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Structure and interactions of ulvan in the cell wall of the marine green algae *Ulva rotundata* (Ulvales, Chlorophyceae)

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ABSTRACT

Ulvan, the sulfated cell-wall polysaccharides from green seaweeds (*Ulva* species), presents structural and functional properties of interest for different applications. Its extraction yield in water varies depending on the species, the period of collect and the mode of conservation of algae. To identify limits of extraction, the structure and interactions of ulvan in the cell wall of *Ulva* were investigated following a sequential solvent extraction. Hot sodium oxalate and hot dilute HCl were the two major extracts. Other solvents affecting protein structure or low hydrogen bonded polymers yielded minor ulvan fractions but affected solid state ^{13}C NMR spectra indicating that proteins likely contributed to cross-link and/or to entrap *Ulva* cell wall polysaccharides. Ulvan-lyase degradation showed that ulvan consisted in a wide continuum of related polysaccharides differing in the extent and type of fine structural characteristics. Besides solvent acidity, particle size of seaweeds was an important parameter controlling the ulvan extraction efficiency. Major ulvan cell wall interactions were discussed with regard to ionic interactions, covalent linkages and peculiar solution behavior of ulvan in various physicochemical environments.

1. Introduction

Green seaweeds (Chlorophyta) belonging to Ulvaceae (*Ulva* and *Enteromorpha* species) represent an important biomass available from proliferating algae in eutrophicated coastal waters (Morand & Briand, 1996) and can easily be cultured (Duke, Litaker, & Ramus, 1989a; Fujita, 1985; Guist & Humm, 1976; Parker, 1981). It contains on the dry weight basis 38–54% of cell wall polysaccharides (Lahaye & Kaeffer, 1997) with a majority of water-soluble ulvan. This polysaccharide displays physico-chemical and biological features of potential interest for diverse applications (Lahaye & Robic, 2007). Notably it forms unusual soft gels in the presence of divalent cations and borate (Haug, 1976; Lahaye & Axelos, 1993). Ulvan is mainly built on disaccharides repeating sequences composed of sulfated rhamnose and glucuronic acid, iduronic acid or xylose (Percival & McDowell, 1967; Quemener, Lahaye, & Bobin Dubigeon, 1997). The two major repeating disaccharides are aldobiuronic acids designated as type A: ulvanobiuronic acid 3-sulfate (A_{3s}) and type B: ulvanobiuronic acid 3-sulfate (B_{3s}) (Fig. 1). Partially sulfated xylose residues at O-2 can also occur in place of uronic acids (Fig. 1). In addition, glucuronic acid can branch at O-2 of rhamnose 3-sulfate (Ray & Lahaye, 1995a, 1995b). Low proportions of galactose, glucose, mannose and protein are also generally found in ulvan. Ulvan fine structure was particularly revealed using liquid state NMR spectroscopy combined with partial acid hydrolysis (Lahaye & Ray, 1996) and enzymatic degradation by an ulvan-lyase (Lahaye, Brunel, & Bonnin, 1997; Lahaye, Inizan, & Vigouroux, 1998a). At the ultrastructural level, isolated ulvan in water solution appears as nano-sized bead-like structures with some fiber like material (Robic, Gaillard, Sassi, Lerat, & Lahaye, unpublished). The bead-like structures form aggregates at low ionic strength, at alkaline pH or in the presence of copper and/or boric acid while at acidic pH, aggregates dissociate. In the algae, ulvan is widespread in the intercellular space and in the fibrillar wall of the two cell layers thick *Ulva* sp. thallus (Bobin-Dubigeon, Lahaye, Guillon, Barry, & Gallant, 1997). In the wall, ulvan is present with (1,4)-linked β-D-glucuronan co-occurring with protein, with a (1,4)-linked β-D-glucan containing (1,4)-linked β-D-xylose, and with cellulose (Lahaye, Baumberger, Quemener, & Axelos, 1996; Lahaye, Jegou, & Buleon, 1994). Models of cell wall summarizing different interactions between *Ulva* wall polymers were proposed (Lahaye et al., 1998a, 1998b).

