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Note

Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (Ulvales, Chlorophyta). Chemical structure of ulvan ☆

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Three major polysaccharide families were solubilized from the cell wall of the marine edible seaweed *Ulva "rigida"*: glucuronorhamnoxyloglycans sulfate, polyuronan and glucoxylans [1]. All these polysaccharides were found closely associated and representative components of these three families (rhamnose, xylose, uronic acid and sulfate) remained present in the residual insoluble polysaccharides. Water-soluble polysaccharides from green seaweeds belonging to Ulvales were all shown to be mainly composed of xylose, rhamnose, glucuronic acid and sulfate and the aldobiouronic acid, β-D-GlcA-(1,4)-L-Rha was a common structure among them [2]. For the sake of simplicity, the glucuronorhamnoxyloglycan sulfate extracted from this algal family will be referred to as ulvan as proposed for that extracted from *Ulva* collected from "green-tides" [3]. A special interest in ulvan is that those from *U. lactuca* [4] and from "green-tides" *Ulva* [3] were able to gel with calcium and boric acid and, as a soluble dietary fibre, to resist fermentation by the human colonic flora [5]. This latter behaviour was related to a basic chemical structure rather than a chemical composition since the constitutive monomers and the aldobiouronic acid, were metabolised by colonic bacteria. Furthermore, desulfation and/or reduction of the uronic acid did not modify the polymer's resistance to colonic bacterial degradation [5]. Sugar linkage analysis of ulvan from *U. lactuca*

☆ Part 2 of the series Cell-wall polysaccharides from the marine green alga *Ulva "rigida"* (Ulvales, Chlorophyta). For Part 1, see ref. [1].

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concluded that the polysaccharide was branched with the presence of terminal xylose, rhamnose and glucuronic acid; 1,4-, 1,2,3- and 1,3,4-linked rhamnose; 1,3-, 1,4- and 1,3,4-linked xylose; 1,4- and 1,3-linked glucuronic acid [6–8]. Sulfation was deduced from infrared spectroscopy and periodate oxidation to occur essentially on O-2 of rhamnose and to a lesser extent on O-2 of xylose [7,9]. Similar results were obtained from the ulvan from *U. pertusa* except that 1,3-linked xylose was sulfated on O-4 [10]. We now report on the sugar linkages and the location of sulfate in ulvan fractions recovered from the *Ulva "rigida"* cell walls [1].

1. Experimental

Polysaccharides.—Ulvan fractions investigated were the oxalate soluble extract (A) and fractions S1F4 and S4F4 recovered from anion exchange chromatography of the 1 and 4 M KOH soluble extracts from *Ulva* species tentatively identified as *U. "rigida"*. The alga was collected at Piriac (Pointe du Castelli, Loire-Atlantique, France) in May 1993 [1].

Reduction of uronic acid.—Uronic acids in ulvan disolved in 8 M aqueous urea (10 mg mL^{-1}, 10 mL) were reduced twice as described [11].

Desulfation of ulvan.—Desulfation of the pyridinium salt of ulvan with dimethyl sulfoxide (DMSO) containing 10% methanol was carried out at 100°C as described [12]. Dry weight recovery yields of 55.0–57.5% were obtained.

Chemical analyses.—Neutral sugars were determined after hydrolysis with 2 M H_2SO_4 for 2 h at 100°C [13]. Reduction and acetylation were carried out as described [14] and the alditol-acetates were analysed by GC using a DB 225 fused silica capillary column (J.W. Scientific, Folsom, CA, USA) operating isothermally at 210°C with H_2 as carrier gas. Uronic acid contents were analyzed colorimetrically by the automated *m*-phenyl phenol method using glucuronic acid as standard [15]. Sulfate contents were determined from 5–10 mg material after hydrolysis with 2 M trifluoroacetic acid for 3 h at 100°C and HPLC analysis of the hydrolysate on a Nucleosil Anion II column (Macherey-Nagel, Düren, Germany) as described [3]. Protein contents were estimated colorimetrically by the Lowry method.

Methylation analysis.—The triethylamine form of ulvan [16] was methylated by lithium dimethylsulfinyl anion and iodomethane [17]. The methylated polysaccharides recovered after extensive dialysis and freeze-drying were hydrolysed with 2 M TFA for 75 min at 120°C in the presence of *myo*-inositol added as standard. The methylated sugars were converted into their corresponding alditols by reduction with $NaBD_4$ for 3 h and acetylated [18]. The products were separated by GC on DB 225 using temperature program: 170°C 15 min, 170–210°C, 5°C min^{-1}, 210°C 13 min and BP-1 (SGE) temperature program; 150°C 10 min, 150–210°C, 2°C min^{-1}. Detection was by a R10-10C Nermag mass spectrometer (MS) using the same columns and conditions. Mass spectra were identified from the literature data [19,20]. Peak areas were corrected by using published molar response factors [21]. Identification of the partially methylated alditol acetates were made from (i) measurement of relative retention time, (ii) methoxyl substitution pattern from GC-MS and (iii) carbohydrate composition of the non methylated polymers.

