Polysaccharides of Wood-destroying Fungus *Fomes fomentarius* (L.) FR. Extracted with Water

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Aqueous extraction of fruit bodies of *Fomes fomentarius* yielded a mixture of polysaccharides, fractionation of which afforded a mannofucogalactan and a glucan. The methods used to solve their structure have been hydrolysis, methylation analysis and periodate oxidation.

General knowledge on the nature of skeletal and reserve polysaccharides of wood-destroying fungi, such as cellulose, callose, chitin and glycogen, was obtained from histochemical reactions and colourations of microscopic preparations. The basic botanical literature comments that the mentioned polysaccharides only resemble the plant and animal polysaccharides, resp.

Chemical investigation of the polysaccharides from wood-destroying fungi began in the middle of our century. The first report of such a bearing was that of *Duff* [1] which describes the isolation of α and $\beta(1\rightarrow3)$-glucans, resp. from the *Polyporus betulinus* fungus. Other about 16 reports deal with structural studies of polysaccharides of fruit bodies and mycelia of random chosen species of wood-destroying fungi. In a series of works, *Lindberg et al.* [2—7] studied the polysaccharides isolated from the fungus of *Polyporus* type, polysaccharides from *Armillaria mellea* and *Pullularia pullulans*, resp. There is only one study on the polysaccharides of the *Fomes* type fungus [8].

Practically all of the studied fungi contain glucans and heteropolysaccharides. From the homopolysaccharides it is $\alpha(1\rightarrow4)$-glucan [9], glycogen [2] and glucans of the $\beta(1\rightarrow3)$-linked type which are widely distributed [2, 4]. Heteropolysaccharides contain units of D-galactose, D-mannose, L-fucose, D-xylose, 3-*O*-methyl-D-galactose and D-glucuronic acid. Mannofucogalactan [2—4], fucoxylomannan [3] and xylomannan [5] are the most frequently appearing heteropolysaccharides.

This communication reports the isolation of, and the results of a structural investigation on the polysaccharides isolated from the fruit bodies of *Fomes fomentarius*.

Experimental

Melting points were determined on a Kofler block, optical rotations were measured on an authomatic polarimeter Bendix—Ericson, type 143 A at room temperature. Evaporations were carried out at reduced pressure at temperatures not exceeding 40°C.

Paper chromatograms were run on Whatman No. 1 and Whatman No. 3 papers using the following solvent systems (v/v): S_1: ethyl acetate—pyridine—water (8 : 2 : 1) and S_2: ethyl acetate—acetic acid—water (18 : 7 : 8). Compounds were detected with aniline hydrogen phthalate [10]. The R_{Gal} values of monosaccharides and R_{MeG} values of methyla-

ted monosaccharides refer to rates of movement relative to that of D-galactose and 2,3,4,6-tetra-*O*-methyl-D-glucose, resp. Chromatograms were quantitatively evaluated with Lange Chromatometer 3. TLC of methylated saccharides was performed on Kieselgel G (10—40 μm) using benzene—acetone (1 : 1) as solvent.

Gas chromatography of methyl derivatives of methyl glycosides was performed on the Chrom 3 apparatus at 164 and 170°C, resp. Carbowax 6000 (10% on Chromosorb) was used as the stationary phase. The retention times of the methylated methyl glycosides are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside. The quantitative evaluation of chromatograms was omitted as the results obtained by evaluation of the areas would have been loaded with error more than that obtained by other methods.

The eluates from the DEAE-cellulose column were analyzed with phenol and sulfuric acid [11] on the FEK 4 colorimeter.

Electrophoresis was carried out on Electrophoresegerät 35 (Zeiss, Jena) in borate buffer pH 9.2 and 11, resp. The polysaccharide concentration was 1—1.5%.

The sedimentation constant was determined on an ultracentrifuge MOM G 110, diffusion coefficient on a Kern LK 30 device. The partial specific volume was determined pycnometrically. Molecular weight was calculated from the sedimentation constant, diffusion coefficient and partial specific volume.

