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Studies on Fungal Polysaccharides. IX.[1] The Acidic Polysaccharide from the Cell Wall of *Rhizopus nigricans*[2]

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Main polysaccharide from the cell wall of *Rhizopus nigricans*, $[\alpha]_D$ −70.5°, is a highly heterogenous glycan, composed of fucose, mannose, galactose, and glucuronic acid in an approximate relative ratio of 11 : 1 : 3 : 10. The results of periodate oxidation, Smith degradation, and partial hydrolysis indicate that the chemical structure of the polysaccharide is in a form oxidizable with periodate and greatly labile to mineral acid.

The alkali-soluble but water-insoluble fragment of the cell wall contains uronic acid, glucosamine, and amino acids with small amounts of fucose, mannose, and galactose.

Increasing interest is being shown in the cell wall of fungi and, in particular, chemistry of the polysaccharide of the cell wall has been studied by several groups of workers in recent years. Preliminary evidence for the presence of acidic polysaccharides in the cell wall of various fungi has only lately been recognized. In 1968, Bartnicki-Garcia[4] demonstrated that D-glucuronic acid polymers, which were separated into two distinct categories by their susceptibility to acid hydrolysis, may comprise a major portion of the cell wall of *Mucor rouxii*.

As a part of studies on fungal polysaccharides, we have examined the relationship between taxonomy and chemical structure of polysaccharides in the cell wall of Mucorales, especially of *Absidia cylindrospora* (*A. cylindrospora*). *Mucor mucedo*, (*M. mucedo*), and *Rhizopus nigricans* (*R. nigricans*). Previous communication[5] reported the preparation of acidic polysaccharides from the cell walls of *A. cylindrospora*, *M. mucedo*, and *R. nigricans* and the present paper describes the separation and characterisation of a polysaccharide from the cell wall of *R. nigricans*.

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 sediments were microscopically free from cytoplasmic materials. Nitrogen and phosphorus contents in the cell wall were 4.1% (by the micro-Dumas analysis) and 1.9% (by the method of Fiske–Subbarow[6]), respectively. Five consecutive extractions were carried out from the purified cell wall according to following procedures.

The defatted cell wall treated with ethanol–ether (1:1) was extracted with 10% trichloroacetic acid (TCA) at 4° in accordance with the procedure of Baddiley, *et al.*[7] After centrifugation, the supernatant was neutralized and then dialyzed against running water. Non-dialyzable material precipitated with ethanol contained 0.14% of phosphorus, and neither glycerol nor ribitol was detected in the acid hydrolysate. This experiment was performed in

1) Part VIII: T. Miyazaki and T. Irino, *Chem. Pharm. Bull.* (Tokyo), **19**, 1450 (1971).
2) A part of this work was presented at the 39th Annual Meeting of the Japanese Biochemical Society, Sakai, November 1967.
3) Location: *20-1, Kitashinjuku 3-chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) S. Bartnicki-Garcia and E. Reyes, *Biochim. Biophys. Acta*, **170**, 54 (1968); S. Bartnicki-Garcia and W.J. Nickerson, *Biochim. Biophys. Acta*, **108**, 32 (1962).
5) T. Miyazaki and T. Irino, *Chem. Pharm. Bull.* (Tokyo), **18**, 1930 (1970).
6) C.H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).
7) J.J. Armstrong, J. Baddiley, J.G. Buchanan, B. Carss, and G.R. Greenberg, *J. Chem. Soc.*, **1958**, 4344.

order to determine whether teichoic acid or similar substance, a well-known bacterial cell wall component, is present or not in the cell wall.

The residue was stirred with 30% potassium chloride. Extraction of this residue with 1N sodium hydroxide at a room temperature gave crude polysaccharides in approximate yield of 12% of the cell wall. The alkali-extracted materials were treated with pronase and then by the Sevag method, followed by DEAE-cellulose column chromatography.

