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Characterization of polysaccharides from *Enteromorpha intestinalis* (L.) Link, Chlorophyta

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Cell-wall mucilaginous polysaccharides from *Enteromorpha intestinalis* were extracted, purified and their physicochemical characteristics determined. The successive extracts, obtained using distilled water at 35°C, distilled water at 75°C then an aqueous solution (0·25%) of ammonium oxalate at 75°C, all contained a sulphated glucoglucuronoxylorhamnan containing about 43% rhamnose, 19·8% sulphate and 17% uronic acid (based on the dry weight of the extracts). This composition is comparable to reported values for other Ulvaceae. However, the successive extracts differ in osidic composition and physical properties. In particular, extracts using oxalate at 75°C contain galactose and have lower values of viscosity and molecular weight with respect to those obtained in the first and second extracts, probably as a result of the breakage of labile interchain bonds during extraction.

INTRODUCTION

The study of anionic polysaccharides extracted from seaweeds is of both physiological and economic interest.

In some cases, their specific physiological function (recently reviewed by Kloareg & Quatrano, 1988) seems to be quite well established: mechanical, hydratation and ionic regulation, spore release and adhesivity, fixation of thalli on their substratum. The acidic polysaccharides may also play a role in the differentiation of *Fucus* embryo cells (Kloareg & Quatrano, 1988). Recent studies also emphasize the important function of the cell wall in ionic regulation, in the Fucales and the Laminariales (Kloareg, 1991), as well as in *E. intestinalis* (Mariani *et al.*, 1990).

The phycocolloid industry is the main economic area utilizing seaweeds. At present, only agars and carrageenans extracted from Rhodophyta, and alginates extracted from Phaeophyta, are used on an industrial scale. Polysaccharides from Chlorophyta may have a potential pharmacological interest because of their anticoagulant (Deacon-Smith *et al.*, 1985; Maeda *et al.*, 1991), antiviral and/or antitumoral (cf. reviews of Hoppe *et al.*, 1979 and Colwell *et al.*, 1985) properties. Their thickening ability could also enable them to be used to control the texture of cosmetics.

Ulvaceae may cause a problem in the summer because it covers beaches. In some cases it must be removed so there is an incentive to find ways for it. *E. intestinalis* grows in waters with a wide range of salt conditions (salinity between 0·5 and 180‰, Koeman & van den Hoek, 1982). It is therefore a good material for aquaculture (Harlin, 1978) and could be produced in quantity if applications were found.

The very large salt range tolerated by *Enteromorpha intestinalis* has resulted in a physiological approach, using this species, to approach the ionic regulation function of cell-wall polysaccharides, for example the work of Black and Weeks (1972) concerning the role of plasmalemma in influx and efflux of different ions, and Ritchie and Larkum (1982*a*, *b*) specifying ion exchange properties of isolated cell walls. In this paper we report a physicochemical analysis of the cell-wall anionic polysaccharides. Studies of the polysaccharides of the very closely related species *E. compressa* (L.) Greville have been reported by McKinnel and Percival (1962*b*) and Medcalf *et al.* (1972). Gosselin *et al.* (1964) analysed an extract from a mixture of the species *E. torta*, *E. compressa* and *E. intestinalis*. Dodson and Aronson (1978) furnished a global composition of the whole cell

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wall but without polysaccharide analysis. Medcalf *et al.* (1972) analysed a parietal extract from the US *E. intestinalis*. In the study described here we analysed a series of extracts from the French *E. intestinalis* species that may possess different chemical features.

EXPERIMENTAL PROCEDURES

Sample collection

Three samples were collected with the following location and dates: Pointe de Bloscon, near Roscoff (Brittany), 22 November 1982, 6 January 1983 and 17 January 1983, in a polyhaline puddle of the upper littoral zone. The fresh sample (200 g) was drained, washed with running water then distilled water, and drained again through linen. The first two samples were kept in one litre of methanol and ethanol respectively. The third was lyophilized. The data presented correspond to the average of the three samples.