The periodate uptake and the formed formic acid produced were determined by the thiosulfate method [12] with amperometric titration [13].

Preparation of the parent material

Fruit bodies of *Fomes fomentarius* (grown on a poplar) were harvested at the same time to prepare a unique material independent on the vegetative period.

Fruit bodies were stored in acetone immediately after harvesting. Before the extraction, the material was desintegrated in a Turmix blendor and then refluxed with acetone 20 hours to extract the colour materials and inactivate the enzymes present. After being air-dried, the material (890 g) was extracted with distilled water (27 l) 2 hours at 75°C. Extracted polysaccharides were recovered from the concentrated solution by precipitation with acidified ethanol (10 ml of concd. hydrochloric acid to 1 liter of ethanol) in the ratio of 1 : 3. The precipitate was filtered of and washed, first with dilute ethanol (60%) followed by 96% ethanol and ether. Yield 10 g of a white powder.

Air-dried material remaining after extraction with distilled water was further extracted with 15 liters of 0.05 M hydrochloric acid 2 hours at 75°C. The extract was worked up as in the preceding experiment. Yield 1.2 g of a white powder.

The residue was further extracted with 15 liters of 4% sodium hydroxide solution at room temperature for 3 hours. The extract was neutralized and worked up as above. Yield 8 g of a white powder.

Fractionation of the polysaccharides extracted with water

a) Chromatography on a DEAE-cellulose column

The polysaccharide mixture (300 mg) was dissolved in 25 ml of distilled water and placed on a DEAE-cellulose column (borate form). The column was eluted with water, followed with sodium borate solutions (0.025; 0.05; 0.10; 0.25; 0.50 M) and then with sodium hydroxide solutions (0.1 and 0.3 N) at a flow time 30 ml/hrs. Only the sodium borate-eluted fractions contained polysaccharides. These fractions were deionized by percola-

tion through Zerolit 225 in H^+-cycle and evaporated several times with methanol to dryness. The separation gave five fractions ($I-V$) in the amount of 55, 64, 55, 32 and 25 mg.

b) Fractional precipitation with cetyltrimethylamonium bromide (CTA-Br)

The polysaccharide mixture (5 g) was dissolved in 1400 ml of distilled water. The pH of the solution was adjusted to 8—8.5 with 0.5 M sodium hydroxide and the solid was precipitated with 200 ml of 0.23 M aqueous solution of CTA-Br. The gelatinous mass was collected on a centrifuge and was washed successively with diluted, then concentrated ethanol and ether. Yield 3.17 g of a brown powder. The supernatant liquid was treated with further amount of CTA-Br (300 ml) and allowed to stand 5 days in refrigerator. The fine precipitate was filtered, washed as in the former case. Yield 127 mg of a brown powder. Further addition of the acidified ethanol gave no precipitate.

c) Chromatography on a Dowex 1X8 ion-exchanger

Polysaccharides (400 mg in 250 ml of water) were applied to the top of the Dowex 1X8 (200/400 mesh) column (45 × 3 cm) in acetate form. The column was eluted with water and then with 4 N acetic acid. The water solution was freeze-dried (yield 208 mg) and the acetic acid-eluate was concentrated and precipitated with ethanol in the ratio of 1 : 3. The precipitate was washed with ethanol and dried (145 mg).

Hydrolysis of saccharides

a) Partial hydrolysis

Dowex 50 W (H^+-form) ion-exchanger (50 g) was added to a solution containing 220 mg of polysaccharide in 30 ml of distilled water. The solution was heated for 4 hours in a flask provided with a reflux condenser. After filtering, the hydrolyzate was evaporated and products of hydrolysis were separated and identified by paper chromatography. During hydrolysis, samples of products were examined at corresponding intervals.