The main fraction (PI) eluted with 0.5M sodium bicarbonate from the column gave one spot on a paper electrophoresis using a borate buffer and a single sedimentation pattern (S_{20}, w=2.0). It had $[\alpha]_D$ −70.5° (c=1.0, H_2O), *ca.* 11000 in molecular weight (by ultracentrifugal analysis), and 0.019 in reducing power (by the Park–Johnson method[8] as fucose). It contained 0.37% of nitrogen (by the micro-Dumas analysis), nil of phosphorus, 45.6% of total uronic acid (by the method of Bitter–Muir[9] as glucuronolactone) and 22.2% of total methylpentose (by the procedure of Dische, *et al.*[10] as fucose). The infrared (IR) spectrum of PI, which precipitated by Cetavlon treatment, showed absorption bands due to carboxylate group at 1640 and 1420 cm^{-1}.

The component sugars of PI were identified as L-fucose, D-mannose, D-galactose, and D-glucuronic acid by paper chromatography of the acid hydrolysate, and the molar ratio was estimated as 11.1:1.1:3.0:9.9 by the methods of Dubois, *et al.*[11] (I, for all sugars), Dische[10] (II, for methylpentose), and Bitter–Muir[9] (III, for uronic acid). The component sugars of 10% TCA-extract and 30% KCl-extract, which were used without further purification described above, were almost qualitatively identical with those of PI.

On periodate oxidation of PI, consumption of periodate from anhydro component sugar unit was 1.08 moles, and the values of formic acid and acetaldehyde liberated from the unit were 0.26 and 0.02 mole. Formaldehyde was not produced. The periodate-oxidized PI was treated by the Smith procedure.[12] Paper chromatographic analysis of the hydrolysate revealed the presence of glycerol and unoxidized componental sugars. By this treatment, the molar ratio changed to 2.0:0.9:0.4:2.4:2.7 (fucose, mannose, galactose, glucuronic acid, and glycerol) when these were estimated by the methods of I, II, III, and O'dea–Gibbons[13] (for polyhydric alcohol).

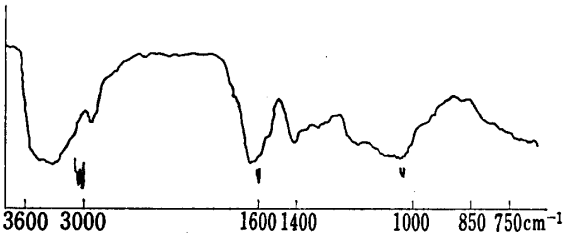

Fig. 1. Infrared Absorption Spectrum of PI

Partial acid hydrolysis was examined by three successive steps and dialyzable fragments released were estimated by the methods of I (as fucose) and III (as glucuronolactone). The released sugar contents by means of the successive treatments with 0.01N sulfuric acid, 90°, 2 hr; 0.01N sulfuric acid, 100°, 3 hr; and 0.05N sulfuric acid, 100°, 3 hr, were 10.9% (by the method of I), 6.1% (by the procedure of III); 34.4% (by the method of I), 25.4% (by the procedure of III), and 44.4% (by the method of I), 44.4% (by the procedure of III) corresponding respectively to each of the non-dialyzable materials. Paper chromatographic examination of these dialyzable fragments released showed the presence of fucose, galactose, and oligouronides, and a large part of mannose remained in the final non-dialyzable fragment. Molar ratios of these released sugars were similar to each other even under different hydrolysis conditions.

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9) T. Bitter and H.M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
10) Z. Dische and L.B. Shettles, *J. Biol. Chem.*, **172**, 515 (1948).
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The oligouronide portions on the paper chromatograms were combined and re-chromatographed under different conditions, and four oligouronides, rglucuronic acid 1.00 (oligo 1), 0.89 (oligo 2), 0.28 (oligo 3), and 0.05 (oligo 4), were isolated. From the results of the quantitative estimation by the methods of I and III and the reducing power of the uronides, oligo 1,2, and 3 would be disaccharides consisting of neutral sugar and glucuronic acid, and oligo 4, would be a hexasaccharide consisting of equimol ratio of neutral sugar and uronic acid residues.

As described above, quantitative estimation of the uronide-area on the paper chromatogram by the methods of I and III gave different results and the content of fucose (22.2%) by the method of Dische, *et al.* was lower than that (39.0%) from paper chromatographic analysis. This may be due to the presence of acid-stable oligouronide which contains fucose.

These result showed that teichoic acid and similar component are undetected, and a lower content of phosphorus is observed.[4] The main alkali-soluble cell wall polysaccharide, PI, is a heterogenous acidic glycan and it is labile to acid.