Infrared spectroscopy.—Infrared spectra were recorded from polysaccharide powders in KBr pellets on a Bruker IRS 25 FT spectrometer.

2. Results and discussion

In order to identify the nature of the uronic acid, determine the sugar linkages and to locate the sulfate position(s) by methylation analysis, the previously isolated ulvan fractions A and S4F4 [1] were reduced and/or desulfated. The methanol/DMSO [12] desulfation method was used as it proved in preliminary experiments (data not shown) to give a better recovery yield and the lowest residual sulfate content compared to the autodesulfation and methanolic-HCl methods [9]. The chemical compositions of the native and modified ulvan fractions were close (Table 1) indicating that their sugar backbone compositions were overall unaffected by the chemical modification. Reduced and desulfated/reduced ulvan demonstrated a marked increase in glucose content, thus glucuronic acid was the uronic acid in this polymer. This result is in agreement with previous data demonstrating the presence of glucuronic acid as a major sugar in water soluble polysaccharides from members of Ulvales [2]. A slight increase in the content of rhamnose was also noticed which probably resulted from a better depolymerization of reduced than of native ulvan. Indeed, the aldobiuronic acid, in ulvan is resistant to acid hydrolysis [22] but after reduction of the polymer, the glucose-rhamnose linkage is hydrolysed more easily and thus a better recovery of rhamnose is obtained.

The glycosidic linkages and the positions of the sulfate groups in ulvan were determined by methylation analysis of native (A, S1F4, S4F4), reduced (S4F4R), desulfated (S4F4D) and desulfated-reduced ulvan fractions (ADR, S4F4DR; Table 2). The neutral sugar composition of the permethylated desulfated-reduced polymers (ADR and S4F4DR) calculated from the partially methylated alditol acetates were in good agreement with the proportions of the sugars determined before methylation. For the other polymers, the results were less quantitative probably because of β-elimination of ulvan during methylation and loss of the degradation products during dialysis of the derivatized samples. Permethylated native ulvan samples (A, S1F4 and S4F4) demon-

Table 1
Chemical composition (mol%) of native ulvan frations A and S4F4 and after desulfation (AD, S4F4D), reduction (S4FR) and desulfation and reduction (ADR, S4F4DR)

	A	AD	ADR	S4F4	S4F4D	S4F4R	S4F4DR
Sulfate	35.9	0.3	3.0	40.6	1.3	35.6	2.8
Uronic acid	24.9	37.1	1.8	20.8	36.2	3.8	3.9
Rhamnose	23.3	39.4	46.2	25.0	43.5	32.0	50.4
4-*O*-Me hexose	1.6	1.7	2.1	1.4	1.2	1.3	1.1
Arabinose	tr [a]	tr	tr	1.0	1.5	1.0	1.6
Xylose	10.9	18.1	15.3	10.6	16.4	11.8	13.9
Galactose	0.4	0.4	1.1	0.3	tr	0.5	tr
Glucose	3.0	2.9	30.5	0.4	tr	13.9	26.4

[a] Trace.

Table 2
Partially methylated alditol acetates (mol%) from native (A, S1F4 and S4F4), carboxyl reduced (S4F4R), desulfated (S4F4D) and desulfated and then reduced (ADR, S4F4DR) ulvan fractions. (mol% measured from neutral sugar analysis)

Partially methylated alditol acetate [a]	A	ADR	S1F4	S4F4	S4F4R	S4F4D	S4F4DR
2,3,4-Rha	1.5	3.8	0.8	0.8	1.5	1.6	1.1
3,4-Rha	0.1	0.5	0.2	0.3	1.5	0.8	0.4
2,3-Rha	1.4	36.5	2.4	2.4	16.9	57.2	41.0
2,4-Rha	1.9	–	–	–	–	–	–
2-Rha	53.0	0.3	55.1	55.2	28.7	0.7	0.4
3-Rha	0.2	8.7	–	–	4.5	14.4	11.4
Rha	12.2	2.0	13.5	13.7	7.3	0.8	0.7
Total Rha	70.4 (64.6)	51.7 (48.5)	72.1 (65.9)	72.3 (64.7)	60.4 (52.8)	75.5 (69.5)	55.0 (54.0)
2,3,4-Xyl	0.2	0.4	–	–	–	–	–
2,3-Xyl	12.6	11.8	13.9	13.8	10.4	20.5	14.8
3-Xyl	5.5	0.3	6.8	6.7	2.7	0.4	0.4
Xyl	0.8	0.1	1.3	1.3	0.4	0.7	0.5
Total Xyl	19.0 (27.8)	12.8 (16.1)	22.0 (34.1)	21.8 (27.4)	13.5 (19.6)	21.6 (26.2)	15.6 (14.8)
2,4,6-Gal	0.7	2.1	0.8	0.8	1.5	1.3	2.1
2,3,4-Gal	1.5	2.3	0.4	0.4	2.2	1.0	1.3
Total Gal	2.1 (5.0)	4.4 (3.4)	1.2 (0.4)	1.2 (4.5)	3.7 (3.0)	2.3 (1.9)	3.4 (1.1)
2,3,4,6-Glc	0.1	8.5	–	–	4.4		5.4
2,3,6-Glc	4.7	22.6	1.0	1.0	16.1	0.5	20.6
Total Glc	4.8 (7.7)	31.1 (32.1)	1.0 (tr)	0.9 (1.0)	20.5 (22.9)	0.5 (tr)	26.0 (28.3)

