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Structural studies of a polysaccharide isolated from the green seaweed *Chaetomorpha anteninna*

E. Venkata Rao* and K. Sri Ramana
Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003 (India)

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ABSTRACT

Extraction of the green seaweed *Chaetomorpha anteninna* with water, followed by fractionation using the copper complex, gave a sulphated heteropolysaccharide, $[\alpha]_D^{27}$ +84°, that contained arabinose (57.7%), galactose (38.5%), rhamnose (3.8%), and sulphate (11.9%). Application of methylation analysis, periodate oxidation, and partial acid hydrolysis indicated a branched structure with 4-linked arabinose, 4- and 3-linked galactose, and 4-linked rhamnose residues. Both the arabinose and galactose residues carried branches. Desulphation and analysis of the polymeric product showed that sulphate groups were located at C-2 of some of the interior arabinose residues, C-4 of some of the non-reducing-end arabinosyl groups, and C-3 of the non-reducing-end galactosyl groups.

INTRODUCTION

Although the constituent sugars of heteropolysaccharides present in several species of Chlorophyceae have been identified, the structural features of only a few members have been reported. In a continuation of our studies[1] of the carbohydrates of Chlorophyceae, we have examined a *Chaetomorpha* species. In general, the mucilages of green seaweeds of the order Chladophorales and Siphonales are proteoglycans. The glycan moieties are usually sulphated heteropolysaccharides composed of arabinose, galactose, and xylose, often with small proportions of rhamnose. The ratios of these sugars vary widely, not only between species but also between different batches of the same species harvested at different times of the year.

Four species of the genus *Chaetomorpha* (order Cladophorales), namely, *C. aerea*, *C. capillaris*, *C. linum*, and *C. melagonium*, have been investigated[2,3]. The mucilages of *C. capillaris* and *C. linum* contain carbohydrate (~43%), sulphate (~15%), and protein (~19%), and the glycan moiety is composed of galactose (1.0 mol), arabinose (~2.5 mol), and xylose (0.7 mol). The glycan part of the mucilages of *C. aerea* and *C. melagonium* is composed of galactose (1.0 mol), arabinose (1.2 mol), and xylose (0.45 mol). Studies[4] of the polysaccharide of *C. moniligera* and certain algae of the orders Cladophorales and Siphonales showed that arabinose and galactose constituted 70–90% of the total cell-wall polysaccharides, and the remaining 30–10% comprised glucose, xylose, and fucose. Apart from carbohydrates, toxic saponins[5], sterols[6,7], fatty

* Author for correspondence.

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acids[8], tocopherols[9], and phospholipids[10] have been isolated from this genus. Apparently, no work has been reported on *C. anteninna*[11], except for the determination[12-15] of some metal ions, and we now report on the structure of a sulphated heteropolysaccharide.

RESULTS AND DISCUSSION

The seaweed *C. anteninna* was treated with alcohol to remove coloring matter and free sugars, then extracted[16] with water. The mucilage, isolated from the aqueous filtrate by repeated precipitation with alcohol, had $[\alpha]_D^{27}$ +102°, and contained 65.2% of carbohydrate, 16.1% of protein, and 13.1% of sulphate. Hydrolysis of the mucilage yielded arabinose (46.7%), galactose (35.1%), glucose (14.9%), and rhamnose (2.9%) (the values relate to total carbohydrate).

Fractionation of the mucilage, using copper complexes[17], gave two fractions, PS-I and PS-II. PS-II had $[\alpha]_D^{27}$ +61° and, on hydrolysis, yielded glucose together with traces of arabinose and galactose. In view of the previous reports on *Caulerpa filiformis*[18], *C. racemosa*[19], *C. sertularioides*[19], *C. taxifolia*[20], *Spongomorpha arcta*[21], and *Chaetomorpha capillaris*[22], PS-II could be a starch-like polysaccharide. PS-II consumed 1.02 mol of periodate per "anhydrosugar" unit and reduction of the product followed by acid hydrolysis gave erythritol, which indicated the presence of mainly 4-linked glucose residues. However, the $[\alpha]_D$ value was too low for a starch-like polymer. Moreover, alpha-amylase did not digest PS-II completely. Thus, PS-II could be a (1→4)-glucan with associated impurities. Further work was not carried out.

