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SOME STRUCTURAL FEATURES OF A NEW SULPHATED HETEROPOLYSACCHARIDE FROM *PADINA PAVONIA*

M. MAGDEL-DIN HUSSEIN, A. ABDEL-AZIZ* and H. MOHAMED SALEM*

Laboratory of Natural Products, National Research Centre, Dokki, Cairo, Egypt and * Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt

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Abstract—An acid-extractable, water-soluble, polysaccharide sulphate, isolated from *Padina pavonia*, comprised variable proportions of glucuronic acid, galactose, glucose, mannose, xylose, and fucose in addition to a protein moiety. Partial acid hydrolysis and autohydrolysis of the free acid polysaccharide yielded several oligosaccharides. Evidence from periodate oxidation studies indicated that the inner polysaccharide portion is composed of (1 → 4)-linked β-D-glucuronic acid, (1 → 4)-linked β-D-mannose and (1 → 4)-linked β-D-glucose residues. The heteropolymeric partially sulphated exterior portion is attached to the inner part and comprises various ratios of (1 → 4)-linked β-D-galactose, β-D-galactose-3-sulphate residues, (1 → 4)-linked β-D-glucose residues, (1 → 2)-linked α-L-fucose 4-sulphate residues and (1 → 3)-linked β-D-xylose residues.

INTRODUCTION

We have reported [1] the isolation from the brown alga *Padina pavonia* of a new sulphated heteropolysaccharide composed of D-glucouronic acid, D-galactose, D-glucose, D-mannose, D-xylose, and L-fucose residues, as well as a protein moiety. We now report further on the structural features of this sulphated heteropolysaccharide.

RESULTS AND DISCUSSION

After partial acid hydrolysis of the new algal polysaccharide with 0.5 N oxalic acid at 100° for 2 hr, the dialysable product A contained glucose, galactose, xylose, and fucose in the molar ratios 1.0:2.3:8.7:17.0 and oligosaccharides. The dialysable materials were fractionated on Amberlite IR-400 resin into netural and acidic fragments (A1 and A2, respectively). The neutral product A1 (found: total carbohydrates, 210 mg) was resolved by preparative paper-chromatography into three oligosaccharides (A_1, A_2, and A_3) having R_{xyl} 0.18, 0.28 and 0.40 (solvent A), respectively. The oligosaccharide A_1 stained brown with aniline hydrogen phthalate, whereas A_2 and A_3 stained yellowish brown and pink, respectively. Acid hydrolysis of A_1–A_3 gave galactose, glucose, xylose and fucose in the molar ratios: A_1 1:2:1:1; A_2 1:0:2:1; A_3 1:1:2:0. After reduction with borohydride, these ratios changed as follows: A_1 0:2:1:1; A_2 0:0:2:1; A_3 1:0:2:0. These results indicated that galactose was the reducing terminus of A_1 and A_2, and glucose of A_3. A_1–A_3 showed negative optical rotations, but the amounts available were too small to determine accurate values. The presence of β-D- and α-L-glycosidic linkages is thereby indicated.

Resolution of the acidic product A2 (Found: total carbohydrates, 105 mg) by chromatography on paper afforded acidic oligosaccharides (A_4–A_6) which stained brown with aniline hydrogen phthalate and had R_{xyl} 0.27, 0.36, and 0.42 (solvent A), and M_{GlcA} values 0.35, 0.59 and 0.69, respectively. The sulphate and monosaccharide (Gal, Glc, Fuc) composition of A_4–A_6, determined after hydrolysis with 0.5 N HCl, were respectively: A_4 3:1:2:1, A_5 2:1:2:1 and A_6 2:1:1:1. Reduction of A_4A_6 with borohydride, followed by acid hydrolysis, gave galactose, glucose and fucose in the molar ratios: A_4 1:1:1; A_5 1:2:0; A_6 1:0:1. These results demonstrated the presence of glucose as the reducing terminus of A_4 and A_6, and fucose of A_5, and the presence of the respective degree of polymerization of the oligosaccharides A_4–A_6 as 4,4, and 3. However, A_4–A_6 showed negative optical rotations, but the amounts available were too small to determine accurate values.