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl rhamnitol etc.

strated the presence of 1,3,4- and 1,2,3,4-linked rhamnose, 1,4- and 1,2,4-linked xylose residues. Reduction of ulvan (S4F4R, ADR, S4F4DR) resulted in the appearance of 1,4-linked and also terminal glucose residues and also increased the proportion of 1,4-linked rhamnose residues in S4F4R (Table 2). This was probably due to partial desulfation of S4F4 during reduction which was supported by the lower relative sulfate molar percentage in S4F4R (Table 1). Desulfation and desulfation and reduction of ulvan fractions (S4F4D, S4F4DR, ADR) demonstrated that O-3 of rhamnose and O-2 of xylose were sites of sulfation and that about 16.8–20.7% of the rhamnose were branched at O-2 and sulfated on O-3. The length and the composition of the side chain is unknown but it is likely that most of them have glucuronic acid as the terminal glucose (reduced glucuronic acid) content is close to that of branched rhamnose (Table 2, ADR). Detection of 2,3,4-tri-*O*-methylrhamnose suggested that some rhamnose were also located at a non-reducing terminal position. Assuming that no desulfation occured during methylation of ulvan, about one third of the xylose and most of the rhamnose residues in these ulvan fractions were sulfated. These results are in agreement with reports indicating that most of the rhamnose residues in *U. lactuca* ulvan resisted periodate oxidation [7,8]. The chemical structure of the three ulvan extracts studied was very close as no major difference was observed between the nature and yield of methylated sugars obtained from them. Minor 1,3- and 1,6-linked galactose residues were also detected.

The infrared spectrum of the native S4F4 ulvan fraction showed strong sulfate

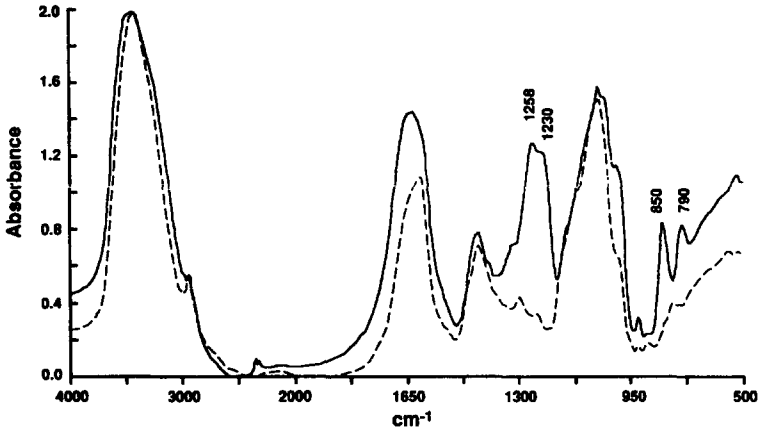

Fig. 1. Infrared spectrum of native (—) and desulfated (—) ulvan fraction S4F4.

absorbances at 1258–1230 cm^{-1} [23] and two other bands at 850 and 790 cm^{-1} (Fig. 1). Desulfation of S4F4 resulted in the disappearance of these absorbances demonstrating that they were associated with sulfate groups (Fig. 1). The previous assumption that the 850 cm^{-1} band was from the axial O-2 sulfate of rhamnose [9] cannot be supported in the light of the present methylation results. In fact, the position of IR peaks between 810–860 cm^{-1} cannot be used with certainty to predict the position of the sulfate substitution [24].

Thus, the present data indicate that ulvan from *Ulva "rigida"* was composed of terminal, 1,4- and 1,2,4-linked rhamnose 3-sulfate, 1,4-linked xylose partially sulfated on O-2 and terminal and 1,4-linked glucuronic acid residues. On a molar ratio basis there were 2.8–3.1 1,4-linked rhamnose, 0.7–0.8 1,2,4-linked rhamnose, 0.4–0.7 terminal glucuronic acid and 1.4–1.9 1,4-linked glucuronic acid per 1,4-linked xylose. Although these results were in agreement with some of the linkages reported in the literature [6–8,10], none of the 1,3-linkages of xylose and glucuronic acid, the branched and non-reducing end xylose were found. Furthermore, no sulfation was deduced to occur on O-2 of rhamnose in contrast with the interpretations of the periodate oxidation and IR results [7,9] and in fact, 3-O-sulfated rhamnose residues in ulvan will also resist periodate oxidation. Such site of sulfation has been suggested in the xylorhamnoglucuronans from a filamentous Ulvale, *Urospora wormskioldii* [25] beside possible 2-O- and 4-O-sulfated rhamnose. The discrepencies observed for the sugar linkages and sulfation pattern between ulvans obtained in this study and those published may also be related to differences in species and/or plant physiological status. Studies are now under way to evaluate these variables on the chemical structure and properties of ulvan.

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