PS-I had $[\alpha]_D^{27}$ +84°, contained 81.8% of carbohydrate, 4.5% of protein, and 11.9% of sulphate, and, on acid hydrolysis, afforded arabinose (57.7%), galactose (38.5%), and rhamnose (3.8%). Comparison of the analytical data before and after the formation of the copper complex revealed a reduction in the proportion of protein and an increase in the carbohydrate content with variation in the proportions of constituent sugars. Thus, the original biopolymer contained protein firmly bound to the glycan moiety and had undergone degradation to give PS-I.

Gel filtration of PS-I on Sephadex G-100 gave a single peak, and PS-I was homogeneous in high-voltage electrophoresis. The sugar components present in PS-I were isolated, after hydrolysis, by preparative p.c., and their $[\alpha]_D$ values indicated them to be L-arabinose, D-galactose, and L-rhamnose. Treatment[23] of PS-I with dry methanolic hydrogen chloride removed ~80% of the total sulphate. Desulphated PS-I (DSPS-I) had $[\alpha]_D^{27}$ +67°, contained 90.2% of carbohydrate and 2.1% of sulphate, and acid hydrolysis yielded arabinose (57.2%), galactose (39.1%), and rhamnose (3.9%).

On periodate oxidation[24], PS-I consumed 0.74 mol of oxidant and liberated 0.091 mol of formic acid per "anhydrosugar" unit. Reduction of the product followed by hydrolysis gave threitol (21.9%), glycerol (33.9%), arabinose (14.8%), and galactose (15.2%). DSPS-I consumed 1.20 mol of periodate and liberated 0.28 mol of formic acid. Reduction of the product followed by hydrolysis gave threitol (23.9%), glycerol (35.1%), arabinose (15.2%), and galactose (7.2%). Thus, ~30% of the sugars in PS-I

were resistant to periodate and ~22% in DSPS-I. Of the unoxidised galactose residues in PS-I, ~50% were attacked by periodate after desulphation. The formation of glycerol from PS-I and DSPS-I suggested the presence of 2-, 6-, or 2,6-linked galactose, or 4-linked arabinopyranose, or terminal arabinofuranose residues. Similarly, the formation of threitol suggested the presence of 4- or 4,6-linked galactose residues.

PS-I and DSPS-I were each methylated twice by the Hakomori method[25]. The products, which showed no i.r. absorption for hydroxyl, were hydrolysed, and the products were characterised by g.l.c. and g.l.c.–m.s. as their alditol acetates on the basis of retention times and fragmentation patterns[26–32]. The results are shown in Table I. The large proportion of 2,3-di-*O*-methylarabinose indicated the original presence of mainly 4-linked arabinopyranose or 5-linked arabinofuranose residues. Arabinopyranose residues were also present as non-reducing groups, as revealed by the formation of 2,3,4-tri-*O*-methylarabinose. The formation of 2,4,6-tri-, 2,3,6-tri-, and 2,3-di-*O*-methylgalactose indicated the galactose to be 3-, 4-, and 4,6-linked. Rhamnose was 4-linked as evidenced by the formation of 2,3-di-*O*-methylrhamnose.

Methylation analysis of DSPS-I revealed the multi-linked nature of the arabinose and galactose residues. Compared with the data for PS-I, the decrease in the proportion of 2,3-di-*O*-methylarabinose and the increase in that of 2,3,4-tri-*O*-methylarabinose from DSPS-I suggested that some of the non-reducing arabinose residues in PS-I were 4-sulphated. Similarly, the decrease in the proportion of unmethylated arabinose and a parallel increase of 2-*O*-methylarabinose indicated that the interior arabinose residues were 2-sulphated. The appearance of 2,3,4,6-tetra-*O*-methylgalactose and the simultaneous loss of 2,4,6-tri-*O*-methylgalactose suggested that the non-reducing galactosyl end-units were 3-sulphated.

Partial acid hydrolysis of PS-I gave six fragments.

Fragment **1** (8.2 mg, R_{Gal} 0.69, $[\alpha]_D^{27}$ +42°), on acid hydrolysis, gave galactose and arabinose in almost equal proportions. Borohydride reduction of **1** revealed that the reducing end-group was galactose. Methylation analysis gave 2,3,4-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylarabinose in almost equal proportions. Thus, **1** was L-Ara*p*-(1→6)-D-Gal.

