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Polysaccharides Elaborated by *Fomes annosus* (Fr.) Cooke

II. Neutral Polysaccharides From the Fruit Bodies. Isolation and Purification of a Fucoxylomannan by Precipitation With the H-Agglutinin From Eel-Serum

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Two polysaccharides, a fucogalactan and a glucan, have been isolated from the water extracts of the fruit bodies from *Fomes annosus*. The fucogalactan consists of chains of (1→6)-linked α-D-galactopyranose residues about 32 % of which are substituted with an α-L-fucopyranose residue in the 2-position. The glucan consists of (1→3)- and (1→6)-linked β-D-glucopyranose chain residues and branch points with (1→3)- and (1→6)-linkages.

From the alkali extracts a fucoxylomannan was isolated by precipitation with purified H-agglutinin from eel-serum. The fucoxylomannan consists of (1→3)-linked β-D-mannopyranose residues, of which about 40 % are substituted in the 4-position with 2-*O*-α-L-fucopyranosyl-D-xylopyranose residues.

In a recent publication[1] studies on an acidic polysaccharide from *F. annosus* (*Polyporus annosus*) was reported. The present communication describes the isolation and structural studies on two neutral polysaccharides from the water extract and one from the alkali extract.

Fruit bodies of *F. annosus* were harvested locally. A homogenate of the fruit bodies was first extracted with water and subsequently with alkali. The water extracts were processed as previously described for a similar mixture,[2] to yield a fucogalactan and a glucan. The fucogalactan had $[\alpha]_{578}+78°$ (*c*, 0.8 water) and yielded on hydrolysis L-fucose and D-galactose in the relative molar proportions 1.0:2.5. The glucan had $[\alpha]_{578}+10°$ (*c*, 1.0 water) and yielded only D-glucose on hydrolysis, demonstrating it to be a β-glucan. The alkali extract was separated into a neutral and an acidic fraction by batch-wise treatments with DEAE-Sephadex (acetate-form).[2] The neutral fraction yielded, on acid hydrolysis, L-fucose, D-xylose, D-mannose, and D-glucose in the relative proportions 1.0:1.0:2.4:3.3. Attempts to purify the polysaccharide mixture by

conventional techniques were unsuccessful. However since polysaccharides containing L-fucose together with D-mannose and D-glucose had not previously been isolated, it was assumed that all L-fucose in the mixture originated from a fucoxylomannan.[9] Part of the neutral fraction was therefore precipitated with H-agglutinin from eel-serum (Fig. 1) to yield a polysaccharide which

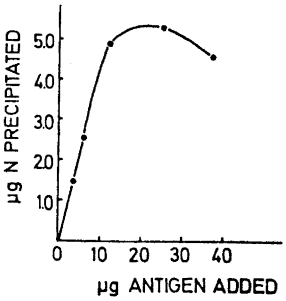

Fig. 1. Precipitation of the crude neutral portion of the alkali extract with purified anti-H hemagglutinin. Total volume was 200 µl.

contained L-fucose, D-xylose, and D-mannose in the relative molar proportions 1.0:1.0:2.5. The value for the optical rotation of the fucoxylomannan, $[\alpha]_{578}$ − 10°, is not very accurate because of the small amount available.

The polysaccharides were methylated by the Hakomori procedure,[3] the fully methylated materials hydrolysed and the mixtures of methylated sugars in the hydrolysates analysed, as their alditol acetates, by GLC−MS.[4] The results of the methylation analyses of the fucogalactan, the glucan, and the fucoxylomannan are given in Tables 1 (column A), 2, and 3, respectively. 3,4-Di-*O*-methyl-D-xylose could be distinguished from 2,3-di-*O*-methyl-D-xylose by reduction with borodeuteride, acetylation and MS.[4]

Part of the fucogalactan was subjected to a mild acid hydrolysis, whereby fucosidic linkages should be preferentially cleaved, and the resulting polymeric material, $[\alpha]_{578}$ + 125° (*c*, 0.8 water), recovered. The methylation analysis (Table 1, column B) revealed that only traces of 2,3,4-tri-*O*-methyl-L-fucose and 3,4-di-*O*-methyl-D-galactose remained whereas the amount of 2,3,4-tri-*O*-methyl-D-galactose had increased.

Table 1. Methyl ethers from the hydrolysate of (A) methylated fucogalactan, (B) methylated partially hydrolysed fucogalactan.

Sugars	T^a	Mol % A	Mol % B
2,3,4-Tri-*O*-Me-L-Fuc	0.65	25	Trace
2,3,4,6-Tetra-*O*-Me-D-Gal	1.25	7	10
2,3,4-Tri-*O*-Me-D-Gal	3.41	42	90
3,4-Di-*O*-Me-D-Gal	6.9	26	Trace

[a] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Table 2. Methyl ethers from the hydrolysate of methylated glucan.

Sugars	T^a	Mol %
2,3,4,6-Tetra-*O*-Me-D-G	1.00	20
2,4,6-Tri-*O*-Me-D-G	1.95	24
2,3,4-Tri-*O*-Me-D-G	2.50	36
2,4-Di-*O*-Me-D-G	5.10	20

[a] See Table 1.