Acid hydrolysis of the non-dialysable, water-insoluble residue B (Found: total carbohydrates, 26.9, protein, 67.3, $SO_4^{=}$, 0.17%) followed by paper chromatography (solvent A), revealed the presence of glucuronic acid, galactose, glucose, mannose, xylose, and fucose in the molar ratios 1.8:1.1:1.1:1.0:1.3:1.8. Furthermore, sample B was subjected to another acid hydrolysis followed by paper chromatography (solvents B and C) to determine its amino acid pattern. The results revealed the presence of cysteine (1.4%), lysine (1.0%), histidine (0.3%), arginine (4.3%), aspartic acid (8.2%), serine (6.7%), glycine (16.1%), glutamic acid (8.8%), threonine (7.9%), alanine (9.4%), proline (0.3%), tyrosine (0.5%), methionine (0.4%), valine (0.9%), phenylalanine (0.3%), isoleucine (0.3%) and leucine (0.6%). These results indicated that sample B comprises a major protein moiety linked to polysaccharide.

Complete hydrolysis of the non-dialysable, water-soluble product C followed by paper chromatography (solvent A) showed the presence of glucuronic acid, galactose, glucose, mannose, xylose and fucose in the molar ratio 7.1:1.0:3.0:3.2:1.8:1.7, respectively. Protein was absent in C whereas sulphate was present in lower proportion (4.6%) as compared to that of the original material (18.6%).

In a further attempt to determine the structure of this polysaccharide, partial hydrolysis with *N*-oxalic acid was investigated. A dialysable fraction D and two non-dialysable fractions (E and F) were obtained. Fraction D contained glucuronic acid, galactose, glucose, manose, xylose and fucose in the molar ratio 2.5:6.4:1.1:1.0:11.5:12.8 and oligosaccharides. The detection of relatively higher proportions of free xylose and fucose in both A and B indicated the presence of these sugar residues near the periphery of the molecule.

Acid hydrolysis of E (found: crude protein, 65.73, total carbohydrates, 28.4, SO_4^{2-}, 0.2%) followed by paper chromatography indicated the presence of glucuronic acid, galactose, glucose, mannose, xylose and fucose in the molar ratio 2.8:1.0:1.1:1.2:2.4:2.0. On the other hand, the amino acid pattern of E was as follows: cysteine (3.2%), lysine (1.3%), histidine (1.5%), arginine (3.3%), aspartic acid (11.8%), serine (6.4%), glycine (17.1%), glutamic acid (5.3%), threonine (4.3%), alanine (5.6%), proline (0.3%), phenylalanine (0.3%), tyrosine (0.4%), methionine (0.2%), valine (0.6%), isoleucine (0.3%) and leucine (0.4%). It is worth noting that the analytical composition of E is, to a great extent, similar to that of B.

Complete acid hydrolysis of another non-dialysable product F (found: crude protein, 0.0, SO_4^{2-}, 1.2%) with sulphuric acid afforded (PC, solvent A) glucuronic acid, mannose and glucose in the molar ratio 1.6:1.0:1.3. These results revealed that the inner portion of the macromolecule comprised glucuronic acid, mannose and glucose residues.

Autohydrolysis of the free acid form of the original polysaccharide afforded a dialysable (G) and non-dialysable product (H). Fraction G contained the free sugars galactose, glucose, xylose, and fucose in the molar ratio: 1.0:1.4:7.0:10.2, and was separated into neutral (G1) and acidic (G2) fragments as previously described. Preparative paper chromatography of G1 gave four neutral oligosaccharides (G_1–G_4) of which G_1 and G_2 stained brown with aniline hydrogen phthalate, whereas G_3 and G_4 stained pink. G_1–G_4 showed negative optical rotations, but the amounts available were too small to determine accurate values. Acid hydrolysis of G_1–G_4 afforded galactose, glucose, xylose, and fucose in the molar ratio G_1 1:1:2:2; G_2 1:2:1:0; G_3 1:1:1:1 and G_4 1:1:2:2. After reduction with borohydride, these ratios changed as follows: G_1 0:1:2:2; G_2 0:2:1:0; G_3 1:1:0:1 and G_4 1:0:2:2. These data indicated that galactose was the reducing terminus of G_1 and G_2 whereas xylose and glucose were of G_3 and G_4, respectively. Acid hydrolysis of the non-dialysable material H gave glucuronic acid, galactose, glucose, mannose, xylose, and fucose in the molar ratio 2.4:1.0:1.8:2.0:1.3:1.2, and 8.97% sulphate.

