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CONSTITUTION OF SARGASSAN, A SULPHATED HETEROPOLYSACCHARIDE FROM *Sargassum linifolium*

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ABSTRACT

The behaviour towards periodate of the brown-algal polysaccharide sargassan before and after partial hydrolysis, alkali treatment, and methanolysis has been studied Evidence is thereby provided that the sargassan backbone is composed of (1→4)-linked β-D-glucuronic acid and β-D-mannose residues Heteropolymeric, partially sulphated branches are attached to the backbone, and these branches comprise various proportions of (1→4)-linked β-D-galactose, β-D-galactose 6-sulphate, and β-D-galactose 3,6-disulphate residues, (1→2)-linked α-L-fucose 4-sulphate residues, and (1→3)-linked β-D-xylose residues

INTRODUCTION

We have reported[1] on the isolation from the brown alga *Sargassum linifolium* of a new sulphated heteropolysaccharide, sargassan, composed of D-glucuronic acid, D-mannose, D-galactose, D-xylose, and L-fucose residues, as well as a protein moiety Evidence was obtained[2] which indicated that the carbohydrate part of sargassan involves a backbone composed of D-glucuronic acid and D-mannose residues, with side chains containing residues of D-galactose, D-xylose, and L-fucose, and having sulphate groups attached to some galactose and fucose residues We now report further on the constitution of the polysaccharide part of sargassan

EXPERIMENTAL

General — Paper chromatography (p c) was performed on Whatman No 1 paper with the following solvent systems *A* 1-butanol–ethanol–water[3] (40 11 19), *B* 1-butanol–pyridine–water[4] (3 1 1 1 5), *C* ethyl acetate–acetic acid–water[5] (3 1 3) Detection was effected with aniline hydrogen phthalate, alkaline silver nitrate, and aniline–xylose reagents[6] Ash content was determined by heating the polysaccharide sample to constant weight at 800° Protein was estimated by the method of Lowry *et al* [7] Complete, acid hydrolysis of polysaccharide material was performed with sulphuric acid[8] Determination of sugars in the acid hydrolysates was done, after

descending p c (solvent *A*) for 48 h, according to the method of Wilson[9], with the appropriate corrections Total carbohydrate content was determined by the phenol–sulphuric acid method[10] The total inorganic sulphate liberated by hydrolysis with hydrochloric acid[11] was determined by barium chloranilate[12] All solutions were concentrated *in vacuo* at 40° in a rotary evaporator

Sargassum linifolium was the brown-algal species used in the present work It was collected by the Edfina Co for Preserved Foods from the Alexandrian coast during 1969 The algae were washed with running water to remove foreign substances, and then air-dried and milled

Preparation of purified sargassan — The preparation was performed as previously described[13], giving material having $[\alpha]_D^{30}$ −78 5° (Found total carbohydrate, 78 2, ash, 12 2, SO_4^{2-} 17 68, protein, 3 85%) On complete, acid hydrolysis, sargassan afforded (p c , solvent *A*), D-glucuronic acid, D-galactose, D-mannose, D-xylose, and L-fucose, in the molar ratios of 4 57 8 40 1 00 2 48 2 53

Degradation of sargassan — Portions (1 g) of sargassan were hydrolysed with 40 ml of 0 5N oxalic acid at 100° for 1 and 2 h, respectively, and with 20 ml of N oxalic acid at 100° for 5 h Each hydrolysate was cooled and dialysed against distilled water (4 × 300 ml) The contents of the dialysis sacs were then centrifuged, and the supernatants were lyophilized to afford products *A*, *B*, and *C*, respectively, for which the following data were obtained *A* (0 3 g) (Found SO_4^{2-}, 7 14%), on complete hydrolysis with acid, gave (p c , solvent *A*) glucuronic acid, galactose, and mannose, in the molar ratios of 3 25 0 85 1 00 *B* (0 2 g) (Found SO_4^{2-}, 3 42%), on complete hydrolysis with acid, gave (p c , solvent *A*), glucuronic acid, galactose, and mannose, in the molar ratios of 3 20 0 48 1 00 *C* (0 05 g) contained a negligible amount of sulphate and, on complete hydrolysis with acid, afforded (p c) only glucuronic acid and mannose, in the molar ratio of 2 8 1 0, and a trace of galactose