The samples were crushed in 600 ml ethanol with a Polytron Kinematica Mixer PT 35. The first and third were exhaustively extracted with ethanol 85% by volume and the second with acetone:toluene 2:1. Lipids were partly removed by extraction with a methanol:chloroform mixture 2:1 by volume for 24 h (Laur, 1970). The decolorized product was washed with 600 ml ethanol, 600 ml acetone, then dried at 40°C. The first and third samples were powdered for 2 min with a Dangoumau crusher.

Dry product (20 g) was extracted as follows: 4 h in distilled water at 35°C, 4 h in distilled water at 75°C (the products of these two successive extractions of 2 × 4 h were mixed), 4 h in ammonium oxalate 0·25% at 75°C followed by two extractions of 4 h in distilled water at 75°C (these three last extractions were mixed together). The proportions used were 50 ml solvent with 0·5% formaldehyde (in order to protect the extracts from microorganisms) to 1 g of dry sample.

Extracts were dialysed (15 kD Union Carbide tubing, sterilized with formaldehyde which diffuses during the dialysis) against distilled water. After filtration on Chardin Millipore, the extracts were concentrated at low pressure (maximum temperature of the water bath: 35°C) then lyophilized.

Aqueous 20–50 g litre^{-1} solutions were chromatographed on DEAE-SEPHADEX A25. The 20 × 2·6 cm column was washed with distilled water. The acidic polysaccharides were eluted by NaCl 2 M then dialysed, concentrated and lyophilized again. The temperature of the column was 20°C.

Selected ionic forms of the chromatographed extracts were prepared by passing 10 ml of 1% polymer solution through a cation exchange resin Dowex AG 50. The capacity of the ionic exchange resin was calculated to be four to five times more than the potential equivalent number present in the polymer solution. The resulting acidic polysaccharide was neutralized by an appropriate base.

General characterization

The protein content was determined according to the method of Lowry *et al.* (1951). Starch was quantified by the method of Shuster and Gifford (1962), rhamnose by the sulphuric cystein procedure of Dische and Shettles (1948) and uronic acids by the Bitter and Muir (1962) carbazole method. The reference uronic acid was D-glucuronic acid which is the usual component of the ulvacean acid polysaccharides (Percival, 1978, 1979; Percival & Smestad, 1972). The total acid group content was measured by precipitation with CPC (N-cetylpyridinium chloride) at pH = 7. Sulphate was determined by CPC at pH = 1·5 (Scott, 1960). A UV/visible Unicam SP 3700 spectrophotometer was used, except for proteins, for which a Varian DMS 90 spectrophotometer was used. Elemental analysis (% C, P, N, S, O, H) was performed at the CNRS Centre (Service central d'analyse, Vernaison, France) on lyophilized chromatographed extracts.

IR spectra were recorded from films deposited on a BaF_2 crystal, using a Beckmann IR II spectrophotometer. Optical rotation at 589 nm of previously filtered (Millipore 0·2 μm) polymer aqueous solutions (1 g litre^{-1}) was determined at 25°C, with a Perkin-Elmer 241 MC polarimeter. Limiting viscosity numbers of previously filtered (Millipore 0·2 μm) polymer solutions were determined at 25°C in 1 M NaCl, with a Fica MS, model 332, viscosimeter.

Molecular weights were determined by measurement of the low angle ($\theta = 4{\cdot}883°$ for water) scattered light intensities, using a Chromatix KMX laser (633 nm) photometer, at 25°C. The refractive index increment for the polymer solutions ($dn/dc = 0{\cdot}111$) was determined for several concentrations, using a differential refractometer (Waters Associates). The apparatus was previously standardized using a KCl solution.

