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STUDIES OF THE PURIFICATION AND SOME PROPERTIES OF SARGASSAN, A SULPHATED HETEROPOLYSACCHARIDE FROM *Sargassum linifolium*

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ABSTRACT

The acid-extractable, water-soluble, polysaccharide material of the brown alga *Sargassum linifolium* has been fractionated by using lead acetate and barium hydroxide, cetylpyridinium chloride, and copper acetate, and by chromatography on DEAE-cellulose and DEAE-Sephadex. A neutral, laminaran-like glucan and a sulphated heteropolysaccharide (sargassan), composed of D-glucuronic acid, D-mannose, D-galactose, D-xylose, L-fucose, and a protein moiety, were obtained. The latter polysaccharide could not be freed from protein. On partial hydrolysis, sargassan afforded three neutral oligosaccharides, together with a dialysable acidic fragment, a non-dialysable water-soluble portion, and the protein moiety. The backbone of sargassan seems to be composed of glucuronic acid, mannose, and galactose residues with partially sulphated side-chains composed of galactose, xylose, and fucose residues. Sargassan showed high anticoagulant activity.

INTRODUCTION

Recently, we reported[1] the isolation from *Sargassum linifolium* of a hitherto unrecognised, acid-extractable, water-soluble, sulphated heteropolysaccharide (sargassan) contaminated with a glucan. Extraction was effected with dilute hydrochloric acid and oxalic acid, and it was shown that the sargassan contained residues of D-glucuronic acid, D-mannose, D-galactose, D-xylose, and L-fucose, and a protein moiety.

We now report on the purification and some properties of sargassan.

EXPERIMENTAL

General. — Chromatography on Whatman No. 1 and 3MM papers was performed with the following solvent systems: *A* 1-butanol–ethanol–water[2] (40:11:19), *B* 1-butanol–acetic acid–water[3] (4:1:5, upper layer), *C* phenol–water[4] (80:20). Detection was effected with aniline hydrogen phthalate (also for electrophoresis), alkaline silver nitrate, and ninhydrin reagents[5]. Paper electrophoresis was effected

with an Elphor apparatus and a pyridine–acetic acid buffer[6] (0.05M) at pH 6.0. A potential of 300 volts, giving a current of 0.2 mamp/strip was applied for 2.5 h at room temperature.

Ash contents were determined by heating polysaccharide samples to constant weight at 800°. Unless otherwise specified, protein was determined by the method of Lowry *et al.*[7]. Complete, acid hydrolysis of polysaccharide samples was performed with sulphuric acid according to the method of Haug and Larsen[8], and hydrolysis of oligosaccharides was achieved[9] with 0.3M hydrochloric acid at 97° for 4 h. Sugars in the hydrolysates were determined, after descending p.c. (solvent *A*) for 48 h, according to the method of Wilson[10], using appropriate correction factors. Total carbohydrate content was determined by the phenol–sulphuric acid method[11] with conventional, graphical calibration. Inorganic sulphate liberated by hydrolysis[12] was determined by barium chloroanilate[13]. All solutions were concentrated *in vacuo* at 40° in a rotary evaporator.

Sargassum linifolium was the brown-algal species used throughout 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, then air-dried for several days, and finally milled.

Preparation of partially purified, acid-extractable, water-soluble polysaccharide material. — The algal material (15 g) was extracted with water (150 ml, adjusted to pH 1.0 with hydrochloric acid) at 80° for 3 h. After neutralizing the clarified extract with saturated, aqueous sodium carbonate, followed by dialysis against distilled water (48 h), the dialysed solution was centrifuged and the residue was discarded. The supernatant was then percolated through a column of Lewatit S-100(H^+) resin, and the effluent was dialysed against distilled water (48 h), then concentrated to half volume, and treated with 4 vol. of ethanol. The precipitate, isolated by centrifugation, was dissolved in water, and trichloroacetic acid solution was added to give a final concentration of 10%. The precipitated proteins were removed by centrifugation and excess trichloroacetic acid was removed from the supernatant by extraction (thrice) with an equal volume of ether. The aqueous layer was then dialysed for 2 days against distilled water. Thereafter, the dialysed solution was concentrated to half of its volume and treated with 4 vol. of ethanol. The partially purified, polysaccharide material (10 g) was isolated by centrifugation, washed with ethanol and then ether, and finally dried under diminished pressure at 40°; it had $[\alpha]_D^{30}$ −70° (*c* 0.5, water) (Found: ash, 11.84; total carbohydrate, 77.59; protein, 3.30; SO_4^{2-}, 15.40%). On complete, acid hydrolysis[8], the polysaccharide material afforded glucuronic acid, galactose, mannose, glucose, xylose, and fucose (p.c., solvent *A*).

