged, washed with acetone and ether, and dried *in vacuo*. Yield, 117.3 mg.

The 0.5 M $NaHCO_3$-eluted polysaccharide (160.3 mg) in H_2O (5 ml) was applied on the column (1.5×30 cm) of DEAE-cellulose (borate form) and treated as above. Yields were as follows: H_2O eluate, 86.7 mg (54.1%); 0.01 M $Na_2B_4O_7$ eluate, 10.8 mg (6.7%), and other fractions were nil. The water-eluted polysaccharide (86.7 mg), corresponding to 6.7% of the crude polysaccharide, was dissolved in H_2O (0.7 ml), centrifuged at 4000 rpm for 10 min, and the supernatant was mixed with 4 volumes of EtOH. The yellowish-brown precipitate (PII) so formed was centrifuged, washed with acetone and ether, and dried *in vacuo*. Yield, 75.0 mg.

Properties of PI and PII——Paper electrophoresis of PI and PII using 0.026 M borate buffer (pH 10.0) showed a single spot (detected with the periodate-Schiff's reagent.[12]). PI was ultracentrifugally pure

12) E. Köiw and A. Grönwall, *Scand. J. Clin. Lab. Invest.*, 4, 279 (1952).

($S_{20,w}$=2.4), $[\alpha]_D+68.1°$ (c=0.66, H_2O), contained 1.4% of nitrogen, 75% of hexose (as mannose, by the $C_6H_5OH-H_2SO_4$ method[5]), and 18.5% of hexosamine (as glucosamine, by the Blix method[6]). Reducing power of PI was 1:0.007 (as fucose, by the Park–Johnson method[9]).

PII, $[\alpha]_D-45.6°$ (c=0.77, H_2O), contained 88% of total sugar (by the $C_6H_5OH-H_2SO_4$ method[5]).

Componental Sugars of PI and PII——PI and PII (*ca.* 5 mg in 1 ml of 1 N H_2SO_4) sealed in a tube were heated at 100° for 8 hr. The hydrolysate was neutralized with $BaCO_3$, filtered, and concentrated to a syrup. A portion of the concentrate was examined by the ascending method of paper chromatography on Toyo Roshi No. 50 filter paper using the following solvent systems (v/v): (1) EtOAc–pyridine–H_2O (10:4:3), (2) BuOH–AcOH–H_2O (5:1:2), (3) EtOAc–pyridine–AcOH–H_2O (5:5:1:3), (4) BuOH–pyridine–H_2O (6:4:3). Sugars were detected by the spray reagents of $AgNO_3$–NaOH,[13] *p*-anisidine–HCl,[14] *p*-dimethylaminobenzaldehyde,[15] and ninhydrin.[16]

Paper chromatographic analysis of the hydrolysate of PI revealed the presence of fucose, mannose, glucose, galactose, glucosamine, and galactosamine. In the case of PII, fucose, galactose, and glucuronic acid, in addition to traces of mannose and glucose, were detected.

Quantitative Estimation of the Component Sugars of PI and PII——Portions of the hydrolysates were spoted on filter papers (Toyo Roshi No. 50) and developed with the solvent system (1) for 10 hr. Developing procedure was repeated 4 times. After air-drying, the areas containing sugars on the paper chromatogram were cut out and quantitatively extracted with constant volume of H_2O, which was filtered through a sintered glass filter. The componental sugars were estimated by the methods of Dubois, *et al.*[5] (for hexoses), Dische[7] (for methylpentose), Blix[6] (for hexosamines), and Bitter–Muir[17] (for uronic acid).

Periodate Oxidation of PI and PII——1) PI (14.9 mg) was dissolved in H_2O (10 ml) and its total volume was made up to 25 ml with 2 ml of 0.22 M $NaIO_4$ and distilled water. The mixture was allowed to stand in the dark at room temperature. The consumption of $NaIO_4$ and the formation of HCOOH, HCHO, and CH_3CHO were determined with this solution by the procedures of Maraprade,[18] Whilstler,[19] O'dea & Gibbons,[11] and Annison.[20] The number of moles of $NaIO_4$ consumed per anhydro sugar unit of PI was as follows: 0.71 (1 hr), 0.74 (3 hr), 0.78 (6 hr), 0.92 (12 hr), 0.95 (24 hr), 1.00 (48 hr), and 1.03 (72 hr). The value of formic acid was 0.10 (1 hr), 0.11 (3 hr), 0.12 (6 hr), 0.20 (12 hr), 0.22 (24 hr), 0.28 (48 hr), and 0.32 (72 hr), and the value of acetaldehyde was 0.003 (2 hr), 0.004 (49 hr) and 0.011 (74 hr). Formaldehyde was not produced.

2) The periodate oxidation of PII (13.1 mg) was examined as described above. Consumption of $NaIO_4$: 0.44 (1 hr), 0.54 (3 hr), 0.72 (6 hr), 0.82 (12 hr), 0.88 (24 hr), and 1.06 (48 hr). The values of HCOOH and HCHO: 0.011 (12 hr), 0.018 (24 hr), and 0.026 (48 hr), and 0.032 (12 hr), and 0.04 (48 hr). No acetaldehyde was produced.