b) Total hydrolysis

Polysaccharide and/or oligosaccharide (*ca.* 5 mg) were hydrolyzed with 1 N sulfuric acid (1 ml) in a sealed tube at 105°C for 20 hours. The cooled solution was neutralized with barium carbonate, filtered, treated with Zerolit 225 (H^+-form) to remove barium ions, evaporated and the hydrolysis products were identified by paper chromatography. Saccharides were characterized as follows:

D-Galactose — m.p. 166—168°C, $[\alpha]_D = +80°$ ($c = 1$ in water), *N*-*p*-nitrophenyl-D-galactosylamine tetraacetate m.p. 97—100°C, $[\alpha]_D = -70°$ ($c = 1$ in chloroform) (lit. [14] reports m.p. 98°C, $[\alpha]_D = -72°$).

D-Mannose — $[\alpha]_D = +16°$ ($c = 1$ in water).

L-Fucose — m.p. 145°C, $[\alpha]_D = -54°$ ($c = 0.7$ in water).

D-Glucose — m.p. 81°C, $[\alpha]_D = +53.3°$ ($c = 0.9$ in water), *N*-*p*-nitrophenyl-D-glucosylamine tetraacetate m.p. 180—183°C, $[\alpha]_D = -105°$ ($c = 0.95$ in chloroform) (lit. [14] reports m.p. 182—183°C, $[\alpha]_D = -101°$).

Methylation analysis

a) Methylation of the polysaccharides

To the polysaccharide (100 mg) were added simultaneously and dropwise with stirring at room temperature (3 days) 20 ml of 30% sodium hydroxide and 15 ml of dimethyl sulfate at a rate such that the mixture remained alkaline throughout.

The mixture was then heated for 30 minutes at 60°C to destroy any excess dimethyl sulfate and the cooled solution was neutralized with 2 N sulfuric acid. The partially methylated product was extracted with chloroform and the dried (Na_2SO_4) extract was concentrated to a syrup. The partially methylated product (100 mg) was further refluxed with methyl iodide (6 ml) and silver oxide (0.6 g), the latter having been added in three portions during 3 days. After filtration, the solid was washed with chloroform and the combined extracts were evaporated under reduced pressure. The resulting product was devoid of OH absorption in the infrared.

b) Methanolysis of the methylated polysaccharides

A part of the methylated product (8 mg) was methanolyzed with 5% methanolic hydrogen chloride in a sealed tube at 105°C for 48 hours. It was then neutralized with silver carbonate and centrifuged. The supernatant liquid was concentrated, dissolved in chloroform and analyzed by gas chromatography.

c) Hydrolysis of the methylated polysaccharides

A part of the methylated polysaccharide (100 mg) was hydrolyzed with 70% sulfuric acid (2.5 ml) for 45 minutes at room temperature. The solution was then diluted with 17 ml of water and hydrolyzed in a sealed tube for 6 hours at 105°C. After neutralization with barium carbonate, the present methyl derivatives of saccharides were analyzed chromatographically on paper in system S_1 and on silica gel plates in system S_3.

d) Methylation of the oligosaccharides

Oligosaccharide (5 mg) was dissolved in 1 ml of *N*,*N*-dimethylformamide and treated with 1 ml of methyl iodide and 1 g of silver oxide with stirring in the dark for 18 hours. The solution was then extracted with chloroform and the combined extracts were concentrated under reduced pressure. Methylated oligosaccharide was methanolyzed and hydrolyzed, resp. The products of methanolysis and hydrolysis were analyzed by gas and paper chromatography, resp.

Reduction of the oligosaccharide

Sodium borohydride (20 mg) was added to a solution of oligosaccharide (4—6 mg) in water (2 ml) and was allowed to react with stirring for 2 hours at room temperature. The solution was then neutralized with acetic acid, deionized on Dowex 50 W (H^+-form) and evaporated several times with methanol to dryness. The reduced oligosaccharide was hydrolyzed as described above and hydrolysis products were identified by paper chromatography in system S_1.