Fractionation of the foregoing polysaccharide. — (*a*) *With lead acetate and barium hydroxide.* A solution of the polysaccharide (10 g) in water (1 litre) was stirred and treated with saturated, aqueous lead acetate. After isolating the precipitate (fraction **1**) by centrifugation, the supernatant was basified to phenolphthalein with barium hydroxide, whereby a precipitate was obtained (fraction **2**). After centrifugation, the remaining supernatant was dialysed for 5 days against distilled water and

TABLE I

FRACTIONAL PRECIPITATION OF THE PARTIALLY PURIFIED, ALGAL, POLYSACCHARIDE MATERIAL WITH LEAD ACETATE AND BARIUM HYDROXIDE

Fraction	*Weight* (g)	*Total carbohydrate* (%)	*Protein* (%)	*Sulphate* (%)	$[\alpha]_D^{30}$ (*degrees*)	*Proportion of monosaccharides* (%)					
						GlcA	*Gal*	*Glc*	*Man*	*Xyl*	*Fuc*
1	3.30	72	7.5	20.20	−96.8	25.71	29.19	5.61	2.14	2.14	19.44
2	5.65	60	6.1	16.66	−80.0	22.98	42.80	2.78	6.32	7.50	17.70
3	0.70	61	5.80	0	−15.0	0	0	100	0	0	0

TABLE II

FURTHER FRACTIONAL PRECIPITATION OF FRACTIONS 1 AND 2 WITH CETYLPYRIDINIUM CHLORIDE

Fraction[a]	*Weight* (g)	*Total carbohydrate* (%)	*Protein* (%)	*Sulphate* (%)	$[\alpha]_D^{30}$ (*degrees*)	*Proportion of monosaccharides* (%)					
						GlcA	*Gal*	*Glc*	*Man*	*Xyl*	*Fuc*
4	0.70	72	4.88	21.42	−161	24.84	28.54	Trace	3.41	10.72	32.47
5	0.85	80	4.27	19.72	−100	29.26	30.25	Trace	3.01	14.37	23.15
6	0.40	84	3.05	16.66	−80	37.25	33.76	Trace	4.44	10.53	14.19
7	0.15	56	2.44	0	−16	0	0	100	0	0	0
8	0.62	80	2.44	18.70	−110	33.96	27.53	Trace	6.10	6.39	26.02
9	0.81	84	2.44	18.20	−84	30.59	34.86	Trace	6.31	8.26	19.98
10	1.00	72	1.83	15.98	−60	23.52	39.83	Trace	7.94	15.35	12.69
11	0.10	54	2.14	0	−16	0	0	100	0	0	0

[a]Fractions 4, 5, 6, and 7 from fraction 1. Fractions 8, 9, 10, and 11 from fraction 2.

then freeze-dried (fraction 3). Fractions **1** and **2** were separately extracted overnight with 0.5M sulphuric acid. The extracts were centrifuged, dialysed against distilled water for 5 days, concentrated to 25 ml, and treated with 4 vol. of ethanol, and the isolated precipitates were washed with ethanol, ether, and dried. The analytical data are recorded in Table I.