Table 3. Methyl ethers from the hydrolysate of methylated fucoxylomannan.

Sugars	T^a	Mol %
2,3,4-Tri-*O*-Me-L-Fuc	0.65	22
3,4-Di-*O*-Me-D-Xyl	1.54	22
2,4,6-Tri-*O*-Me-D-Man	2.10	34
2,6-Di-*O*-Me-D-Man	3.35	22

[a] See Table 1.

A similar partial hydrolysis could not be performed with the purified fucoxylomannan because of the small amount available and was therefore performed on the crude neutral fraction. A comparison of the methylation analyses on the original crude neutral fraction and on the partially hydrolysed product showed a decrease in 2,3,4-tri-*O*-methyl-L-fucose and 3,4-di-*O*-methyl-D-xylose and a corresponding increase in 2,3,4-tri-*O*-methyl-D-xylose due to the partial hydrolysis. This demonstrates that the terminal L-fucose residues are linked to the 2-position of D-xylose residues.

DISCUSSION

The methylation analysis of the glucan shows that it consists of (1→3)- and (1→6)-linked chain residues with branch points with (1→3)- and (1→6)-linkages. From the low optical rotation of the polysaccharide it may be inferred that most of the D-glucose residues are β-linked. Glucans of this type are common in fungi.[5]

A methylation analysis on the fucogalactan demonstrates that it contains (1→6)-linked D-galactopyranose residues, about 30 % of which are substituted in the 2-position. The branches consisted of single L-fucopyranose residues, as demonstrated by the methylation analysis on the partially hydrolysed material. The high optical rotations of the original material ($[\alpha]_{578}+78°$) and the degraded ($[\alpha]_{578}+125°$) indicate that the D-galactopyranose residues are α-linked. The increase in optical rotation after the hydrolysis of the L-fucopyranosyl linkages suggests that these also have the α-configuration.

The fucogalactan (Fig. 2) from *F. annosus* thus has a structure similar to that of the fucogalactans isolated from *P. borealis* [6] and *P. ovinus*.[7]

The neutral fraction of the alkali extracts contained a complex mixture of polysaccharides. The fucoxylomannan was successfully isolated by precipitation with the H-agglutinin from eel-serum (Fig. 1). By this technique, 370 μg of fucoxylomannan was isolated which was sufficient for sugar analysis and methylation analysis. Methylation analysis (Table 3) demonstrated that all sugar residues are pyranosidic. About 40 % of the (1→3)-linked D-mannose residues are substituted in the 4-position with side chains. A methylation analysis on the degraded crude neutral fraction indicated that these side-chains are terminated by 2-*O*-α-L-fucopyranosyl-D-xylopyranose residues. The

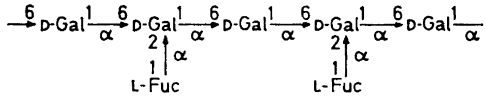

Fig. 2. Proposed structure for the fucogalactan from *F. annosus*. (All sugar residues are pyranosidic.)

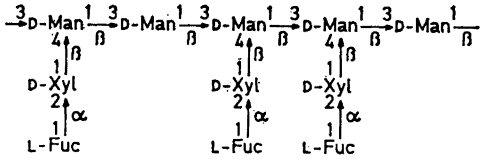

Fig. 3. Proposed structure for the fucoxylomannan from *F. annosus*. (All sugar residues are pyranosidic.)

optical rotation of the crude neutral fraction increased from +16° to +25° during the mild acid hydrolysis, suggesting that the terminal L-fucose residues are α-linked. This finding is further corroborated by the fact that the fucoxylomannan is precipitated by the H-agglutinin from eel-serum, since this protein does not react with β-L-fucopyranosides.[8] From the low optical rotation of the purified fucoxylomannan (~ −10°), it may be inferred that the D-mannose and D-xylose residues are β-linked. This polysaccharide (Fig. 3) resembles the fucoxylomannan isolated from *Polyporus pinicola*,[9] but the degree of branching in the former is lower.

The purification of polysaccharides by immunological techniques has previously been used in the isolation of *Pneumococcus* Type 1 polysaccharide [10] and of hog blood-group substance.[11] This method will, no doubt, become increasingly important in the future, when antibodies or lectins become more generally available.

EXPERIMENTAL

The methods used for structural analyses were the same as previously described.[2]