During partial acid hydrolysis of PI, molar ratios of the released sugars were similar in spite of different hydrolysis conditions and the isolated four oligouronides may be composed of glucuronic acid and fucose or other sugar residue from the results of the quantitative estimations. Presence of oligouronide such as glucuronosylfucose residue (Rglucuronic acid 1.00) has recently been demonstrated by Bartnicki-Garcia, *et al.*[4] in the cell wall of *Mucor rouxii*, therefore, it should be said that the above fact is interest in view of taxonomical relationship.

Periodate oxidation and Smith degradation of PI oxidized large parts of the component sugars except mannose, and formic acid and glycerol were produced. It is reasonable to assume the presence of (1→6) or (1→2)-linked galactopyranosyl residue and some branching residue of (1→3)-linked component sugar units.

The alkali-soluble but water-insoluble fragment of the cell wall, which amounts to 6.3% of the cell wall, contains uronic acid, glucosamine, and amino acids with small amounts of fucose, mannose, and galactose. It is presumably mucopeptide.

Paper chromatographic examination of the acid hydrolysate of alkali-extracted cell wall and infrared spectrum analysis of the material suggests that the main portion corresponding to 53% of the cell wall is chitin.

Detailed structual elucidation of PI, oligouronides, and the alkali-soluble but water-insoluble fragments, which obviously contains uronic acid and glucosamine, will be described in a later communication.

Experimental

Preparation of Cell Wall Materials——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. The culture flasks were shaken once a day to obtain the aerobic 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 employed 3 times at a pressure of 400 kg/cm^2 and it was usually sufficient to achieve total cell rupture. After the breakage procedure, the cell wall was separated immediately from cytoplasmic debris and intracellular soluble materials by repeated centrifugation at 3000 rpm for 5 min to prevent enzymic degradation of the cell wall. The supernatant was discarded and the residue was resuspended in an aqueous solution of 0.5% sodium dodecylsulfate, and followed with vigorous stirring for 24 hr. This treatment was repeated 4 times. The sedimented cell wall preparation was washed with distilled water until free of sodium dodecylsulfate and dried over P_2O_5 *in vacuo*. Yield, white greyish fine powder, 0.36 g/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): Extract I: The finely powdered cell wall (3.81 g) was extracted with acetone and then with EtOH: ether (1:1, v/v) for

2) Extraction with 10% TCA: Extract II: The dried fat-free cell wall was stirred with 10% TCA (100 ml) for 24 hr at 4° and this treatment was performed 3 times. After centrifugation, the supernatant was neutralized with 1N NaOH and then dialyzed against distilled water for 2 days. Care was taken to keep the temperature below 4° during the all stages of this procedure. The internal solution was concentrated under a reduced pressure and mixed with 4 volumes of EtOH. The precipitate formed was collected by centrifugation and dried with acetone and ether. Yield, 0.12 g.

3) Extraction with 30% KCl: Extract III: The above-mentioned residue was washed with distilled water by repeated centrifugation. The residue was stirred with 30% KCl (250 ml) for 2 days, and centrifuged. The supernatant was dialyzed against distilled water for 3 days, the internal solution was concentrated, and mixed with 4 volumes of EtOH. The precipitate formed was collected by centrifugation and washed with acetone and ether. Yield, 0.04 g.

4) Extraction with 1N NaOH at a Room Temperature: Extract IV: The residue left after extraction with 30% KCl was extracted with 1N NaOH (100 ml) for 24 hr at a room temperature, and centrifuged. This treatment was repeated 3 times. The mixture became slimy and brown. The supernatant was neutralized with 1M AcOH, dialyzed against distilled water for 2 days, and then concentrated. Water-insoluble material was appeared on dialysis. As the internal solution was found to become foamy during concentration *in vacuo*, octanol (2 ml) was added to the solution. To the concentrate, 4 volumes of EtOH was 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 polysaccharides, 0.46 g (corresponding to 12% of the cell wall) and water-insoluble material, 0.24 g.

5) Extraction with 1N NaOH at 100°: Extract V: The residue from the alkali treatment was suspended in 200 ml of 1N NaOH, kept at 100° for 12 hr, and then treated as described above. Yield of the material obtained by this treatment, 0.03 g.