Potentiometry was conducted as follows: the polyosidic acid solution (prepared as described above) was set in a thermostated vessel, at 25°C, under nitrogen atmosphere, in order to prevent atmospheric CO_2 dissolution. The polyosidic acid was then titrated by a base of selected normality, using a Gilmont micrometric burette. The pH was determined with a Tacussel Isis 200 1/100 pHmeter. Conductimetry was measured with a Tacussel CD 78 conductimeter. Activity coefficients of protons were determined using Manning's theory (1974).

Sugar analysis

Lyophilized chromatographed extracts (50 mg) were hydrolyzed with 20 ml of 0·33 M sulphuric acid at 100°C.

The efficiency of the hydrolysis was quantified by HPLC (15 min to 24 h); an internal reference allowed the percentage of hydrolysed polysaccharide to be calculated. The optimum hydrolysis time was 6 h. The hydrolysate was neutralized by barium hydroxide.

HPLC was performed using a Waters Associates model 510 chromatograph and a refractometric R 401 detector; 25 cm long Merck Lichrosord NH_2 5m column was employed. The solvent used was aqueous acetonitrile 80% by volume and the flow rate was 1 ml min^{-1}. The hydrolysates were previously filtered on Whatman GF/C 1·2 μm.

After sylilation of monosaccharides (Sweeley *et al.*, 1963), GLC was carried out using an Intersmat IGC 121 FL. Separations were made with a 3-m × 3-mm glass column provided with a Supelcoport 100–120 mesh containing 5% OV 17. The fluent gas was helium at 1·6 bar, and the temperature rise was programmed at 2°C min^{-1} from 150°C to 180°C; injection and detection systems were maintained at 275°C.

For HPLC as well as for GLC, the relative amounts of the sugars were evaluated from the peak areas.

RESULTS AND DISCUSSION

Extraction yields

In our extracts, variations in yield were noticed from one sample to the other, probably because of the preservation or grinding method. Lower yields (15·0% of dry weight of the thalli) was obtained for the third sample for which thalli were lyophilized instead of being placed in alcohol. The extraction yield of the second sample (16·6%) was lower than that of the first one (20·0%) probably because the second sample had not been powdered with a Dangoumau crusher. The proportion of each successive extract (on average) was: distilled water at 35°C, 16%; distilled water at 75°C, 46%; and aqueous ammonium oxalate (0·25%) solution at 75°C, 38%.

General characterization of the crude extracts

Compositions, presented in Table 1, are expressed as a percentage of the dry extract. Sulphate and uronic acid contents are comparable to those measured in other Ulvaceae (see Table 4), except for the low value (3·6% dry weight of the extract) obtained by Maeda *et al.* (1991) in *E. intestinalis* from Japan.

The presence of contaminating molecules, not eliminated by dialysis, particularly starch, was observed in our crude extracts. Starch has been quantified: the relatively low contamination by starch, even in hot water, probably results from extraction temperatures below 80°C.

Rhamnose is usually the main component of ulvacean polysaccharides (Percival, 1978, 1979). The sugar analysis (see below) demonstrated that all the methyl-pentoses determined were rhamnose. The lower value of methyl-pentoses obtained in extracts in distilled water at 35°C can be explained in two ways. Either the sugar composition of this extract was different, or more non-acidic macromolecules were present. Lower values (total acid groups, sulphate and uronic acids) were also noticed in the first extracts. This observation favours the second hypothesis. It can be seen that the molar ratios between total acid groups and rhamnose were very similar in all the extracts.

The neutralization curves confirmed the presence of two acidic functions assigned to carboxyl and sulphate groups. Activity coefficient for protons were always less than 1 thus confirming the polyelectrolytic nature of the extracts.

Characterization of chromatographed extracts

The NaCl-eluted fraction did not contain detectable starch. The protein content was 4% of the dry weight for extracts in distilled water at 35 and at 75°C and 3% for the extracts in oxalate at 75°C. Percival and McDowell (1967) also obtained polysaccharide fractions containing proteins not eliminated by purification. Our results do not allow us to specify if these are contaminant molecules or the peptide part of proteoglycans.