Periodate oxidation

The polysaccharide (100 mg) was dissolved in distilled water (10 ml), and the solution was cooled to 5°C. To this solution was added a 0.23 M solution of sodium metaperiodate

(5.5 ml), and the volume was adjusted to 50 ml with water. The solution was kept at 5°C in the dark throughout the oxidation to minimize side reactions. Aliquots of the sample (1 ml) were removed at intervals and analyzed for periodate consumption and liberation of formic acid. Analytical data were extrapolated to zero time. When the oxidation was over (after 17 days), the solution was neutralized with lead carbonate. The precipitate formed was separated by centrifugation and the Pb^{2+} ions were eliminated with Zerolit 225 (H^+-form). After concentration, the product was hydrolyzed with 0.5 M sulfuric acid and neutralized with barium carbonate. Saccharides present in hydrolysate were determined by paper chromatography in system S_1.

Results

Subsequent extraction of fruit bodies of *Fomes fomentarius* with distilled water, 0.05 M hydrochloric acid and 4% sodium hydroxide yielded three polysaccharide fractions. Acid hydrolysis of the water-extracted fraction yielded galactose and glucose as predominant components together with smaller amounts of mannose and fucose. Fractions obtained on acidic and alkali extractions, resp. gave glucose as a predominant component together with smaller amount of mannose and traces of fucose on hydrolysis. None of the three fractions was homogeneous in ultracentrifuge and free boundary electrophoresis. Only the water-extracted fraction was further investigated.

The following fractionation methods were investigated for separating the polysaccharide mixture: chromatography on a DEAE-cellulose column, precipitation with cetyltrimethylamonium bromide and separation on a Dowex 1X8 ion-exchanger. Chromatography on DEAE-cellulose gave five fractions, two of them were homogeneous. However, the separation ability of DEAE-cellulose was low and could not readily give workable amounts of homogeneous fractions. Fractional precipitation with CTA-Br gave heterogeneous fractions of a very similar composition. Separation on Dowex 1X8 ion-exchanger afforded two fractions of a different composition in a relatively short time. The water-eluted fraction (*A*) was apparently homogeneous when examined by boundary electrophoresis and sedimentation in ultracentrifuge and furnished galactose, mannose, fucose and traces of glucose on acid hydrolysis. Acid hydrolysis of the fraction eluted with 4 N acetic acid (*B*) released glucose as a main component together with other saccharides present in fraction *A*.

Heteropolysaccharide A was well soluble in water, had $M_{s,D} = 22{,}200$, DP = = 140 and $[\alpha]_D = +72°$ ($c = 0.4$ in water). The molar ratio of D-galactose : D-mannose : L-fucose was 2.78 : 1.03 : 1.37.

During partial hydrolysis D-mannose, L-fucose and 3-*O*-α-D-mannopyranosyl-L-fucose were released first, 6-*O*-β-D-galactopyranosyl-D-galactose later. The disaccharides were characterized as follows:

3-*O*-α-D-Mannopyranosyl-L-fucose — $R_{Gal} = 0.85$, $[\alpha]_D = -4°$ ($c = 0.8$ in water) (lit. [4] reports $[\alpha]_D = +0.5°$), on acid hydrolysis gave D-mannose and L-fucose in an equimolar ratio. After reduction with sodium borohydride and hydrolysis of the reduced product D-mannose was determined by paper chromatography in system S_1. Hydrolysis of the methylated disaccharide afforded 2,3,4,6-tetra-*O*-methyl-D-mannose — $R_{MeG} = 0.98$ and 2,4-di-*O*-methyl-L-fucose — $R_{MeG} = 0.66$ identified by comparison with authentic samples.

6-*O*-β-D-Galactopyranosyl-D-galactose — $R_{Gal} = 0.48$, $[\alpha]_D = +25°$ ($c = 1$ in water) (lit. [2] reports $[\alpha]_D = +28°$), on acid hydrolysis gave D-galactose only. Hyd-

rolysis of the methylated product afforded 2,3,4,6-tetra-*O*-methyl-D-galactose — $R_{MeG} = 0.90$ and 2,3,4-tri-*O*-methyl-D-galactose — $R_{MeG} = 0.66$ identified by both paper and gas chromatography on the basis of comparison with authentic samples.