(*b*) *With cetylpyridinium chloride.* A solution of fraction **1** (3 g), described in (*a*), in water (300 ml) containing sodium sulphate (1.2 g) was successively treated with 30-ml portions of 2.5% aqueous cetylpyridinium chloride to afford three precipitates (fractions **4–6**). Each precipitate was isolated by centrifugation in the presence of Celite. The remaining supernatant was dialysed against distilled water and freeze-dried (fraction **7**). Polysaccharide material was recovered from each of the precipitated fractions by several extractions of the Celite with 4M potassium chloride. Excess cetylpyridinium chloride was then removed by precipitation with M potassium thiocyanate (10 ml), followed by dialysis of the supernatant against distilled water. The dialysed solution was concentrated to 25 ml and treated with 4 vol. of ethanol, and the precipitate was isolated and dried.

Fraction **2** was similarly treated with cetylpyridinium chloride to afford fractions **8**, **9**, and **10**, as well as the supernatant fraction **11**.

The analytical data for fractions **4–11** are recorded in Table II.

(*c*) *With copper acetate and ethanol.* A solution of fraction **2** (1 g), described in (*a*), in water (100 ml) was successively treated with 25-ml portions of 7% aqueous copper(II) acetate and ethanol to afford two precipitates. The remaining supernatant was then treated with an excess of ethanol to give a further precipitate. Each of the isolated fractions was macerated for 1 min in a blender at ~0° with ethanol containing 5% of hydrochloric acid. Thereafter, the polysaccharide fractions were washed with ethanol, until the washings were free from chloride ions, and dried under diminished pressure at 40°.

(*d*) *By column chromatography on DEAE-cellulose and DEAE-Sephadex.* A solution of the partially purified polysaccharide (0.3 g) in water (30 ml) was added to a column (3 × 26 cm) of DEAE-cellulose (Cl^-). The column was eluted with water until the effluent was free from carbohydrate (fraction **12**), and then in succession with the solvent gradients: water → 0.5M sodium chloride (1 litre of each, fraction **13**) and 0.5 → 2.0M sodium chloride (1 litre of each, fraction **14**). Finally, the column was eluted in succession with 0.25 (fraction **15**) and 0.5M sodium hydroxide (fraction **16**). Fractions (15 ml) were analysed by the phenol–sulphuric acid method[11]. After dialysis against distilled water, each fraction was freeze-dried. The analytical data for fractions **12–16** are given in Table III.

Fractionation of the partially purified, polysaccharide material by column chromatography on DEAE-Sephadex (A-50, Cl^-) was also achieved in the same manner.

Preparation of purified sargassan. — A solution of the partially purified polysaccharide (20 g) in 0.3% aqueous sodium sulphate (2 l) was treated with 2.5% aqueous cetylpyridinium chloride (800 ml) as previously described. The isolated,

TABLE III

FRACTIONATION OF THE PARTIALLY PURIFIED, ALGAL, POLYSACCHARIDE MATERIAL BY CHROMATOGRAPHY ON DEAE-CELLULOSE

Fraction	*Tube No.*	*Weight* (mg)	*Total carbohydrate* (%)	*Protein* (%)	*Sulphate* (%)	$[\alpha]_D^{30}$ (*degrees*)	*Proportion of monosaccharides* (%)					
							GlcA	*Gal*	*Glc*	*Man*	*Xyl*	*Fuc*
12	1–3	21	52	2.44	0	−12	0	0	100	0	0	0
13	4–37	102	78	1.83	17.68	−60	24.07	49.79	Trace	2.48	7.81	15.87
14	38–64	104	76	1.83	17.68	−80	27.43	35.22	Trace	4.63	10.98	17.78
15	65–67	25	74	4.27	18.70	−94	27.43	31.34	Trace	4.63	14.59	22.00
16	68–87	41	84	4.27	18.70	−108	32.82	19.05	Trace	9.21	14.25	25.05

acidic polysaccharide fraction (17 g), which was then used in the succeeding experiments, had $[\alpha]_D$ −78.5° (water) (Found: total carbohydrate, 78.2; ash, 12.2; SO_4^{2-}, 17.68; protein, 3.85%). On complete, acid hydrolysis, pure sargassan afforded (p.c., solvent *A*) glucuronic acid, galactose, mannose, xylose, and fucose in the molar ratios 4.57:8.40:1.00:2.48:2.53.