Elemental analysis is presented in Table 2. Our values of nitrogen content are comparable to those measured by Medcalf *et al.* (1972) in four Ulvaceae species: 2·5, 1·6, 0·6 and 0·8 in *Ulva lactuca*, *Enteromorpha linza*, *E. intestinalis* and *E. compressa* respectively. Nitrogen content multiplied by Attwater's coefficient (supposed valid for algal proteins) gave results (distilled water at 35°C: 12%; ammonium oxalate at 75°C: 10%) differing from those obtained with the Lowry method. It thus appears that the nitrogen content of these extracts was not only proteinaceous.

Table 1. General characterization of crude extracts (% dry weight)

Extract	Starch	Rhamnose	Total acid groups	$-OSO_3^-$	Uronic acids
D. w. 35°C	1·1	27·2	21·5	8·7	11·4
D. w. 75°C	3·1	43·0	31·3	19·9	16·8
Am. oxal. 75°C	1·6	42·5	31·3	19·6	17·8

Table 2. Elemental analysis of the chromatographed distilled water (35°C) and ammonium oxalate extract (% dry weight of the fraction)

Extract	C	H	N	O	S	P
D. w. 35°C	32·2	5·6	2·0	47·5	5·0	0·2
Am. oxal. 75°C	31·2	5·2	1·8	45·7	5·5	0·2

Table 3. Sugar proportions (molar ratio) in the distilled water (35°C) and ammonium oxalate chromatographed extracts

Sample	Galactose	Xylose	Rhamnose	Glucose
D. w. 35°C	—	1	4·7	1·3
Oxalate 75°C	0·2	1	3·4	0·6

Sugar composition of the chromatographed extracts

GLC analysis led to the identification of rhamnose, xylose, glucose and ribose, in the first (distilled water at 35°C) and the last (ammonium oxalate at 75°C) extracts. Moreover, mannose and galactose were identified respectively in both. HPLC development allows control of GLC results. Rhamnose, glucose, xylose and galactose were confirmed, ribose and mannose were not. Nevertheless, it should be noted that the mannose proportion determined with GLC was exactly the same (molar ratio: 0·2) as that found by Medcalf *et al.* (1972) for *E. intestinalis* (Table 4, Ref. 4). Galactose was confirmed only in the last extract. The presence of glucose in starch-free polyosidic extracts has been demonstrated previously (Percival, 1978; Medcalf *et al.*, 1972). The sugar composition (GLC results), presented in Table 3, is a typical ulvacean one. The proportions of the different monosaccharides measured by HPLC confirmed the proportions obtained with GLC, but resolution was better with GLC. Furthermore, data obtained with GLC, in previous works, allow comparisons (Table 4). The main sugars are always rhamnose, xylose and glucose (see also Dodson & Aronson, 1978; Maeda *et al.*, 1991 and Yamamoto, 1980; in these studies, the sugars are not quantified and so we did not include them in Table 4). Mannose, however, appears to be one of the major sugars in the polysaccharide from *E. compressa* and *Ulva arasakii* from Japan, and galactose one of the major sugars in *E. intestinalis* and *Blidingia minima* from Japan (Maeda *et al.*, 1991). In an extract at 23°C (the others extracts are contaminated by glucans) from a mixture of *Enteromorpha torta*, *E. compressa* and *E. intestinalis*, Gosselin *et al.* (1964) measured the following molar ratio: rhamnose 5/xylose 3/glucose 1.

IR spectrophotometry

Infrared spectra of sodium polysaccharidic extracts were quite similar (Fig. 1). The peaks at 1100 cm^{-1} and 1060 cm^{-1} corresponded to hydroxyl groups. The large peak at 1240 cm^{-1} was characteristic of sulphate groups (S=O linkage). The marked band at 820 cm^{-1} (-C-O-S linkage) indicated that sulphate groups were equatorial. The large band towards 1640 cm^{-1} corresponded to hydroxyl groups or to crystallization water. Low bands were noticed at 1720 and 1750 cm^{-1} and could correspond to the -C=O and -NH groups from proteins. The band at 1740 cm^{-1} probably corresponded to crystallization water (for references, see Bellamy, 1968).