A sample of the polysaccharide consumed 1.1 moles of periodate and released 0.43 moles of formic acid per average saccharide unit. Hydrolysis of the periodate-oxidized polysaccharide, followed by paper chromatographic examination of the hydrolysate in system S_1, indicated that D-galactose and L-fucose were the reducing sugars present.

A sample of the polysaccharide was fully methylated and had $[\alpha]_D = +49.5°$ ($c = 0.4$ in chloroform). Hydrolysis products of the methylated polysaccharide were separated by using chromatography on Whatman No. 3 paper. The following methyl derivatives of monosaccharides were identified by both paper and TL chromatography:

2,3,4,6-Tetra-*O*-methyl-D-mannose — $R_{MeG} = 0.98$, after demethylation with hydrobromic acid [15] D-mannose was identified by paper chromatography in system S_1.

2,3,4,6-Tetra-*O*-methyl-D-galactose — $R_{MeG} = 0.90$, $[\alpha]_D = +120°$ ($c = 0.5$ in water) (lit. [16] reports $[\alpha]_D = +114°$), demethylation revealed D-galactose as parent sugar.

2,3,4-Tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-L-fucose — $R_{MeG} = 0.66$, demethylation revealed D-galactose and L-fucose. This fraction represents a mixture of both methyl derivatives as their standards have the same chromatographic movement in system S_1.

2,4-Di-*O*-methyl-D-galactose — $R_{MeG} = 0.36$, $[\alpha]_D = +84°$ ($c = 0.5$ in water) (lit. [17] reports $[\alpha]_D = +86°$), demethylation revealed D-galactose.

The methanolysis products of the methylated polysaccharide examined by gas chromatography were as follows: methyl 2,3,4-tri-*O*-methyl-L-fucoside — $R_t = 0.46$; methyl 2,3,4,6-tetra-*O*-methyl-D-mannoside — $R_t = 1.00$; methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside — $R_t = 1.15$; probably methyl 2,4-di-*O*-methyl-L-fucoside — $R_t = 1.92$; methyl 2,3,4-tri-*O*-methyl-D-galactoside — $R_t = 3.55$ and 5.85, and methyl 2,4-di-*O*-methyl-D-galactoside — $R_t = 15.85$.

Glucan containing fraction B was difficultly soluble in water, soluble in alkali and had $[\alpha]_D = +66°$ ($c = 1$ in 1 N sodium hydroxide). On acid hydrolysis gave D-glucose, D-galactose, D-mannose and L-fucose in the molar ratio of 10 : 1 : 1 : 1.

The methylated fraction had $[\alpha]_D = +87.5°$ ($c = 1$ in chloroform) and showed no hydroxyl absorption in the infrared. After hydrolysis of the methylated product, the resulting methylated sugars were examined by both paper and TL chromatography. The following methyl derivatives were identified: 2,3,4,6-tetra-*O*-methyl-D-glucose — $R_{MeG} = 1.00$; 2,3,6-tri-*O*-methyl-D-glucose — $R_{MeG} = 0.86$; 2,3,4-tri-*O*-methyl-D-galactose — $R_{MeG} = 0.66$ (traces) and 2,4-di-*O*-methyl-D-galactose — $R_{MeG} = 0.36$ (traces).

The methanolysis products of the methylated fraction examined by gas chromatography were as follows: methyl 2,3,4-tri-*O*-methyl-L-fucoside — $R_t = 0.48$; methyl 2,3,4,6-tetra-*O*-methyl-D-glucoside — $R_t = 0.69$ and 1.00; methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside — $R_t = 1.17$ and methyl 2,3,6-tri-*O*-methyl-D-glucoside — $R_t = 2.70$ and 3.61.

The polysaccharide consumed 0.83 moles of periodate and released 0.18 moles of formic acid per average saccharide unit. Only traces of D-galactose were found in the hydrolysis product of the periodate-oxidized polysaccharide.

