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SOME STRUCTURAL FEATURES OF SARGASSAN, A SULPHATED HETEROPOLYSACCHARIDE FROM *Sargassum linifolium*

A. FOUAD ABDEL-FATTAH, M. MAGDEL-DIN HUSSEIN, AND H. MOHAMED SALEM

Laboratory of Microbiological Chemistry, National Research Centre, Dokki, Cairo, and Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza (Egypt)

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ABSTRACT

The acid-extractable, water-soluble, sulphated heteropolysaccharide, sargassan, contains residues of D-glucuronic acid, D-mannose, D-galactose, D-xylose, and L-fucose, and a protein moiety. Partial, acid hydrolysis of sargassan and autohydrolysis of the free-acid polysaccharide have been studied. Several acidic and neutral oligosaccharides were subsequently isolated. Evidence is advanced for the presence of ester sulphate on some galactose and fucose residues. It is concluded that the carbohydrate moiety of sargassan involves a backbone chain of D-glucuronic acid and D-mannose residues, and side chains involving residues of D-galactose, D-xylose, and L-fucose with sulphate attached to some galactose and fucose residues.

INTRODUCTION

Recently, we reported[1] the isolation of a hitherto unrecognised, acid-extractable, water-soluble, sulphated heteropolysaccharide, sargassan, from *Sargassum linifolium*. It has been shown that sargassan contains residues of D-glucuronic acid, D-mannose, D-galactose, D-xylose, and L-fucose, sulphate, and a protein moiety.

In a preceding paper[2], it was shown that sargassan could not be obtained free from protein, and some results of partial, acid hydrolysis were reported.

We now report further on the elucidation of the structure of sargassan.

EXPERIMENTAL

General. — Details are given in the preceding paper[2]. The following additional solvents were used for p.c.: *F* 1-butanol–ethanol–water[3] (4:1:5) and *G* 1-butanol–ethanol–water–ammonia[4] (40:10:49:1). The chromatographic mobilities of oligosaccharides relative to glucuronic acid, galactose, xylose, and fucose are designated R_{GlcA}, R_{Gal}, R_{Xyl}, and R_{Fuc}, respectively. The distance migrated during ionophoresis by the sulphated fragments relative to glucuronic acid is designated M_{GlcA}. Hydrolysis of sulphated oligosaccharides was effected with 0.5M hydrochloric acid[5]. The degree of polymerisation (d.p.) of oligomers was determined by the method of Timell[6].

The sargassan investigated herein is the purified material described in the preceding paper[2].

*Partial, acid hydrolysis of purified sargassan with 0.5*N *oxalic acid.* — The details of this hydrolysis are given in the preceding paper[2]; the hydrolysis was performed for 2 h. The acidic material was subjected to preparative, paper electrophoresis on Whatman 3MM paper to give fractions *D*, *E*, and *F*. P.c. (solvent *A*) of *D*, *E*, and *F* resolved *F* only into three syrupy, acidic oligosaccharides (*F1*, *F2*, and *F3*).

Methylation and hydrolysis of acidic oligosaccharides. — Using the method of Perila and Bishop[7], each oligosaccharide (0.5–2 mg) was shaken with methyl iodide (0.2 ml), *N*,*N*-dimethylformamide (0.2 ml), and silver oxide (0.2 g) at room temperature in the dark for 18 h. Thereafter, the reaction mixture was filtered and the residue was washed with chloroform. The combined filtrate and washings were dried ($CaCl_2$) and evaporated. The methylated oligosaccharide was heated with 1% methanolic hydrogen chloride (2 ml) at 100° for 7 h. After removal of solvent, the residual syrup was hydrolysed with 0.5M hydrochloric acid (2 ml) at 100° for 6–8 h. Volatile acid was removed by repeated evaporation with water under diminished pressure. The resulting methylated sugars were subjected to p.c. (solvents *B* and *C*) with 2,3,4,6-tetra-*O*-methyl-D-glucose as marker.