Sargassan (2 g) dissolved in water (20 ml) was converted into the free-acid form with Lewatit S-100(H^+) resin The resulting solution was dialysed against stirred, distilled water at 100° The dialysate was replaced by fresh, distilled water after 1, 3, and 5 5 h Thereafter, the solution remaining in the dialysis sac was neutralised with ammonia and freeze-dried to give *D* (0 32 g) (Found SO_4^{2-}, 8 42%) Complete, acid hydrolysis of *D* gave (p c) glucuronic acid, galactose, and mannose, in the molar ratios of 3 01 1 36 1 00

Desulphation of sargassan — Sargassan (1 61 g) was treated with sodium hydroxide according to the method of Rees[14] Thereafter, the alkaline solution was neutralised with Lewatit S-100(H^+) resin, dialysed for several hours against distilled water, and then freeze-dried to give *E* (1 2 g) (Found SO_4^{2-}, 4 46%) On complete, acid hydrolysis, *E* gave (p c) glucuronic acid, galactose, mannose, xylose, and fucose, in the molar ratios of 3 73 3 97 1 00 1 14 2 45

Sargassan (1 g) was shaken with 0 1M methanolic hydrogen chloride[3] (50 ml) at room temperature for 26 h The residual, undissolved material was dissolved in water, dialysed against distilled water, and then freeze-dried to give *F* (0 61 g) (Found SO_4^{2-}, 4 51%) On complete hydrolysis with acid, *F* gave (p c , solvent *A*) glucuronic acid,

galactose, mannose, xylose, and fucose, in the molar ratios of 2 29 4 17 1 00 0 75 1 20

Periodate oxidation of sargassan and the modified polysaccharides A–F — (*a*) *In buffered solution* Sargassan (400 mg) was dissolved in 0 2M acetate buffer (pH 3 7, 125 ml), 0 32M sodium metaperiodate (125 ml) was added, and the mixture was set aside at 2° in the dark At intervals, aliquots (3 ml) were removed and the reduction of periodate was measured[15]

(*b*) *In unbuffered solution* Sargassan and polysaccharides *A–F* were separately oxidised with 16mM sodium metaperiodate (250 ml) at 2° in the dark[3] Aliquots (5 ml) were withdrawn at intervals and used for the determination of consumed periodate[15] and released formic acid[16] At the end of the oxidation process, the released formaldehyde was determined[17]

Reduction of the periodate-oxidised materials[3] — At the end of the oxidation process, the residual periodate was reduced with ethylene glycol and, after 2 h, the solution was dialysed against several changes of distilled water and concentrated to ~100 ml To this solution, potassium borohydride (1 g) was added and the mixture was kept overnight Thereafter, excess borohydride was decomposed with acetic acid and the solution was treated with Lewatit S-100(H^+) resin, neutralised with ammonium hydroxide, and concentrated The polyalcohol was precipitated by the addition of 4 vol of ethanol

Hydrolysis of the polyalcohols — Hydrolysis was effected by the method of Drummond *et al* [18] Each hydrolysate was treated with Lewatit S-100(H^+) resin and subjected to p c (solvents *A*, *B*, and *C*), using the appropriate reference compounds

RESULTS AND DISCUSSION

No significant difference was noted between the efficiency of oxidation of sargassan by periodate in buffered or unbuffered solution The data for unbuffered conditions are recorded in Table I The data indicate that oxidation of sargassan with periodate stopped after 24 h and the reduction of periodate was then 0 58 mole per "anhydro-sugar" unit The low reduction of periodate is partly due to sulphated residues and also indicates the presence of (1→3)-linked units and branch points

From the amount of formaldehyde and formic acid released during the oxidation, average degrees of polymerisation (d p) of 52 9 and 8 4 for the whole sargassan molecule and each of its branches, respectively, were calculated Since it has been demonstrated earlier[2] that the carbohydrate part of sargassan involves a backbone composed of D-glucuronic acid and D-mannose residues, the release of formaldehyde provides evidence that mannose is the aldehydic end of the sargassan molecule On the basis of the d p of sargassan and its monosaccharide composition, it may be deduced that the backbone is composed of 12–13 glucuronic acid residues and 3–4 mannose residues, whereas 25 galactose, 7 xylose, and 7 fucose residues are incorporated into the branches An average number of 4–5 branches for each sargassan molecule may be deduced by dividing the total number (39) of galactose, xylose, and fucose residues by the average d p (8 4) of each branch