Optical rotation

The rotary power of starch is strongly positive. Our values (−45·32°, −65·35° and −78·23°, respectively for successive extracts) agreed with the absence of starch

Table 4. Sugar proportions (molar ratio), sulphate and uronic acid contents (% dry weight of the extract) in polysaccharidic extracts from Ulvaceae

Species (references)[a]	Rhamnose	Xylose	Glucose	Galactose	Mannose	Sulphate (%)	Glucuronic acid (%)
Ulva lactuca (1, 2, 3, 5, 6, 7 and 8)	4·2	1·3	1	—	—	20	17·5
Ulva lactuca (4)	2·8	1	0·2	0·3	T[b]	19·8	22
Ulva lactuca (9)	6·9	3·5	1	—	T	18	
Enteromorpha compressa (3, 5 and 6)	8	2·7	1	—	—	18·3	16
Enteromorpha compressa (4)	4·1	1	0·2	0·3	—	12·7	19
Enteromorpha intestinalis (4)	6·2	1	0·2	0·2	0·2	14·1	20
Enteromorpha linza (4)	5	1	T	0·2	T	14·4	19

[a](1) Haq & Percival, 1966 (2) McKinnel & Percival, 1962*a* (3) Mckinnel & Percival, 1962*b* (4) Medcalf *et al.*, 1972 (5) Percival, 1964*a* (6) Percival, 1964*b* (7) Percival, 1978 (8) Percival & McDowell (9) Percival & Wold, 1963.
[b]Trace.

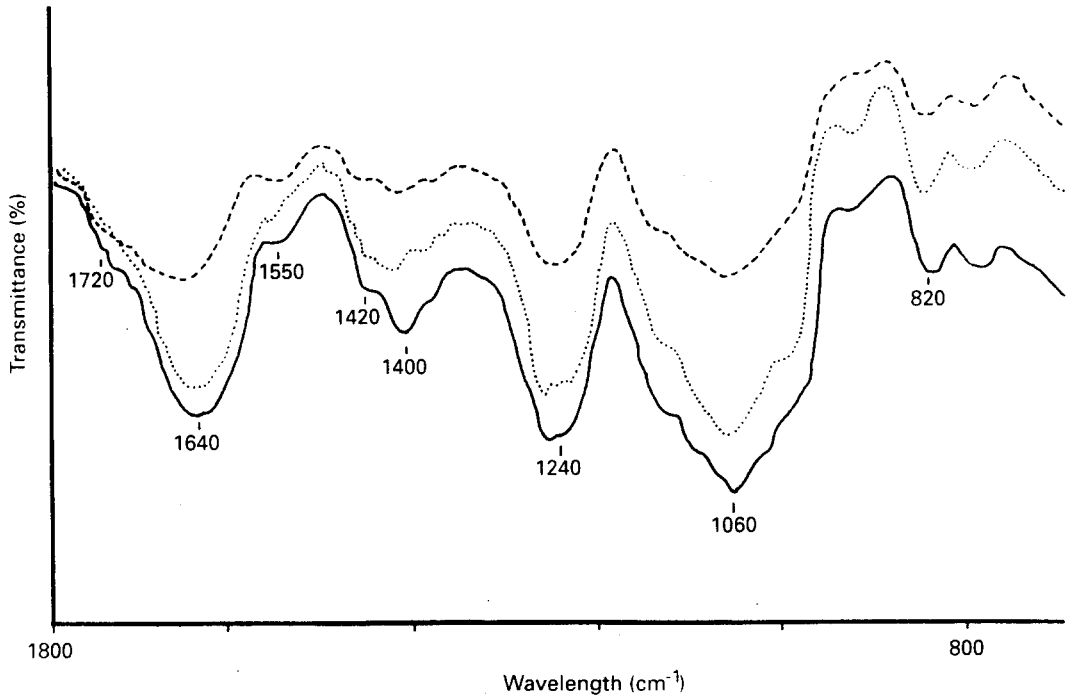

Fig. 1. Infrared spectra, between 800 and 1800 cm^{-1}, of chromatographed sodium polysaccharides. (·····) Distilled water at 35°C extract; (- - - -) distilled water at 75°C extract; (——) ammonium oxalate at 75°C extract.