Partial, acid hydrolysis of purified sargassan with N *oxalic acid.* — (*a*) Sargassan (1 g) was hydrolysed[8] with N oxalic acid (20 ml) at 100°. After 1 h, 5 ml of the hydrolysate were treated with ethanol (20 ml) and centrifuged. The precipitate was returned to the original solution, and the supernatant was neutralised with calcium carbonate, filtered, treated with Lewatit S-100(H^+) resin, and freeze-dried (product *G*). After 2-h hydrolysis, another 5 ml of the hydrolysate were treated as described above to give *H*. After 5-h hydrolysis, the remaining hydrolysate (10 ml) was treated with 3 vol. of ethanol (precipitate *J*, 0.14 g) and the supernatant was treated as described above to give *I*.

(*b*) Sargassan (1 g) was hydrolysed in N oxalic acid (20 ml) at 100° for 5 h. Thereafter, the hydrolysis mixture was cooled and dialysed against distilled water (4 × 300 ml), and the combined dialysates were neutralized with calcium carbonate, filtered, treated with Lewatit S-100(H^+) resin, and concentrated to 50 ml (product *K*). The non-dialysable material was isolated by freeze-drying (product *L*, 0.05 g).

Fractionation of acidic oligosaccharides. — Product *K* was fractionated into neutral and acidic fragments by chromatography on Amberlite IR-400(AcO^-) resin, as previously described[2]. The acidic fragment (*M*) afforded a single band on electrophoresis, but p.c. (solvent *A*) yielded three acidic oligosaccharides (*M1*, *M2*, and *M3*) with the following R_{GlcA}, R_{Gal}, R_{Xyl}, and R_{Fuc} values: *M1* 0.57, 0.09, 0.05, 0.04; *M2* 0.10, 0.15, 0.08, 0.07; *M3* 1.79, 0.27, 0.15, 0.13.

Reduction and hydrolysis of acidic oligosaccharides. — The method[7] of Perila and Bishop was used as described in the preceding paper[2].

Autohydrolysis of sargassan. — A solution of sargassan (2 g) in water (20 ml) was converted into the free-acid form with Lewatit S-100(H^+) resin. The resulting solution was dialysed against distilled water (200 ml) maintained at 100° with stirring.

The dialysate was replaced by fresh, distilled water after 1, 3, and 5.5 h. Thereafter, the combined dialysates were concentrated *in vacuo* to ~50 ml (product *N*). The solution remaining in the dialysis sac was neutralized with ammonia and freeze-dried (product *O*, 0.32 g). Product *N* was separated into neutral and acidic fragments by chromatography on Amberlite IR-400(AcO^-) resin, as described before. On paper electrophoresis, the acidic fragments afforded five products. Preparative p.c. (solvent *A*) of the neutral fragments gave four oligosaccharides (*P1–P4*) having the following R_{Xyl} and R_{Fuc} values: *P1* 0.13, 0.12; *P2* 0.33, 0.29; *P3* 0.49, 0.44; *P4* 0.65, 0.58. The reducing units were identified by treatment with borohydride as in the preceding experiments.

Desulphation of sargassan. — Sargassan (1.61 g) dissolved in water (250 ml) was reduced with sodium borohydride (0.2 g) at room temperature for 48 h. Sodium hydroxide (10 g) and sodium borohydride (3 g) were added to the solution which was then kept at 80° for 2 h. Thereafter, the solution was neutralised with Lewatit S-100(H^+) resin, dialysed for several hours against distilled water, and freeze-dried (product *R*). The presence of 3,6-anhydrogalactose in *R* was detected by the resorcinol[9] and 5-hydroxymethyl-2-furaldehyde[10] tests.

RESULTS AND DISCUSSION

After partial hydrolysis of purified sargassan[2] with 0.5N oxalic acid at 100° for 2 h, the dialysable material contained galactose, xylose, and fucose in the molar ratios 1.12:1.00:1.04, and oligosaccharides. The mixture was fractionated on Amberlite IR-400(AcO^-) resin into neutral and acidic material, and the latter was further fractionated by preparative, paper electrophoresis into three syrupy, acidic products *D*, *E*, and *F* having M_{GlcA} values 0.3, 0.68, and 0.98, respectively. Product *F* yielded three acidic oligosaccharides (*F1*, *F2*, and *F3*) on paper chromatography. Product *D* was immobile on paper chromatography and *E* moved as a single spot. All the acidic fragments stained brown with aniline hydrogen phthalate and were found to be oligomers composed of either galactose or fucose residues.