Ulvan is usually extracted using hot water often containing a calcium chelating agent such as sodium oxalate. Under these conditions, the extraction yield varies from 8% to 30% of the algal dry yield (Lahaye & Robic, 2007) and the ulvan extraction efficiency varies between 15% and 70% according to the seaweed species,

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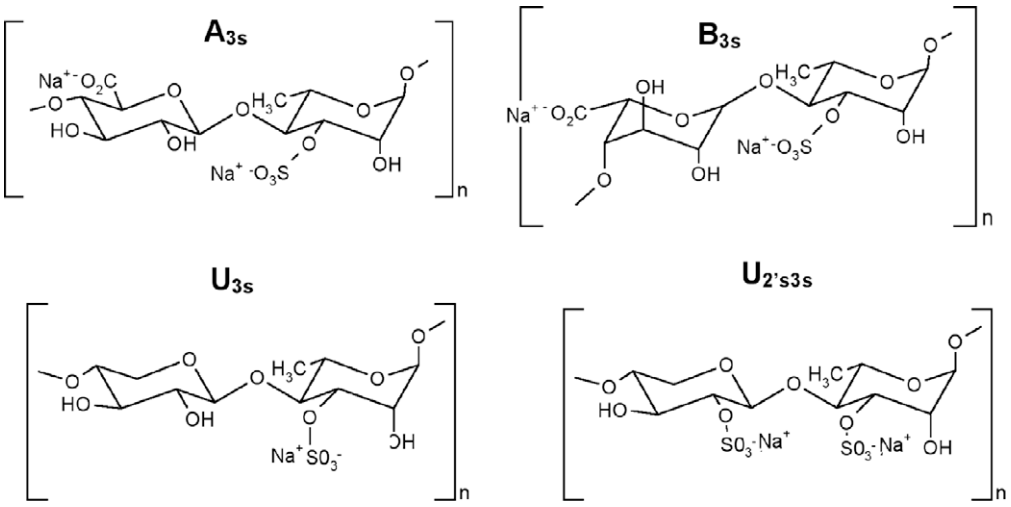

Fig. 1. Structure of the main repeating disaccharides in *Ulva* ulvan: ulvanobiuronic acids A_{3S} and B_{3S} and ulvanobioses U_{3S} and $U_{2'S,3S}$.

its period of collect and its post-collect treatment (Robic, Sassi, & Lahaye, 2008, Robic, Sassi, Dion, Lerat, & Lahaye, 2009). Little ulvan was extracted by alkali (Ray & Lahaye, 1995a). In order to identify conditions improving ulvan extraction efficiency, *Ulva rotundata* cell wall material was sequentially extracted by different solvents. The yield of extracts, the chemical composition, the chemical structure and the macromolecular properties of soluble extracts and insoluble residues were determined and discussed with regard to the nature of interactions between ulvan and its cell wall partners. Alternative extraction conditions were then evaluated on the basis of ulvan recovery and macromolecular distribution.

2. Materials and methods

2.1. Material

Ulva rotundata was collected at Pleubian (3°4′12.61″ W, 48°51′35.39″ N, Côte d'Armor, France) on May 2007 and frozen immediately without rinsing. Before extraction, the seaweeds were thawed at room temperature, rinsed with seawater and dried at 50 °C in ventilated oven (Thirode Pulsair Polycuiseur, HMI-Thirode). The seaweeds were ground with a grinder (FastPrep®-24, MP) and particles sieved through 100 μm were used for analysis.

2.2. Preparation of alcohol insoluble residue

The seaweed flour was boiled for 60 min in 70% boiling ethanol (10% w/v) and recovered by centrifugation (16,000*g*, 15 min). The pellet was dispersed in 70% ethanol at room temperature for 16 h and recovered by centrifugation. This operation was repeated twice more for 8 and 16 h until the filtrate was colorless and free of sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and then with 96% ethanol. The final material was dried overnight at 40 °C under vacuum and was referred to as alcohol insoluble residue (AIR).