found by direct determination. These values were always less than −30°, as in all previous data for Chlorophyta by other authors for sulphated glucuronoxylorhamnans (Carlberg & Percival, 1977; Fisher & Percival, 1956, 1957; Medcalf *et al.*, 1972; Percival, 1964*a*,*b*; 1978; Percival & Smestad, 1972; Percival & Young, 1971). However, it should be noted that, when indicated, these authors measured the rotatory power at different temperatures and in different ionic forms.

Viscometry and molecular weight

Reduction of intrinsic viscosity of the successive sodium polysaccharidic extracts seemed to depend upon the extraction procedure: 61 ml g^{-1} for distilled water at 35 C; 37 ml g^{-1} for distilled water at 75°C; 24 ml g^{-1} for ammonium oxalate at 75°C; these values are slightly lower than those measured by Percival (1964*b*) and Percival & Wold (1963): 150, 110 and 95 ml g^{-1} in three fractions from *U. lactuca*, or by Yamamoto (1980): 111 ml g^{-1} in *Ulva pertusa*, 155 ml g^{-1} in *Ulva conglobata* and 55 ml g^{-1} in *Enteromorpha prolifera*. The intrinsic viscosity correlated with the molecular weight which decreased strongly with successive extractions. This decrease (502 kD for distilled water at 35°C, 286 kD for distilled water at 75°C and 189 kD for ammonium oxalate at 75°C) could correspond, by temperature elevation and/or by oxalate breaking of ionic interactions, to breaking of interchain bonding. Such a phenomenon was pointed out by Medcalf & Larsen (1977*a*,*b*): fucans with high uronic acid and xylose contents are attached on sulphated fucans. This bonding is labile at 70°C and pH = 2.

CONCLUSION

The successive extracts were all sulphated glucuronoxylorhamnan with, approximately, rhamnose 43%, sulphate 19·8% and uronic acid 17% (dry weight extract). The low values obtained for the crude extracts in 35°C distilled water probably results from the presence of contaminating macromolecules. These macromolecules could correspond to glycoproteins analogous to those extracted by Jónsson *et al.* (1985) from *Enteromorpha prolifera*. Elemental analysis showed about 2% nitrogen content for chromatographed extracts. Nitrogen not eliminated by purification is known in ulvacean polysaccharidic extracts (Percival & McDowell, 1967), but does not indicate the presence of a covalent bond between polysaccharide and protein.

The successive extracts share some characteristics but were not identical. The rotatory powers differed strongly, but were of the same order of magnitude as those determined by other authors for sulphated glucuronoxylorhamnans. Sugar contents of the first and last extracts were different; the presence of galactose was mainly noticed in the last extract. Such differences in sugar content were already encountered in the Ulva-

ceae (Percival, 1964*b*). Differences between molecular weight and viscosity of the first and the last extracts may correspond to breaking of labile interchain binding as established for fucans by Medcalf & Larsen (1977*a*,*b*).

The polysaccharidic extracts had a typical ulvacean sugar composition and physical properties. A cell-wall acid polysaccharide containing galacturonic acid was described by Maeshige (1962) in *Monostroma nitidum* Wittrock, but the genus *Monostroma* is no longer included in the Ulvaceae family (Bliding, 1968). Our results should be added to the morphological, anatomical, cytological and chemical data, and point to an extreme homogeneity of this family.

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