Discussion

The mannofucogalactan

It is apparent from the molar ratios of monosaccharides revealing on acid hydrolysis and from the disaccharide consisting of D-galactose units revealing on partial hydrolysis that D-galactose represents the main part of the heteropolysaccharide. The identified methyl derivatives of D-galactose as well as the periodate-resistant D-galactose units clearly show that the backbone of the polysaccharide is formed by D-galactose units linked together with (1→6)-glycosidic bonds. Some of the D-galactose units are found as branch points substituted in the 3-position by side chains. D-Galactose is also present as non-reducing end-group. From the observed values of optical rotations of the polysaccharide and disaccharide composed of D-galactose units it was concluded that the glycosidic linkage was in the β-form.

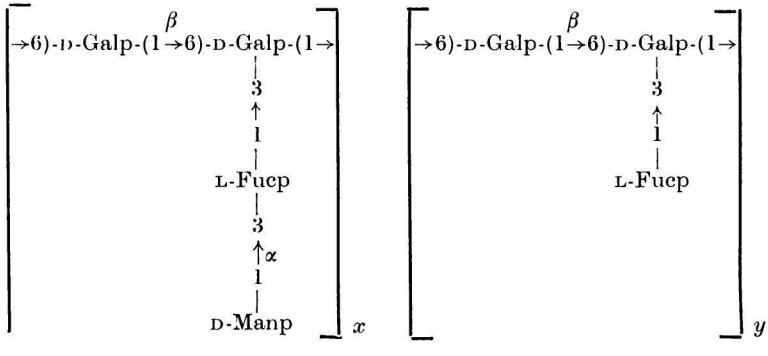
D-Mannose and L-fucose were split off in the first stage of hydrolysis together with mannosylfucose. As the main chain is composed of D-galactose units linked together with relatively stabile $\beta(1\rightarrow6)$-glycosidic bonds and D-galactose appears in the hydrolysate only after some time, D-mannose and L-fucose form side chains.

2,3,4-Tri-*O*-methyl-L-fucose and 2,4-di-*O*-methyl-L-fucose identified on methylation analysis indicate that L-fucose is involved in both non-reducing end-groups, and as chain units linked through C-1 and C-3. 2,3,4,6-Tetra-*O*-methyl-D-mannose, as the only identified methyl derivative, indicates that D-mannose is present exclusively as terminal, non-reducing residue. It can be suggested from the predominant amount of L-fucose that some of the side chains are formed by single L-fucose units and others by mannosylfucose. The side chains are attached to the D-galactose units in the 3-position.

From the optical rotation of the disaccharide it may be concluded that the D-mannopyranosidic linkage is in the α-form. The structural studies give no information on the configuration of the linkage by which the L-fucose and mannosylfucose are attached to the galactan chain.

The methylation analysis indicated that all of the saccharide units were present in pyranose form.

The obtained values of periodate consumption (1.1 moles) and of produced formic acid (0.43 moles) are in reasonably good agreement with the values (1.06; 0.53) calculated for a polysaccharide with the following general structure:



$x = 3y$

The described polysaccharide resembles the polysaccharide of *Polyporus gigauntes* reported by *Lindberg et al.* [2]. However, in that polysaccharide, L-fucose was attached to the 2-position of D-galactose units and was not found as non-reducing end-group.

The glucan

The results of structural studies show that the fraction *B* is a mixture of a glucan and the described mannofucogalactan *A* which could not be fully separated and gives rise to heterogeneity of this fraction.

From the methylation analysis and periodate oxidation follows that the glucan is a linear chain of D-glucose units linked by (1→4)-glycosidic bonds as the main hydrolysis product of the methylated polysaccharide is 2,3,6-tri-*O*-methyl-D-glucose. On the basis of the observed values of optical rotation and the absorption band at 891 cm^{-1} in the infrared it is concluded that the glycosidic linkages are in the β-form.

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