Thus it may be concluded that the present polysaccharide involves, in addition to a protein moiety, a portion composed of D-glucuronic acid, D-mannose, and D-glucose residues and other portions involving residues of D-galactose, D-glucose, D-xylose and L-fucose, The D and L nature of the monosaccharides have been assumed by analogy with other fucans.

Treatment of the algal polysaccharide P with alkali provided evidence for the location of sulphate groups on D-galactose residues. Sulphate ions were detected in the dialysable products after such treatment, whereas the non-dialysable material I gave positive tests (resorcinol and 5-hydroxymethyl-2-furaldehyde) for 3,6-anhydrogalactose. This result indicated the presence of galactose 3- and/or 6-sulphate residues. Acid hydrolysis of I afforded (PC, solvent A) glucuronic acid, galactose, glucose, mannose, xylose, and fucose in the molar ratios 3.5:1.0:1.1:1.3:1.7:1.8. As mentioned before [1] the molar ratios of these sugars in the original material P are 2.8:1.0:1.2:1.2:1.5:1.5. The decrease in the galactose content, on desulphation with alkali, accords with the formation of 3,6-anhydrogalactose. On the other hand, the fucose content did not decrease and this indicated the alkali stability of sulphate groups attached to fucose residues and suggested the presence of fucose 4-sulphate moieties in the polysaccharide molecule. (The alkali-treated polysaccharide material contains 5.7% of SO_4^{2-}). This result indicates the presence of (1 → 2)-linked fucose 4-sulphate and/or (1 → 3)-linked fucose 4-sulphate in the present polymer.

Periodate oxidation of P was carried out under unbuffered conditions to facilitate titration of the released formic acid. The results indicated that the oxidation of the original polymer stopped after 24 hr and the reduction of periodate was then 0.47 mol per 'anhydro-sugar unit'. The low reduction of periodate is partially due to sulphated residues and also indicated the presence of (1 → 3)-linked units and branch points.

From the amount of formaldehyde and formic acid released during periodate oxidation, values of 50 and 100 for the average degree of polymerization for the whole polysaccharide molecule and each of its branches were calculated. On the basis of the degree of polymerization of the polysaccharide and its composition, it may be deduced that, on average P is composed, of *ca* 15 glucuronic acid residues, 6–7 mannose residues, 6–7 glucose residues, 5–6 galactose residues, 8 xylose residues, and 8 fucose residues.

Reduction of the resulting polyaldehyde with KBH_4, followed by hydrolysis (solvents A, D and E) revealed the presence of erythronic acid, glyceric acid, glucuronic acid, erythritol, threitol, mannose, glucose, galactose, xylose, fucose, glycolic aldehyde, glycerol and propane-1,2-diol. The formation of erythronic acid demonstrated the presence of (1 → 4)-linked glucuronic acid residues whereas the detection of glyceric acid indicated the presence of glucuronic acid as a non-reducing end to the chains. The formation of erythritol demonstrated the presence of (1 → 4)-linked mannose and/or (1 → 4)-linked glucose residues whereas the formation of threitol referred to the presence of (1 → 4)-linked galactose. The detection of six unreduced sugars indicated that some of these sugar residues are resistant towards periodate oxidation. The formation of glycolic aldehyde in the hydrolysate confirmed the presence of (1 → 4)-linked sugar residues. The resistance of some galactose residues to oxidation may be due to the presence of sulphate groups. This result, together with the demonstration of the presence of (1 → 4)-linked galactose residues, provides confirmation for the presence of galactose 3-sulphate residues. The previous detection of sulphate-free xylose in the exterior portion and the resistance of a higher proportion of the xylose residues towards periodate oxidation, suggest the presence of (1 → 3)-linked xylose residues. On the other hand, the detection of only a trace of glycerol in the polyalcohol hydrolysate confirmed the presence of mannose or glucose as the reducing terminus of the polysaccharide. The appearance of an immune portion of fucose in the polyalcohol hydrolysate accords with the previous demonstration of the presence of (1 → 2)-linked fucose 4-sulphate and/or (1 → 3) linked fucose 4-sulphate residues in the present polymer. Finally, the minute proportion of propane-1,2-diol detected in the polyalcohol hydrolysate indicated the possibility of a small proportion of sulphate-free (1 → 2)-linked fucose residues. However, the presence of fucose residues attached with the last-named glycosidic linkage may indicate the presence of 1 → 2)-linked fucose 4-sulphate residues.