Fragment **2** (6.9 mg, R_{Gal} 0.51, $[\alpha]_D^{27}$ +9°), on hydrolysis, gave galactose and arabinose in the ratio 1.0:1.8. Borohydride reduction followed by acid hydrolysis showed galactose to be the reducing unit. Methylation analysis gave 2,3,4-tri-*O*-methylarabinose (1.0 mol), 2,3-di-*O*-methylarabinose (0.8 mol), and 2,3,6-tri-*O*-methylgalactose (1.2 mol). Thus, **2** was L-Ara*p*-(1→4)-L-Ara*p*-(1→4)-D-Gal*p*.

Fragment **3** (7.2 mg, R_{Gal} 0.42, $[\alpha]_D^{27}$ +14°), on hydrolysis, gave galactose and arabinose in the ratio 2.2:1.0. Borohydride reduction showed arabinose to be at the reducing end. Methylation analysis gave 2,3,4,6-tetra-*O*-methylgalactose (1.0 mol), 2,4,6-tri-*O*-methylgalactose (0.9 mol), and 2,3-di-*O*-methylarabinose (1.2 mol). Thus, **3** was D-Gal*p*-(1→3)-D-Gal*p*-(1→4)-L-Ara*p*.

Fragment **4** (4.7 mg, R_{Gal} 0.31, $[\alpha]_D^{27}$ +17°), on hydrolysis, gave galactose and arabinose in almost equal proportions. Borohydride reduction showed arabinose to be at the reducing end. Methylation analysis gave 2,3-di-*O*-methylarabinose (1.0 mol),

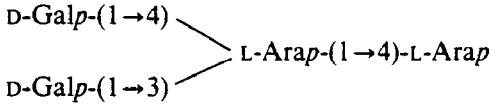
TABLE I

Methylation analysis of PS-I and DSPS-I

Methylated sugar	T[a]		*Approximate molar ratio*		*Major mass-spectral peaks* (m/z)	*Mode of linkage*
	(a)	(b)	*PS-I*	*DSPS-I*		
2,3,4-Tri-*O*-methylarabinose	0.71	0.53	2	4	161, 117, 101, 43	L-Ara*p*-(1-
2,3-Di-*O*-methylrhamnose	0.97	0.91	1	1	203, 161, 143, 117, 101, 87, 43	-4)-L-Rha*p*-(1-
2,3,4,6-Tetra-*O*-methylgalactose	1.23	1.17	-	2	205, 161, 145, 129, 117, 101, 87, 71, 45, 43	D-Gal*p*-(1-
2,3-Di-*O*-methylarabinose	1.30	1.06	9	6	189, 129, 117, 101, 87, 43	-4)-L-Ara*p*-(1-
2-*O*-Methylarabinose	2.01	1.91	2	3	261, 117, 43	-3,4)-L-Ara*p*-(1-
Arabinose	2.44	1.97	2	1	289, 259, 217, 187, 145, 139, 115, 103, 85, 43	-2,3,4)-L-Ara*p*-(1-
2,4,6-Tri-*O*-methylgalactose	2.33	2.03	4	2	233, 201, 161, 129, 117, 101, 87, 45, 43	-3)-D-Gal*p*-(1-
2,3,6-Tri-*O*-methylgalactose	2.39	2.20	4	4	233, 129, 117, 113, 101, 99, 87, 45, 43	-4)-D-Gal*p*-(1-
2,3-Di-*O*-methylgalactose	5.70	4.77	2	2	261, 201, 117, 101, 43	-4,6)-D-Gal*p*-(1-

[a] Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on (*a*) 3% ECNSS-M at 170° and (*b*) 3% OV-225 at 165°.

2-*O*-methylarabinose (0.8 mol), and 2,3,4,6-tetra-*O*-methylgalactose (1.7 mol). Thus, the structure **4** is proposed.



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Fragment **5** (4 mg, R_{Gal} 0.20, $[\alpha]_D^{27}$ +32°), on hydrolysis, gave arabinose only. Methylation analysis gave 2,3,4-tri-*O*-methylarabinose (1.0 mol) and 2,3-di-*O*-methylarabinose (3.3 mol). Hence, **5** was L-Ara*p*-(1[→4)-L-Ara*p*-(1]$_2$→4)-L-Ara*p*.

Fragment **6** (5.3 mg, R_{Gal} 0.09, $[\alpha]_D^{27}$ +5.9°), on hydrolysis, yielded galactose and arabinose in the ratio 1.0:4.2. Borohydride reduction showed arabinose to be at the reducing end. Methylation analysis gave 2,3,4,6-tetra-*O*-methylgalactose (1.0 mol) and 2,3-di-*O*-methylarabinose (4.4 mol). Thus, **6** was D-Gal*p*-(1[→4)-L-Ara*p*-(1]$_3$→4)-L-Ara*p*.