After the above five steps of extraction, the residue was washed with distilled water until free of NaOH and dried with acetone and ether. Yield of the residue, 2.05 g.

Properties of 10% TCA Extract and 30% KCl Extract——1) Extract II (380 mg) was stirred with 10% TCA (4 ml) for 5 hr at 4°. Insoluble material was separated by centrifugation at 4000 rpm for 5 min and 3 volumes of EtOH was added to the supernatant. The precipitate was washed 4 times with EtOH and then dried *in vacuo*. Yield, 180.4 mg. Sephadex G-100 was allowed to swell for 30 min in 0.05M NaCl solution, poured into a tube (3×40 cm) and then washed with water until free from Cl^-. The precipitate (100 mg) was dissolved in water (10 ml) and passed through the column at a flow rate of 1.0 ml/min, and this was followed by elution with water. Fractions (1.0 ml) were collected automatically. An aliquot of each fraction (0.2 ml) was mixed with 1.8 ml of water, followed by C_6H_5OH–H_2SO_4 method[11] and the optical density was read at 485 mμ on a colorimeter.

The main fraction (42 mg) was electrophoretically pure and contained 0.14% of phosphorus. Paper chromatographic examination of the hydrolysate of this polysaccharide (*ca.* 10 mg in 2 ml of 1N H_2SO_4 at 100° for 8 hr) showed the presence of fucose, mannose, galactose, and glucuronic acid, and neither ribitol nor glycerol was detected. These component sugars of the fraction were identical with those of Extract II.

2) Extract III was hydrolyzed as described above. The component sugars of Extract III were fucose, mannose, galactose, and glucuronic acid. It contained 0.85% of phosphorus.

Protease Digestion, Separation, and Purification of Alkali-extracted Polysaccharides——Extract IV (420 mg) was dissolved in 50 ml of H_2O and adjusted to pH 7.8 with $NaHCO_3$. To the solution was added pronase (50 mg), 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 20 ml and submitted to the method of Sevag. After this procedure, the aqueous layer was concentrated to a small volume under a reduced pressure and 4 volumes of EtOH containing 0.1% AcOK was added to this concentrate. A precipitate (360 mg) was obtained.

Separation of the polysaccharide was carried out on a DEAE-cellulose column using H_2O, $NaHCO_3$, and NaOH. A solution of the polysaccharide (360 mg) in H_2O (10 ml) was applied to the column (3×41 cm) and stepwise elution was effected with H_2O, 0.05M, 0.1M, 0.2M, and 0.5M $NaHCO_3$, and then with 0.1N 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 and 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 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 follows: H_2O eluate, 12.2 mg (3.4%); 0.05M $NaHCO_3$ eluate, 90.0 mg (25.7%); 0.1M- and 0.2M $NaHCO_3$ eluate, trace; 0.5M $NaHCO_3$ eluate, 165.6 mg (44.8%), and 0.1N NaOH eluate, trace. Patterns on electrophoresis of these eluates and Extract IV are shown in Fig. 2. The polysaccharide (165.6 mg) from 0.5M $NaHCO_3$ eluate was dissolved in H_2O (3 ml), centrifuged at 4000 rpm for 10 min, and the supernatant was mixed with 4 volumes of EtOH. The greyish white precipitate (PI) so formed, corresponding to the main

polysaccharide of the cell wall, was collected by centrifugation, washed with acetone and ether, and dried *in vacuo*. Yield, 131 mg.

Properties of PI——Paper electrophoresis using 0.026M borate buffer (pH 10.0) showed a single spot (detected with the periodate-Schiff reagent[14]) and a single sedimentation pattern was given (in H_2O, after centrifugation for 144 min at 59780 rpm, Spinco E), $[\alpha]_D$ −70.5° (c=1.0, H_2O), IR ν_{max}^{KBr} cm^{-1} 1640 and 1420, N: 0.37%, P: 0%, total uronic acid: 45.6% (by the method of Bitter–Muir[9] as glucuronolactone), and total methylpentose: 22.2% (by the procedure of Dische, *et al.*[10] as fucose). It had *ca.* 11000 of molecular weight (by ultracentrifugal analysis) and reducing powder of PI was 1:0.019 (by the Park–Johnson method[9] as fucose). An insoluble cetyltrimethylammonium salt with PI was formed.