Acidic saccharide D (R_{Xyl} 0.0; R_{Fuc} 0.0; d.p. >4; OSO_3^-, 28.71%) contained galactose. After methylation and hydrolysis, it afforded 2,3-di-, 2,3,4-tri-, and 2,3,4,6-tetra-*O*-methyl-D-galactose (paper chromatography). Detection of the last-named, methylated sugar indicated the presence of a sulphate-free galactose residue as the non-reducing terminus. The formation of 2,3-di-*O*-methyl-D-galactose demonstrated the presence of galactose 6- and/or 4-sulphate residues. However, the detection of 2,3,4-tri-*O*-methyl-D-galactose might be due to contamination of *D* with oligosaccharide material containing galactose 6-sulphate at the non-reducing terminus.

Acidic saccharide E (R_{Xyl} 0.09; R_{Fuc} 0.08; d.p. 3; 3 OSO_3^- groups) contained galactose. After methylation and hydrolysis, paper chromatography revealed 2,3-di- and 2,3,4-tri-*O*-methyl-D-galactose, the former indicating the presence of galactose 6- and/or 4-sulphate residues, and the latter the presence of galactose 6-sulphate at the non-reducing terminus.

Acidic saccharide F1 (R_{Xyl} 0.29; R_{Fuc} 0.25; d.p. 2; 3 OSO_3^- groups) contained galactose. Methylation and hydrolysis gave 2,3-di- and 2,4-di-*O*-methyl-D-galactose, the former indicating the presence of galactose 6- and/or 4-sulphate residues, and the latter the presence of galactose 3,6-disulphate as the non-reducing terminus.

Acidic saccharide F2 (R_{Xyl} 0.46; R_{Fuc} 0.39; d.p. 2; 2 OSO_3^- groups) contained fucose. Methylation and hydrolysis gave 3-*O*-methyl-L-fucose and 2,3-di-*O*-methyl-L-fucose. The former demonstrated the presence of fucose 2- or 4-sulphate as the reducing terminus, and the latter the presence of fucose 4-sulphate as the non-reducing moiety.

Acidic saccharide F3 (R_{Xyl} 0.54; R_{Fuc} 0.47; d.p. 1; 1 OSO_3^- group) contained fucose, and on methylation and hydrolysis gave 2,3-di-*O*-methyl-L-fucose only. *F3* was fucose 4-sulphate.

The above-mentioned, acidic oligosaccharides showed negative optical rotations, but the amounts available were too small to record accurate values. The presence of β-D- and α-L-glycosidic linkages is thereby indicated.

In a preceding paper[2], it was concluded that sargassan contained a backbone chain composed mainly of D-glucuronic acid and D-mannose residues, with a small proportion of D-galactose, and side chains involving D-galactose, D-xylose, and L-fucose residues. The presence of portions of side chains composed of partially sulphated D-galactose or L-fucose residues has now been established.

In further investigating the backbone structure, sargassan was partially hydrolyzed with N oxalic acid[8] at 100°. Three alcohol-soluble fragments (*G*, *H*, and *I*) and an alcohol-insoluble residue (*J*) were obtained. *G*, *H*, and *I* contained galactose, xylose, and fucose in the molar ratios 1.13:1.0:1.18, 1.47:1.0:1.47, and 1.90:1.0:1.45, respectively. *I*, obtained after acid treatment for 5 h, also contained traces of glucuronic acid and mannose, indicating fragmentation of the backbone chain. Most of *I* was immobile in paper chromatography, indicating the unsuitability of ethanol for precipitating the backbone portion of sargassan.

Acid hydrolysis of *J*, followed by paper chromatography, indicated it to be a protein and to contain the 17 amino acids previously found[2] in the protein moiety of sargassan.

In a further attempt to isolate the backbone portion of sargassan, partial hydrolysis with N oxalic acid for 5 h at 100° was investigated. Dialysable (*K*) and non-dialysable fractions (*L*) were obtained.