2.3. Sequential extraction on AIR

AIR were suspended in deionized water (dry solid content: 6.8% w/v) and stirred for 30 min at 60 °C with 0.5 mL amyloglucosidase (Sigma, 6000 units mL^{-1}). Amyloglucosidase (0.5 mL) was added again and incubation was continued further for 30 min at 60 °C. The suspension was then brought to 85 °C and to 0.05 M sodium oxalate. It was stirred for 2 h at 85 °C, then diluted by its volume with deionized water prior to centrifugation (16,000*g*, 15 min). The pellet (Res-Ox) was washed 5 times with deionized water and finally with 70% ethanol and dried at 40 °C overnight. The extract and water washes were combined, dialyzed against deionized water (Mw 3 kDa) and freeze-dried (E Ox).

Res-Ox were extracted by sodium chlorite solution (0.5%) adjusted to pH 3.4 with acetic acid (dry solid content: 2% w/v) according to Fry (1988). The suspension was stirred at 60 °C for 15 min then cooled to room temperature and centrifuged (16,000*g*, 15 min). The pellet was washed 4 times with deionized water. The extract and water washes were combined, adjusted to pH 8.5 with ammonia, dialyzed against deionized water and freeze-dried (E Chlo). The pellet was dispersed in DMSO (2% w/v) and stirred for 60 min at 100 °C. After centrifugation (16,000*g*, 15 min), the pellet (Res-Chlo/DMSO) was rinsed with DMSO once and 4 times with deionized water. Res-Chlo/DMSO was finally washed with 70% ethanol and dried at 40 °C overnight. The DMSO extract and water washes were combined, concentrated to dryness on a rotavapor, solubilized in water and freeze-dried (E DMSO).

Res-Chlo/DMSO was extracted by phenol/acetic acid/water according to Fry (1988). The extract was freeze-dried (E PAW). The residue (Res-PAW) was washed 4 times with 70% ethanol and dried at 40 °C overnight.

Res-PAW was stirred in HCl 0.05 M (1% w/v) at 85 °C for 30 min and the suspension was centrifuged (16,000*g*, 15 min). The extraction was repeated twice. The last pellet (Res-HCl) was washed 4 times with water and with 70% ethanol and dried at 40 °C overnight. The three extracts and the water washes were combined, dialyzed against deionized-water and freeze-dried (E HCl).

2.4. Alternative extractions of ulvan

AIR extraction with hot HCl. AIR was stirred in HCl 0.05 M (1% w/v) at 85 °C for 30 or 60 min. After centrifugation (16,000*g*, 15 min), the extract was neutralized with NaOH and freeze-dried. For the 30 min extraction, the extraction was repeated twice. All extracts were treated individually and are referred to as 30A, 30B, 30C and 60A.

AIR extraction with acidified ammonium oxalate. AIR was stirred in ammonium oxalate 0.02 M (1% w/v) brought to pH 4.6 with oxalic acid for 60 min at 85 °C. After centrifugation (16,000*g*, 15 min), the extract was neutralized with NaOH and freeze-dried. The extract is referred to as A-Ox.

In each case, the residues were washed with water and with 70% ethanol and dried at 40 °C overnight.

2.5. Chemical analysis

Dry solid content corresponded to the weight of samples determined after 24 h at 103 °C. Organic matter was quantified gravimetrically after 12 h at 550 °C. Sulfate content was measured according to Quemener et al. (1997). Protein content was estimated by the Folin-Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) in water-soluble extracts and as N Kjelhdahl 6.25× in extraction residue. Total uronic acid content was measured by colorimetry (Thibault, 1979) using glucuronic acid as standard. Quantification of neutral sugars was performed by gas-liquid chromatography (GC) after sulfuric acid degradation and derivation as alditol acetates (Englyst & Cummings, 1988; Hoebler, Barry, David, & Delort-Laval, 1989). Extracts were dissolved in sulfuric acid 2 M and hydrolysed (2 h, 100 °C). Insoluble samples (raw *Ulva*, AIR and residues) were dispersed in 13 M sulfuric acid for 30 min at 25 °C, prior to dilution to 1 M and hydrolysis (2 h, 100 °C). Standard monosaccharide solutions and inositol (internal standard) were used for sugar identification and weight response factor determination.