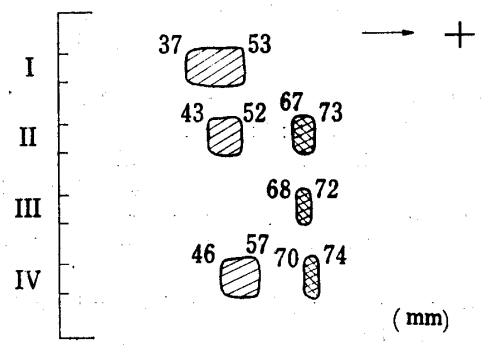

Fig. 2. Paper Electrophoresis Pattern

I: H_2O eluate
II: extract IV
III: PI
IV: 0.05M $NaHCO_3$ eluate
(1% borax, 1 mA/cm, 1.5 hr, Toyo Roshi No. 51, detection with the periodate–Schiff reagent)

Quantitative Estimation of the Componental Sugars of PI——PI (*ca.* 5 mg/ml 1N H_2SO_4) was heated in a sealed tube 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 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), and (3) EtOAc–pyridine–AcOH–H_2O (5:5:1:3). Sugars were detected by the spray reagents of $AgNO_3$–NaOH,[15] *p*-anisidine-HCl,[16] and ninhydrin.[17] Paper chromatographic analysis of the hydrolysate of PI revealed the presence of fucose, mannose, galactose, and glucuronic acid. Portions of the hydrolysate were spoted on a filter paper (Toyo Roshi No. 50), which was developed with the solvent system (1) for 8 hr. Development was repeated 5 times. After air-drying, the areas containing sugars on the paper chromatogram were cut out and quantitatively extracted with constant volume of H_2O, filtered through a sintered glass filter. The componental sugars were estimated by the methods of Dubois, *et al.*[11] (I, for all sugars), Dische[10] (II, for methylpentose), and Bitter–Muir[9] (III, for uronic acid). Content (in μg): fucose (215), mannose (25), galactose (65), and glucuronic acid (450 by the method of I and 245 by the procedure of III).

Periodate Oxidation of PI——PI (14.8 mg) was dissolved in H_2O (10 ml) and its total volume was made up to 25 ml with 2 ml of 0.22M $NaIO_4$ and distilled water. The mixture was allowed to stand in the dark at a room temperature. The amount of periodate consumed and quantity of HCOOH, HCHO, and CH_3CHO released were measured with this solution by the procedures of Maraprade,[18] Whilstler,[19] O'dea–Gibbons,[13] and Annison.[20] The number of moles of $NaIO_4$ consumed per anhydro-sugar unit of PI was as follows: 0.37 (1 hr), 0.53 (3 hr), 0.64 (6 hr), 0.74 (12 hr), 0.97 (24 hr), 1.21 (48 hr), and 1.28 (72 hr). The value of HCOOH was 0.05 (1 hr), 0.10 (3 hr), 0.15 (6 hr), 0.23 (12 hr), 0.32 (24 hr), 0.39 (48 hr), and 0.44 (72 hr) and the value of CH_3CHO was 0.02 (2 hr), 0.03 (48 hr), and 0.03 (72 hr).

Subsequently, PI (25 mg) was oxidized with $NaIO_4$ as described above, followed by Smith degradation. To destroy the excess periodate, ethylene glycol (0.4 ml) was added after 40 hr and the solution was dialyzed against running water for 24 hr. The non-dialyzable solution was concentrated to about 25 ml and $NaBH_4$ (*ca.* 30 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 0.2N H_2SO_4 (2 ml) in a boiling water bath for 6 hr. The syrup (SO_4^{2-} free) was obtained by the usual manner and paper chromatographic analysis of the hydrolysate using the solvent system (1) showed five spots corresponding to glycerol, fucose, mannose, galactose, and glucuronic acid. 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), as described above. All sugars, methylpentose, uronic acid, and polyhydric alcohol were determined by the methods of I, II, III, and O'dea–Gibbons.[13] Contents of the products of Smith degradation were as fol-

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lows (in μg): fucose (90), mannose (45), galactose (20), glycerol (67.5), and glucuronic acid (420 by the method of I and 125 by the procedure of III).