P.c. of sargassan was performed on Whatman No. 3MM paper, using *D* 0.05M disodium hydrogen phosphate–propan-2-ol[14] (11:9) and *E* 0.2M acetate buffer (pH 4.0)–95% ethanol[15] (4:3). Ethylenediaminetetra-acetic acid was added to solvent *E* to give a final molarity of 0.0011 and a final pH of 4.8. The air-dried chromatograms were dipped into formalin–ethanol[14] (1:4) for 15 min and again air-dried, and then sprayed with toluidine blue reagent[14] (40 mg of toluidine blue in 100 ml of 80% acetone). The chromatograms were rinsed repeatedly with very dilute acetic acid until the background became almost discoloured, and then rinsed with distilled water and air-dried at room temperature. Samples of heparin, chondroitin sulphate, and hyaluronic acid were used as markers.

Partial, acid hydrolysis of sargassan. — The purified heteropolysaccharide (1.01 g) was hydrolysed with 0.5N oxalic acid (40 ml) for 1 h at 100°. The hydrolysate was cooled and dialysed against distilled water (4×300 ml). The dialysates were combined, neutralized with calcium carbonate, centrifuged, concentrated to 10 ml, and treated with Lewatit S-100(H^+) resin to give product *A*. The insoluble, non-dialysable material (0.1 g, product *B*) was dried under diminished pressure at 40°. The supernatant was lyophilized to give product *C* (0.3 g).

Product *A* was transferred to a column (2×9 cm) of Amberlite IR-400(AcO^-) resin. The column was washed with water until the effluent gave a negative phenol–sulphuric acid test. This procedure yielded neutral fragments, which were separated by preparative p.c. (solvent *A*) into three neutral oligosaccharides. Further elution with 0.25M sulphuric acid (1 litre) furnished acidic material having M_{GlcA} 0.49. This material is reported on in the following paper.

Reduction and hydrolysis of the neutral oligosaccharides. — Following the procedure of Perila and Bishop[9], samples of the oligosaccharides (1–2 mg) were dissolved in water (0.5 ml) and treated with sodium borohydride (0.05 g) in water (1 ml) at room temperature for 18 h. The solutions were neutralized with acetic acid, treated with Lewatit S-100(H^+) resin, and evaporated, and methanol was then thrice distilled from the residue. The resulting, reduced oligosaccharides were hydrolysed with 0.3M hydrochloric acid (2 ml) at 97° for 4 h, followed by quantitative p.c. (solvent *A*).

Assay for anticoagulation activity. — The method[16] described for heparin sodium was used on a 1% aqueous solutions of sargassan. The time required for the clotting of blood plasma was 72 h, compared with that of 1 h for a standard solution of heparin.

RESULTS AND DISCUSSION

Lead acetate has been used for the elimination of alginates and proteins as insoluble complexes from algal fucoidan extracts[17]. Subsequent addition of barium hydroxide to the filtrate brings about the separation of a fucoidan–lead hydroxide complex. In the present work, this method was adopted in an attempt to fractionate the acid-extractable, water-soluble, polysaccharide materials, isolated from *Sargassum linifolium*, into a glycuronan fraction and a fucoidan-like fraction. The results in Table I indicate that the attempt was largely unsuccessful, but two major fractions (**1** and **2**) were obtained which had the same monosaccharide composition and similar, high contents of sulphate. A small amount of a sulphate-free glucan was isolated, the optical rotation (−15°) of which suggests that it was a laminaran-like polysaccharide[18].

Further fractionation of either fraction **1** or **2** with cetylpyridinium chloride afforded three major, sulphated-polysaccharide fractions and a minor, neutral-glucan fraction (Table II) which was possibly laminaran. Precipitation with cetylpyridinium chloride did not fractionate the sulphated heteropolysaccharide but almost completely removed the glucan. The sulphated polysaccharide fractions contained high proportions of glucuronic acid, galactose, and fucose, but they were similar in their qualitative monosaccharide composition. Furthermore, refractionation of either fraction **1** or **2** did not remove protein.