Fraction *K* contained galactose, xylose, and fucose in the molar proportions of 1.81:1.00:1.42. The acidic material (*M*), M_{GlcA} 0.78, in fraction *K*, separated from neutral compounds by chromatography on Amberlite IR-400(AcO^-) resin, was resolved by preparative, paper chromatography into three acidic oligosaccharides (*M1*, *M2*, and *M3*).

The sulphate and monosaccharide compositions of *M1–M3*, determined after hydrolysis with 0.5M hydrochloric acid, were as follows:

	OSO_3^-	*GlcA*	*Gal*	*Man*	*Xyl*	*Fuc*
M1	1	3	1	2	1	—
M2	1	2	1	—	1	2
M3	2	1	1	—	1	1

Reduction of *M1–M3* with borohydride, followed by acid hydrolysis, gave monosaccharides in the following molar proportions:

	GlcA	*Gal*	*Man*	*Xyl*	*Fuc*
M1	3	1	2	—	—
M2	2	1	—	1	1
M3	1	1	—	—	1

These results demonstrated the presence of xylose as the reducing terminus of *M1* and *M3*, and fucose of *M2*.

Hydrolysis of *L* with M sulphuric acid afforded (paper chromatography) glucuronic acid and mannose in the molar ratio 2.8:1.0, and a trace of galactose. This result indicated that the backbone chain of sargassan involves mainly residues of D-glucuronic acid and D-mannose. Furthermore, *L* contained a negligible amount of sulphate.

Autohydrolysis of the free-acid form of sargassan afforded a dialysable (*N*) and a non-dialysable product (*O*). Fraction *N* contained galactose, xylose, and fucose in the molar proportions 1.0:5.0:5.48, and was separated into neutral (*P*) and acidic fragments by chromatography on Amberlite IR-400(AcO^-) resin. Paper chromatography of *P* gave four neutral oligosaccharides (*P1–P4*), of which *P1* stained brown with aniline hydrogen phthalate, whereas *P2–P4* stained pink. *P1–P4* showed negative optical rotations, but the amounts available were too small to determine accurate values.

Acid hydrolysis of *P1–P4* gave galactose, xylose, and fucose in the following molar ratios: *P1* 3:1:1; *P2* 2:2:1; *P3* 1:2:1; *P4* 0:2:1. After reduction with borohydride, these ratios changed as follows: *P1* 2:1:1; *P2* 2:1:1; *P3* 1:1:1; *P4* 0:1:1. These results indicated that galactose was the reducing terminus of *P1*, and xylose of *P2–P4*.

Acid hydrolysis of the non-dialysable material *O* gave glucuronic acid, galactose, and mannose, in the molar ratios 3.01:1.36:1.00, and 8.42% of sulphate.

Autohydrolysis of the free-acid form of sargassan thus demonstrated the presence of side chains involving portions composed of D-galactose, D-xylose, and L-fucose residues. The autohydrolysis conditions were, however, insufficient to remove galactose from the sargassan backbone.

Treatment of sargassan with alkali provided evidence for the location of sulphate groups on D-galactose and L-fucose residues. Sulphate ion was detected in the dialysable products after such treatment. The non-dialysable material (*R*) gave positive tests (resorcinol and 5-hydroxymethyl-2-furaldehyde) for 3,6-anhydro-

galactose. This result indicated the presence of galactose 3- and or 6-sulphate in sargassan.

Acid hydrolysis of *R* afforded (paper chromatography) glucuronic acid, galactose, mannose, xylose, and fucose, in the molar ratios 3.73:3.97:1.00:1.14:2.45, respectively. As mentioned above, the molar ratios of these sugars in sargassan are 4.57:8.40:1.00:2.48:2.53. The significant decrease in the galactose content on desulphation of sargassan with alkali accords with the formation of 3,6-anhydrogalactose. On the other hand, the insignificant change in fucose content indicates the alkali-stability of the sulphate group attached to fucose and suggests the presence of fucose 4-sulphate moieties in sargassan; the alkali-treated sargassan contains 4.46% of sulphate.

Thus, it may be concluded that sargassan involves, in addition to a protein moiety, a backbone chain composed of D-glucuronic acid and D-mannose residues, and side chains involving residues of D-galactose, D-xylose, and L-fucose with sulphate groups attached to some galactose and fucose residues.

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