Purification of the anti-H hemagglutinin from pooled eel-serum. Blood from 54 eels, obtained from the local fishmarket, was collected from the heart and allowed to clot overnight at +4°. The complement was inactivated by heating each serum at 56° for 20 min. The individual sera were then tested for anti-H activity against human O-

erythrocytes and sera with hemagglutinating titers $\geq 1/64$ were pooled (147 ml from 27 eels). The serum pool was treated with human A_1-erythrocytes to absorb any anti-A active proteins. Hemagglutination titers after absorption were $< 1/1$ against A_1-erythrocytes and 1/128 against O-erythrocytes, respectively. The serum pool was applied to a small column of insoluble A+H-substance (1.5 g) mixed with celite (0.75 g).[12] The material, which passed through the column, had no hemagglutinating activity against O-erythrocytes. The column was then washed with 0.05 M phosphate buffered (pH 7.3) saline (0.9 % aqueous sodium chloride) until the optical density of the eluate at 280 nm was less than 0.050. Specific elution was effected by irrigation with 300 ml of 0.01 M L-fucose in saline (phosphate buffered, pH 7.3) followed by 0.05 M fucose in saline (phosphate buffered, pH 7.3). The bulk of the protein (~ 95 %) was eluted with the 0.01 M L-fucose solution. After concentration by ultrafiltration the eluate was freed from remaining L-fucose by repeated gelfiltration on a Sephadex G-25 column. Yield of purified anti-H hemagglutinin was 27.1 mg. The purity and the specificity of the eluted material was assayed by geldiffusion and immunoelectrophoresis against several rabbit antisera to eel-serum and by direct precipitation with purified hog A+H bloodgroup substance. As can be seen from Fig. 4a, the purified anti-H hemagglutinin gives only one precipitin line with eel-serum antiserum. Identical results were obtained with two additional anti-

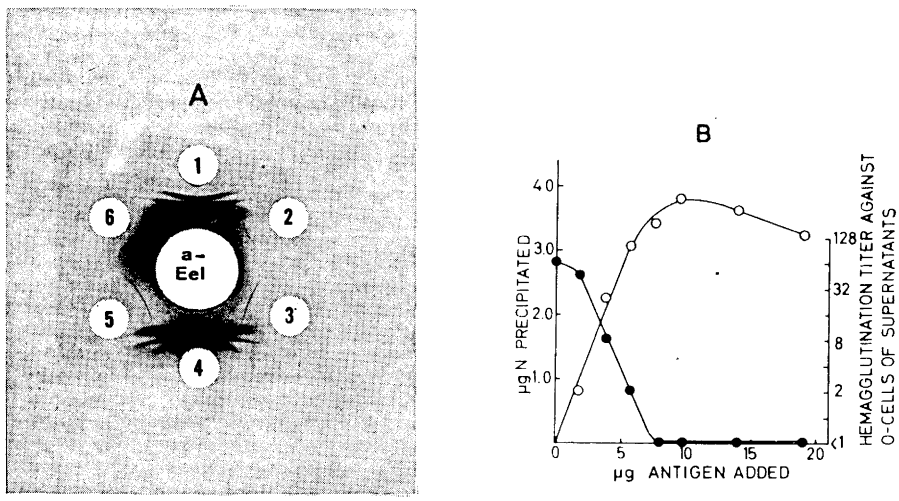

Fig. 4. (A) Immunodiffusion of purified anti H hemagglutinin and of eel-serum against rabbit anti eel-serum. Central well: rabbit anti eel-serum, undiluted. Peripheral wells: (1) eel-serum diluted ½; (2) material eluted in first 55 ml of 0.01 M L-fucose, 260 μg N/ml; (3) material eluted in next 240 ml of 0.01 M L-fucose, 213 μg N/ml; (4) eel-serum diluted 1/8; (5) same as 2, 130 μg N/ml; (6) material eluted with 0.05 M L-fucose, 52 μg N/ml. (B) Precipitation of purified anti H hemagglutinin with hog blood group A+H substance. 4.1 μgN of material eluted with 0.01 M L-fucose was added per tube. The total volume was 200 μl. (○) precipitin curve; (●) hemagglutination titer of supernatants against human O-erythrocytes.

sera to eel-serum. Similarly on immunoelectrophoresis only one precipitin arc located in the γ-region of serum was obtained. Quantitative precipitin analysis against hog blood group A+H substance is shown in Fig. 4b. Approximately 90 % of the purified anti-H hemagglutinin was precipitated at equivalence as calculated from the nitrogen values. Since supernatants from the equivalence zone did not agglutinate O-erythrocytes (Fig. 4b) the hemagglutinating and precipitating activities appeared to be identical.

Isolation of the fucoxylomannan. Purified anti-H hemagglutinin (1.2 ml, 1.3 mg N/ml) was added to the crude neutral fraction of an alkali extract from *Fomes annosus* (0.9 ml; 6 mg/ml) and incubated at + 4° for one week. The precipitate was collected by centrifugation and washed 5 times with 2 ml of 0.9 % aqueous sodium chloride (saline). The washed precipitate was dissolved in 1 ml of 0.05 M L-fucose in saline and applied to a Sephadex G 150 column (150 × 2 cm) equilibrated with 0.05 M L-fucose in saline. The eluent was monitored by measuring the optical density at 280 nm. It had previously been demonstrated that the L-fucose-containing polysaccharides were eluted before the anti-H hemagglutinin and therefore the fractions eluted before the protein peak was pooled. The polysaccharide solution was dialysed against distilled water, and concentrated. The resulting polysaccharide was gelfiltered on a Sephadex G15 column (50 × 2 cm) to remove any remaining L-fucose. Yield of polysaccharide 370 μg, $[\alpha]_{578}$ −10° (*c*, 0.03 water).

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