TABLE I

PERIODATE OXIDATION OF SARGASSAN AND THE SAMPLES[a] OF DEGRADED AND DESULPHATED POLYSACCHARIDE

Time (h)	*Periodate reduced (mole/"anhydro-sugar" unit)*						
	Sargassan	*Sample A*	*B*	*C*	*D*	*E*	*F*
1	0 205	0 475	—	—	0 259	0 269	0 279
2	—	0 567	0 544	0 471	—	0 385	—
3	0 270	—	—	—	0 388	—	0 419
5	0 340	0 717	0 725	0 634	0 582	—	0 488
6	—	—	—	—	—	0 561	—
7	0 380	0 779	0 815	0 865	—	—	—
8	—	—	—	—	0 711	0 619	0 620
9	0 444	0 858	0 906	1 022	—	—	—
10	0 481	—	—	—	—	—	—
24	0 580	0 930	1 040	1 100	0 906	0 677	0 837
48	0 580	0 930	1 040	1 100	0 906	0 677	0 837

[a] *A* and *B* obtained by treatment of sargassan with 0 5N oxalic acid at 100° for 1 and 2 h respectively *C* obtained by partial hydrolysis of sargassan with N oxalic acid at 100° for 5 h, *D* autohydrolysed sargassan, *E* alkali-treated sargassan, *F* methanolysed sargassan

Reduction of the sargassan polyaldehyde with potassium borohydride, followed by hydrolysis and paper chromatography, revealed the presence, *inter alia*, of erythronic acid, glyceric acid, glucuronic acid, erythritol, and mannose 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 the non-reducing end of the backbone The formation of erythritol demonstrated the presence of (1→4)-linked mannose residues, and the detection of glucuronic acid and mannose in the polyalcohol hydrolysate indicated that some of the sugar residues in the backbone are resistant towards oxidation by periodate

Threitol was also detected in the hydrolysate of the polyalcohol, suggesting that (1→4)-linked galactose residues are present in the sargassan molecule, and the formation of glycolic aldehyde in the hydrolysate provided substantial evidence for the presence of (1→4)-linked glucuronic acid, mannose, and galactose residues

Considerable amounts of galactose, xylose, and fucose were also detected in the polyalcohol hydrolysate, indicating that high proportions of these sugar residues are resistant towards oxidation by periodate The resistance of some galactose residues 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 evidence for the presence of galactose 3,6-disulphate residues in the branches The presence[2] of sulphate-free xylose in the branches and the resistance of a high proportion of the xylose residues towards periodate oxidation suggest the presence of (1→3)-linkages between such sugar units The traces of glycerol detected in the polyalcohol hydrolysate indicate that mannose is the reducing terminus of the sargassan molecule and/or the presence of a small number of (1→4)-linked xylose residues The resistance

towards oxidation by periodate of a high proportion of fucose residues is attributed to the presence of sulphate, possibly on C-4 On treatment of sargassan with alkali, there was no diminution in the fucose content, thus demonstrating that the fucose residues cannot be linked through HO-4 since this would necessitate the attachment of sulphate groups on C-2 or C-3, both of which would be alkali-labile (vicinal, *trans*-hydroxyl groups are necessary for alkali lability[19]) These results are consistent with the presence of (1→2)-linked fucose residues However, the minute proportion of propane-1,2-diol detected in the sargassan polyalcohol hydrolysate indicated the possible presence of a small proportion of sulphate-free, (1→4)-linked fucose residues

The product (*A*), obtained by partial hydrolysis of sargassan with 0 5N oxalic acid for 1 h at 100°, reduced 0 93 mole of periodate per "anhydro-sugar" unit (*cf* 0 53 mole for sargassan) This result may be attributed to the partial removal of branches containing periodate-resistant xylose, sulphated fucose, and sulphated galactose residues, and also to the increased exposure of the backbone units to oxidation The amount of formaldehyde and formic acid released was consistent with average d p values of 21 and 1 22 for *A* and each degraded branch, respectively From the monosaccharide content of *A*, it is concluded that there are 13 glucuronic acid residues and 4 mannose residues in the backbone and 4 galactose residues in the degraded branches Consequently, about 3 branches are present in degraded sargassan *A*