The results of the present studies collectively revealed that the polysaccharide part of the algal macroheteromolecule appears to be constructed of an inner portion composed of (1 → 4)-linked β-D-glucuronic acid, (1 → 4)-linked β-D-mannose and (1 → 4)-linked β-D-glucose residues. To this inner portion, heteropolymeric paritally sulphated exterior chains are attached, comprising various proportions of (1 → 4)-linked β-D-galactose, β-D-galactose 3-sulphate residues, (1 → 4)-linked β-D-glucose residues, (1 → 2)-linked α-L-fucose 4-sulphate residues and (1 → 3)-linked β-D-xylose residues. Finally, fucose and xylose residues appear to be nearer to the periphery of the molecule

EXPERIMENTAL

Details of analytical methods are given in the preceding paper [1]. The following additional solvents were used for PC: D, n-BuOH-C_5H_5N-H_2O (3:1.1:1.5) [2]; E, EtOAc–HOAc–H_2O (3:1:3) [3]. Ionophoresis [4] of the acidic oligosaccharides was carried out on Whatman No. 1 paper in C_5H_5N–HOAc buffer (0.05 M, pH 6) for 2 hr at 300 V. Hydrolysis of the neutral and acidic oligosaccharides was effected with the methods of refs. [5] and [6], respectively. The degree of polymerization of the oligomers was determined by the method of ref. [7]. The polysaccharide investigated herein is the purified material described in the preceding paper [1].

Partial acid hydrolysis with 0.5 N *oxalic acid.* The purified polysaccharide P [1] (1 g) was hydrolysed with 0.5 N oxalic acid (40 ml) for 2 hr at 100° and 3 products: A 0.35 g; B 0.09 g; and C 0.55 g were recovered [8]. From product A, 3 neutral and 3 acidic oligosaccharides were separated [8].

Partial hydrolysis with oxalic acid. The polysaccharide P (2 g) was hydrolysed [9] with N oxalic acid (80 ml) for 5 hr. After cooling, the resulting hydrolysate was dialysed against dist. H_2O (4 × 300 ml) for 48 hr and 3 degraded products were obtained; these were the dialysable material D and the non-dialysable portions E and F. The insoluble non-dialysable product E (0.18 g) and the supernatant F (0.60 g) were isolated by centrifugation.

Autohydrolysis of the free-acid polysaccharide. A solution of the polysaccharide P (2 g) in H_2O (80 ml) was treated as described previously [8]. The conc. combined dialysates (50 ml, product G) were separated into neutral (G1) and acidic (G2) fragments and the soluble material H was recovered from the dialysis sac (1.5 g).

Alkaline desulphation of P. The polysaccharide material P (1.5 g) was treated with NaOH according to the method of ref. [10]. Thereafter, the alkaline soln was neutralized with Lewatit S-100 (H^+) resin, dialysed against dist. H_2O for several hours, and then freeze-dried to give product I (1.2 g) (Found. SO_4^{2-}, 5.7%). The presence of 3,6-anhydrogalactose in I was determined by the resorcinol [11] and 5-hydroxymethyl-2-furaldehyde [12] tests.

Periodate oxidation of P. The polysaccharide sample was oxidized with 16 mM $NaIO_4$ (250 ml) at 2° in the dark. Aliquots (5 ml) were withdrawn at intervals and used for determination of consumed IO_4^- [13] and released formic acid [14]. At the end of oxidation, the released formaldehyde was determined [15].

Reduction of the periodate-oxidised material. After oxidation, the residual IO_4^- was reduced with ethylene glycol, and after 2 hr the soln was dialysed against several changes of dist. H_2O and concd to *ca* 100 ml. To this soln, KBH_4 (1 g) was added and the mixture was kept overnight. Thereafter, excess borohydride was decomposed with HOAc and the solution was treated with Lewatit S-100 (H^+) resin, neutralized with NH_3 and concd. The polyalcohol was pptd with 4 vol. EtOH.

Hydrolysis of the polyalcohol. This was effected by the method of ref. [16]. The hydrolysate was treated with Lewatit S-100 (H^+) resin and subjected to PC (solvents A, D and E) using appropriate reference compounds.

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