The isolation of **5** and **6** and formation of large amounts of 2,3-di-*O*-methylarabinose suggested the backbone of the molecule to be made up of 4-linked arabinose residues. The isolation of **6** suggested that galactose also was present in the main chain. Methylation analysis indicated PS-I and DSPS-I to have branched structures with both arabinose and galactose at the branch points. The isolation of **4** showed branching at C-3 of 4-linked arabinose residues. The isolation of **1** and the formation of 2,3-di-*O*-methylgalactose in the methylation analysis of PS-I showed branching also at C-6 of 4-linked galactose.

The major difference between the sulphated polysaccharide from *C. anteninna* and those from *C. capillaris*, *C. linum*, *C. aerea*, and *C. melagonium* was the absence of xylose from the former. There are differences also in the proportions of the component sugars, the $[\alpha]_D$ value, and the sulphate content. Arabinose and/or galactose form part of many green-seaweed polysaccharides, as was also found with *C. anteninna*.

EXPERIMENTAL

Plant material. — *C. anteninna*, a local green alga collected on the coast of Visakhapatnam (India), was identified in the Department of Botany, Andhra University, Visakhapatnam. The alga was washed with water, air-dried, and milled.

General methods. — Solvents were evaporated at <40° unless otherwise stated. P.c. was performed on Whatman No. 1 and 3MM papers, using *A*, 6:4:3 1-butanol–pyridine–water; *B* 4:1:5 1-butanol–ethanol–water; and *C*, 2:1:2 ethyl acetate–pyridine–water. Detection was effected with (*1*) alkaline silver nitrate and (*2*) aniline hydrogen phthalate. Total carbohydrate content was determined by the phenol–sulphuric acid method[33]. The homogeneity of the polysaccharide was tested by paper electrophoresis in borate buffer (pH 10.8). I.r. spectra were recorded with a Shimadzu 408 spectrometer, optical rotations with a Jasco Dip-181 digital polarimeter, and u.v. and visible spectra

with an ECIL spectrophotometer, Model GS 865A. All g.l.c. and g.l.c.–m.s. analyses involved alditol acetates[34]. G.l.c. was performed with a Hewlett–Packard model 5730A gas chromatograph fitted with a flame-ionisation detector and glass columns (1.83 x 6 mm) packed with (*a*) 3% of ECNSS-M on Gas-Chrom Q (100–200 mesh) and (*b*) 3% of OV-225 on Gas-Chrom Q (100–200 mesh). G.l.c.–m.s. was performed at 70 eV with a Chemito 3800 gas chromatograph coupled with a Jeol-D-300 mass spectrometer. Sulphate was determined by the barium chloroanilate method[35].

Isolation of the polysaccharide[16]. — The powdered seaweed (100 g) was extracted with ethanol at room temperature, then dried, and a suspension in water (6.5 L) was stirred overnight at room temperature, and then at 60° for 2 h. The slurry was filtered through a muslin cloth, centrifuged, and diluted with cold ethanol (4 vol.) with continuous stirring. The resulting light-green precipitate was collected by centrifugation, dissolved in water, reprecipitated with ethanol, and collected by centrifugation. The process of dissolution in water and precipiation with ethanol was repeated twice more. The resulting, almost white precipitate was then dissolved in water, dialysed, and freeze-dried (yield, 2.1 g).

Fractionation of the polysaccharide[17]. — A solution of the polysaccharide (7 g) in water (700 mL) was centrifuged, the supernatant solution was basified with M sodium hydroxide (100 mL), and Fehling's solution (*A* + *B* equal parts; total volume, 125 mL) was added with continuous stirring until the precipitation was complete. The precipitate (PS-I) was collected and then washed with water, a suspension in water was stirred, and cold M hydrochloric acid was added to decompose the copper complex. The solution was added to ethanol (4 vol.), and the polysaccharide was collected by centrifugation and then slurried in water. The slurry was acidified and the polysaccharide was precipitated with ethanol. The procedure was repeated thrice. The resulting polysaccharide was dialysed against distilled water for 36 h and freeze-dried to give PS-I (4.3 g). The filtrate from PS-I was neutralised with acetic acid, dialysed against distilled water for 36 h, concentrated, and poured into ethanol (4 vol.) to give PS-II (0.9 g).