Refractionation of fraction **2** with copper acetate and ethanol failed to give a neutral-glucan fraction. The first two fractions were almost free of glucan, which was, however, co-precipitated with the third fraction. Also, this refractionation did not remove protein.

Chromatography of the partially purified, acid-extractable, water-soluble polysaccharide on a DEAE-cellulose column afforded the laminaran-like fraction and four major, sulphated, glucan-free fractions (Table III). Chromatography on DEAE-Sephadex effected a similar fractionation. The acidic fractions were levorotatory, contained variable amounts of protein, sulphate, and total carbohydrate, and different proportions of the same monosaccharides.

Earlier results[1] demonstrated the presence of half-ester sulphate in sargassan. The specific rotation (−70°) of the polysaccharide indicates the presence of at least a majority of α-L-glycosidic and/or β-D-glycosidic linkages. The present results and those previously reported[1] provide substantial evidence for the presence, in *Sargassum linifolium*, of a hitherto unrecognised, sulphated heteropolysaccharide, sargassan, composed of residues of glucuronic acid, mannose, galactose, xylose, and fucose, as well as a protein moiety.

Partial hydrolysis of sargassan, with 0.5N oxalic acid at 100° for 1 h, released galactose, xylose, and fucose in the molar ratios 1.02:1.00:1.02. The dialysable products, after separation from an insoluble residue (*B*) and non-dialysable, water-soluble material (*C*), were fractionated on Amberlite IR-400 resin into neutral and acid fragments. The acid fragment migrated towards the anode as a single band in

paper electrophoresis and stained brown with aniline hydrogen phthalate. Fractionation of the neutral fragments by paper chromatography gave three neutral oligosaccharides, which contained the following molar ratios of galactose, xylose, and fucose: **1** (R_{Xyl} 0.13, R_{Fuc} 0.12) 3:1:1; **2** (0.23, 0.20) 2:1:1; **3** (0.41, 0.36) 1:1:1. After treatment with borohydride, the molar ratios of galactose, xylose, and fucose in the reduced oligosaccharides were as follows: **1** 2:1:1; **2** 1:1:1; **3** 1:0:1. Thus, galactose is the reducing unit of **1** and **2** and xylose is the reducing unit of **3**, and the respective d.p. of the oligosaccharides is 5, 4, and 3.

Each of the neutral oligosaccharides showed negative rotation but, because of the small amounts available, accurate values could not be recorded.

Acid hydrolysis of the non-dialysable, insoluble residue (*B*), followed by paper chromatography (solvent *A*), revealed no carbohydrate material. This observation, coupled with a nitrogen content of 44.5%, indicates that *B* is the protein moiety of sargassan. Two-dimensional paper chromatography (solvents *B* and *C*) of a hydrolysate of *B* revealed a qualitative amino acid composition similar to that of the crude, dried weed, namely, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, valine, leucine, isoleucine, histidine, lysine, arginine, phenylalanine, tyrosine, tryptophane, proline, and methionine.

Hydrolysis of the non-dialysable, water-soluble material (*C*) with sulphuric acid[8], followed by paper chromatography (solvent *A*), afforded glucuronic acid, galactose, and mannose in the molar ratios 3.80:1.00:1.17, a trace of xylose, and 7.14% of sulphate.

Thus, the backbone of sargassan seems to be composed of glucuronic acid, mannose, and galactose residues, with side chains composed of neutral and partially sulphated residues of galactose, xylose, and fucose.

In paper chromatography (solvents *D* and *E*), sargassan migrated as an elongated spot which stained purple with toluidine blue. Similar behaviour and mobility was shown by heparin and chondroitin sulphate.

Sargassan had higher anticoagulating activity than heparin. Under conditions where plasma coagulated after 1 h in the presence of standard heparin solution, coagulation did not occur until after 72 h in the presence of sargassan.

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