Partial Hydrolysis of PI——PI (27.6 mg) was heated with 0.01N H_2SO_4 (15 ml) at 90° for 2 hr and the hydrolysate was dialyzed against distilled water (400 ml) for 24 hr. This dialysis procedure was performed 3 times. The external solution (1200 ml) of the hydrolysate was evaporated to about 30 ml *in vacuo* and its total volume was made up to 100 ml with distilled water. An aliquot of this solution (1 ml) was estimated for sugar content by the methods of I (as fucose) and III (as glucuronolactone). Sugar content of the solution was 3.0 mg (by the method of I) and 1.7 mg (by the procedure of III). The residue was neutralized with $BaCO_3$, followed by paper chromatographic examination using the solvent system (1). Fucose, galactose, and glucuronic acid (approx. in molar ratio of 6: 1: 9) were detected. The internal solution was concentrated to dryness and weighed.

A part of the non-dialyzable material was hydrolyzed with 1N H_2SO_4 at 100° for 8 hr and treated as described above (molar ratio of fucose: mannose: galactose: glucuronic acid=9: 1: 3: 8).

Other part of the non-dialyzable material (18.9 mg) was treated with 0.01N H_2SO_4 (12 ml) at 100° for 3 hr, and treated by the same procedure as described above. Sugar content of the external solution of the hydrolysate was 6.5 mg (by the method of I) and 4.8 mg (by the procedure of III). Paper chromatographic analysis of the hydrolysate revealed the presence of fucose, mannose, galactose, and glucuronic acid in approx. molar ratio of 5: 0.1: 2: 10.

The non-dialyzable material (9.0 mg) (molar ratio; fucose: mannose: galactose: glucuronic acid= 8: 1.5: 6: 10) was heated with 0.05N H_2SO_4 (10 ml) at 100° for 3 hr. The external solution of the hydrolysate had sugar content 4.0 mg (by the method of I) and 4.0 mg (by the procedure of III). It was composed of fucose, mannose, galactose, and glucuronic acid in approx. molar ratio of 6: 0.5: 5: 12. The residue in the internal solution was 3.4 mg, which was composed of fucose, mannose, galactose, and glucuronic acid (molar ratio; 4: 2: 1: 10).

The portions of dialyzable fragments corresponding to uronic acid on paper chromatograms using the solvent system (1) were combined and freed from Ba^{2+} with Amberlite IR-120 (H^+). It was submitted to paper chromatography using the solvent system (3) and four spots were detected. Rglucuronic acid of each of the four spots was as follows: oligo 1 (1.00), oligo 2 (0.89), oligo 3 (0.28), and oligo 4 (0.05). The sugar content (μg) by the method of I was 33 (oligo 1), 67 (oligo 2), 55 (oligo 3), and 270 (oligo 4). The uronic acid content (μg) by the procedure of III was 11, 22, 26, and 151, and the reducing power (μg) by the Park–Johnson method[8] was 18, 31, 25, and 40, respectively.

Componental Sugars in the Water-insoluble Fraction Separated from the Alkali Extract——The alkali-soluble but water-insoluble fraction (6.6 mg) was heated with 1N H_2SO_4 at 100° for 8 hr, and the water-soluble material in the reaction mixture was examined by paper chromatography using the solvent system (1). Glucosamine and small amounts of fucose, mannose, and galactose were detected. The water-insoluble material (3.0 mg) in the hydrolysate, containing 54% of total uronic acid (by the procedure of III), was further hydrolyzed with 5N HCl at 100° for 8 hr. Paper chromatographic examination of the hydrolysate using the solvent system (3) revealed glucosamine and several amino acids.

Examination of the Alkali-extracted Cell Wall Residue——The alkali-extracted residue was hydrolyzed with 1N HCl at 100° for 8 hr and then with 4N HCl for 8 hr in a boiling water bath. Paper chromatographic examination of the hydrolysates using the solvent system (1) and (3) gave no sugar except obviously glucosamine. IR spectrum of the residue was very similar to that of the authentic sample of chitin.

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