Reduction of *A* with potassium borohydride, followed by hydrolysis, gave (p c) erythronic acid, threitol, galactose, and glycerol in proportions higher (determined by visual examination of chromatograms) than for sargassan Thus, hydrolysis of sargassan with oxalic acid removes some branches, so that more glucuronic acid residues in the backbone become susceptible to oxidation by periodate In addition, periodate-resistant residues of xylose, sulphated fucose, and sulphated galactose were also detached from some branches, leaving some unsubstituted galactose residues at the ends of these branches The presence of unsubstituted galactose residues in sargassan branches has been demonstrated earlier[2] Such changes brought about by partial, acid hydrolysis of sargassan increased the susceptibility of the degraded product (*A*) towards oxidation by periodate and hence led to higher proportions of erythronic acid, threitol, and glycerol in the hydrolysate of the corresponding polyalcohol

The above results were further confirmed by investigation of the product *B*, obtained by partial hydrolysis of sargassan with 0 5N oxalic acid for 2 h at 100° *B* consumed 1 04 mole of periodate per "anhydro-sugar" unit, indicating complete cleavage of all sugar residues The formaldehyde and formic acid released indicated average d p values of 17 50 and 1 16 for *B* and for each degraded branch, respectively Thus, *B* comprises a backbone of 12 glucuronic acid and 4 mannose residues, respectively, together with 2 galactose residues as 2 degraded branches Traces of glucuronic acid, mannose, and galactose were, however, detected in the hydrolysate of the polyalcohol derived from *B*

Partial hydrolysis of sargassan with N oxalic acid for 5 h at 100° afforded

product *C*, which consumed 1 1 mole of periodate per "anhydro-sugar" unit, indicating the absence of branch points The amounts of formaldehyde and formic acid released indicated a linear chain composed of 11–12 units This result indicates that a mannose residue is the reducing end of the sargassan molecule The hydrolysate of the polyalcohol derived from *C* contained traces of glucuronic acid and mannose

Autohydrolysis of sargassan gave a product *D* which consumed 0 906 mole of periodate per "anhydro-sugar" unit The average d p values of *D* and of each degraded branch were 22 and 1 4, respectively Consequently, the backbone of *D* comprises 12 glucuronic acid and 4 mannose residues, respectively, and 4 degraded branches composed of 6 galactose residues The hydrolysate of the polyalcohol derived from *D* contained higher proportions of resistant, and lower proportions of oxidation, products than for the partially hydrolysed sargassan *A*

The alkali-treated sargassan sample (*E*) consumed 0 677 mole of periodate per "anhydro-sugar" unit, and was more susceptible towards periodate than the parent polysaccharide This result might be due to the alkaline degradation of the sargassan The average d p values for *E* and each branch were 37 and 5, respectively The hydrolysate of the polyalcohol derived from *E* contained a high proportion of fucose This result is indicative of the presence of alkali-resistant sulphate groups at C-4 of some fucose residues

Desulphation of sargassan by methanolysis gave a product (*F*) which consumed 0 84 mole of periodate per "anhydro-sugar" residue The increased susceptibility towards periodate could be due to the appearance of additional glycol groups on galactose and fucose residues consequent on desulphation and/or to the loss of some of the resistant xylose residues The average d p values of *E* and of each branch were 30 and 4, respectively. The hydrolysate of the polyalcohol derived from *F* contained small proportions of fucose and galactose, indicating that methanolysis conditions were sufficient to desulphate most of the sulphated fucose and galactose residues in the sargassan molecule

The results of the present and previous studies[2] indicate that the polysaccharide part of the sargassan molecule appears to be constructed of a backbone composed of (1→4)-linked β-D-glucuronic acid and β-D-mannose residues To this backbone, heteropolymeric, partially sulphated branches are attached, comprising various proportions of (1→4)-linked β-D-galactose, β-D-galactose 6-sulphate, and β-D-galactose 3,6-disulphate residues, (1→2)-linked α-L-fucose 4-sulphate residues, and (1→3)-linked β-D-xylose residues The fucose and xylose residues appear to be nearer to the periphery of the branches, whereas the galactose residues are nearer the backbone of the polysaccharide

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