Smith Type Degradations of PI and PII——1) PI (9.9 mg) was oxidized with $NaIO_4$ as described above. To destroy the excess periodate, ethylene glycol (0.2 ml) was added after 48 hr and the solution was dialyzed against running water for 24 hr. To the non-dialyzable solution concentrated to a small volume was added $NaBH_4$ (*ca.* 15 mg) with continous stirring overnight and then the excess $NaBH_4$ was decomposed by acidification with AcOH. The reaction mixture was dialyzed against distilled water for 2 days, concentrated to a syrup, and followed by hydrolysis with 0.2 N H_2SO_4 (1 ml) in a boiling water bath for 6 hr. The solution was neutralized with $BaCO_3$, filtered, and concentrated to a syrup. Paper chromatographic analysis of the hydrolysate using the solvent system (1) showed six spots corresponding to glycerol, erythritol, fucose (and threitol), mannose, glucose, and galactose. In order to estimate relative molar ratio of these main products, the syrup was spotted on a filter paper and multiple development was carried out 5 times with the solvent system (1). After air-drying, areas corresponding to the spots on the paper chromatogram were quantitatively extracted with constant volume of distilled water, which was filtered through a sintered glass filter. Hexoses, methylpentose, and polyhydric alcohols were determined by the methods of Dubois, *et al.*,[5] Dische,[7] and O'dea & Gibbons.[11] Content of the products of Smith type degradation was as follows (in μg): Fuc (22.5), Man (27), Glc (12.8), Gal (19.5), Gly (299), and tetrahydric alcohol (113).

2) PII (7.0 mg) was oxidized with $NaIO_4$ for 36 hr. Subsequent procedure was the same as described above. Paper chromatographic analysis of the hydrolysate using the solvent system (1) revealed four spots corresponding to glycerol, fucose, galactose, and glucuronic acid. Content of these products was as follows (in μg): Fuc (55), Gal (20), Glc A (105), and Gly (70).

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18) L. Maraprade, *Bull. Soc. Chim. France*, **1**, 833 (1934).
19) R.L. Whilstler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
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Gradual Acid Hydrolysis of PI——PI (53.0 mg) was heated with 0.01 N H_2SO_4 at 90° for 1 hr and followed by dialysis, and then the external solution (1600 ml) of the hydrolysate was evaporated to about 20 ml *in vacuo*. After neutralization with $BaCO_3$ and filtration, the filtrate was concentrated to a syrup. Paper chromatographic analysis of the syrup using the solvent system (1) showed a trace of fucose. The internal solution was concentrated to dryness, which was heated with 0.1 N H_2SO_4 (10 ml) at 90° for 3 hr. The dialyzable fragments from the hydrolysate, which was treated with the same procedure as described above, gave four components, fucose, mannose, glucose, and galactose (molar ratio, 1:1:1:2). A part of the non-dialyzable material was hydrolyzed with 1 N H_2SO_4 at 100° for 8 hr, and mannose, glucose, galactose, and hexosamine were detected in a molar ratio of 4.14:0.78:1.0:1.3. Other part of the non-dialyzable material was treated with 0.5 N H_2SO_4 (10 ml) in a boiling water bath for 3 hr and then dialyzed. The external solution was concentrated to a small volume, neutralized with $BaCO_3$, and filtered. The filtrate (free from barium ions by Amberlite IR-120 (H^+)) was evaporated to a syrup and 10 volumes of MeOH was added to the syrup. The precipitate (FI) thereby formed was washed 7 times with MeOH (8 ml) and the supernatant of MeOH-soluble fragments was examined by paper chromatography using solvent systems (1) and (4). Presence of mannose, glucose, galactose, glucosamine, and galactosamine was revealed in addition to N-acethylglucosamine and N-acethylgalactosamine.[15] The molar ratio of mannose, glucose, galactose, hexosamine, and N-acethylhexosamine (by the Reissing method[21]) was approximately 8:3:2:0.36:1.2.

Characterisation of FI——The MeOH–insoluble fragment (31.6 mg) was electrophoretically pure and had $[\alpha]_D + 14.3°$ (c=0.57, H_2O), IR ν_{max}^{KBr} cm^{-1}: 1640, 1550 (acetamido group). Content of hexosamine was 3.4% (as glucosamine, by the Blix method[6]). After hydrolysis of the material with 1 N H_2SO_4 at 100° for 5 hr, paper chromatographic examination of the hydrolysate showed the presence of mannose, glucose, galactose, and hexosamine in a molar ratio of 8:1:2:0.5. FI (14.3 mg) was oxidized with periodate as described above. The number of moles of $NaIO_4$ consumed per anhydro sugar unit of the material was as follows: 0.90 (1 hr), 1.05 (3 hr), 1.30 (6 hr), and 1.30 (24 hr). The values of formic acid and formaldehyde were 0.23 and 0.03 (3 hr), 0.23 and 0.04 (12 hr), and 0.23 and 0.04 (24 hr), respectively. After 24 hr, the reaction mixture was passed through columns of Amberlite IRA-400 (OH^-) and Amberlite IR-120 (H^+). The eluate was concentrated to an adequate volume at below 30° *in vacuo*. To this concentrate was added $NaBH_4$ (10 mg) and the mixture was stirred overnight. The excess of $NaBH_4$ was decomposed with a few drops of AcOH and the solution was passed through columns of Amberlite IRA-400 (OH^-) and IR-120 (H^+). The eluate was evaporated to dryness *in vacuo* and the residue was hydrolyzed with 0.1 N H_2SO_4 at 100° for 6 hr, as described above. Paper chromatographic analysis revealed two spots corresponding to glycerol and tetrahydric alcohol, and no hexoses. Content of Gly was 23.0 μg and of tetrahydric alcohol 11.0 μg, showing the relative ratio of 8:3.

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21) J.L. Reissig, J.L. Strominger, and L.F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).