A solution of PS-I (50 mg) in water was applied to a column (80 x 2.8 cm) of Sephadex G-100 and eluted with water. Fractions (15 mL/h) were monitored for carbohydrate content by the phenol–sulphuric acid method. Only one peak was observed (fractions 16–39). The fractions were combined, dialysed, and freeze-dried (yield, 45 mg). PS-I moved as a single component in high-voltage electrophoresis in borate buffer (pH 10.8).

Hydrolysis and sugar analysis. — The polysaccharide (5 mg) was hydrolysed with 0.5M sulphuric acid for 16 h at 100°. The hydrolysate was neutralised ($BaCO_3$), filtered through Celite, decationised with Dowex 50W-X8 (H^+) resin, and concentrated to a small volume. The filtrate was subjected to p.c. (solvents *A*–*C*) and also analysed by g.l.c. as the alditol acetates.

Desulphation of PS-I[23]. — A suspension of PS-I (1 g) in dry methanolic 0.05M hydrogen chloride (250 mL) was shaken at room temperature for 24 h in a stoppered bottle. The insoluble material was collected by centrifugation, washed with methanol (3 x 100 mL), dialyzed, and freeze-dried (yield, 690 mg).

Periodate oxidation[24]. — PS-I (20 mg) and DSPS-I (15 mg) were each oxidised with 0.1M sodium metaperiodate (20 and 15 mL, respectively) in the dark. The consumption of oxidant, which was monitored spectrophotometrically, became constant after 68 h. The excess of periodate was decomposed with ethylene glycol, the solution was dialysed, and then concentrated at room temperature, and the product was reduced with sodium borohydride overnight at room temperature. The mixture was neutralised with acetic acid, dialysed, and freeze-dried. Each product was hydrolysed with 0.5M sulphuric acid for 16 h, and the neutralised hydrolysate was examined by p.c. (solvents *A* and *B*) and by g.l.c. (column *a*) as the alditol acetates.

Methylation analysis of PS-I and DSPS-I. — PS-I (10 mg) and DSPS-I (5 mg) were each methylated twice by the Hakomori method[25]. Each product was hydrolysed with aqueous 90% formic acid for 1 h at 100°. The formic acid was removed by codistillation with water and each residue was hydrolysed with 0.5M sulphuric acid for 16 h at 100°. Each hydrolysate was neutralised ($BaCO_3$), then centrifuged, and the supernatant solution was concentrated to dryness. The products were analysed by g.l.c. (columns *a* and *b*) and g.l.c.–m.s. (column *b*) as the alditol acetates (Table I).

Partial acid hydrolysis. — PS-I (500 mg) was hydrolysed with 25mM sulphuric acid (50 mL) for 2 h at 100°. The hydrolysate was neutralised ($BaCO_3$), filtered through Celite, concentrated to a small volume under reduced pressure, and passed through a column of Dowex 50W-X8 (H^+) resin. The column was eluted with water until it was free from oligosaccharides and the eluate was concentrated to a small volume. P.c. (solvent *A*) revealed arabinose, galactose, rhamnose, and six higher saccharides (**1–6**) that were isolated by p.c. on Whatman No. 3MM paper in solvent *A*.

Each higher saccharide (0.5 mg) was hydrolysed with 0.5M sulphuric acid for 16 h at 100°. The hydrolysate was neutralised and examined by p.c. (solvents *A–B*) and by g.l.c. (column *a*) as the alditol acetates.

Each higher saccharide (1 mg) was treated with sodium borohydride (10 mg) in water (3 mL). The solution was neutralised with acetic acid, and boric acid was removed as methyl borate. The product was hydrolysed with 0.5M sulphuric acid for 16 h at 100°, and the hydrolysate was neutralised ($BaCO_3$), filtered, and concentrated under diminished pressure. The residue was acetylated and analysed by g.l.c. (column *a*).

Each higher saccharide was methylated by the Kuhn procedure[36]. To a solution of each of **1–6** (3 mg) in *N,N*-dimethylformamide (2 mL) was added silver oxide (400 mg). The mixture was stirred for 30 min, methyl iodide (1 mL) was added, and stirring was continued for 48 h in the dark at room temperature. Chloroform (15 mL) was added, and the mixture was vigorously stirred, filtered through Celite, washed with water (4 x 25 mL), dried (Na_2SO_4), and concentrated to dryness. Each product was hydrolysed under the conditions described above, and the alditol acetates were prepared and analysed by g.l.c. (columns *a* and *b*).

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