2.6. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX 400 spectrometer at a carbon frequency of 100.62 MHz. For ^{13}C NMR, about 70 mg sample was dissolved in 1 mL of D_2O (99.9%) and 1 mL of H_2O in 10 mm tubes. Traces of acetone as internal reference (^{13}C δ = 31.45 ppm) permitted to calibrate the carbon chemical shifts. For ^{1}H NMR, 10 mg samples were dissolved in 1 mL of D_2O (99.9%), freeze-dried twice to remove exchangeable protons and then dissolved in 0.5 mL of D_2O (100%) in 5 mm tubes with a trace of acetone as an internal reference (^{1}H δ = 2.22 ppm). The experiments were carried out at 333 K.

The solid-state NMR spectra were performed on fresh algae, dried and rehydrated ulvan, rehydrated AIR and rehydrated residues with D_2O (100 mg of sample for 100 μL D_2O for rehydration to 50% and 100 mg of sample for 200 μL D_2O for rehydration to 66%). Spectra were recorded on a Bruker DMX 400 spectrometer operating at a proton frequency of 400.13 MHz and carbon frequency of 100.62 MHz. A double resonance H/X CPMAS 4 mm probe was used for cross polarization (CPMAS) and single pulse excitation (SPEMAS) magic angle spinning experiments. The magic angle spinning (MAS) rate was fixed at 7 kHz and each acquisition was recorded at room temperature (294 K). CPMAS experiments were realized using a 90° proton pulse of 5 μs, a contact time of 1 ms at 45 kHz and a 5 s recycling time for an acquisition of 25 ms during which dipolar decoupling (TPPM) of 50 kHz was applied. The SPEMAS experiments used a 90° carbon pulse of 4 μs, a 1 s recycling delay and acquisition time. A composite dipolar decoupling (WALTZ 16) of 31.2 kHz was applied during the entire sequence. Chemical shifts were calibrated with external glycine, assigning the carbonyl carbon at 176.03 ppm.

2.7. Enzymatic degradation

Crude ulvan-lyase was prepared as a 60–80% ammonium sulfate precipitate of a liquid culture from a Gram negative ulvanolytic bacterium isolated from marine sediments. This crude preparation was purified by steric exclusion chromatography on Superdex 200-FPLC (GE Healthcare) equilibrated and eluted at 0.5 mL/min with Tris HCl 20 mM pH 7.5 using a ÄKTA™ purifier 10XT (GE Healthcare). Elution was monitored at 280 nm. The protein content (Bradford, 1976), the neutral sugars and uronic acids contents (Thibault, 1979; Tollier & Robin, 1979) and the ulvan-lyase activity were followed in 1 mL fractions. The enzyme activity was measured at 235 nm on laboratory ulvan sample solution at 2.5 g/L in water pH 7.5 according to Voragen, Rombouts, and Pilnik (1971).

The chromatographic peak containing the most active fractions associated to a low sugars content (2% of the initial sugar content of the protein extract) was used for further experiments (Total activity = 0.15 nkat/mL, specific activity = 0.013 nkat/μg).

The different extracts (2% w/v in deionized water, pH 7.5) were degraded at room temperature by addition of ulvan-lyase (0.36 nkat). After 8 h, the same quantity of enzyme was added and incubation was continued for an additional 15 h. Degradation was stopped by heating the reaction solution in a boiling water-bath for 10 min. Enzyme resistant ulvan was precipitated in 4 vol. of ethanol and oligosaccharides were recovered from the ethanol supernatant after evaporation, re-dissolution in water and freeze-drying. The fractions insoluble in ethanol were re-dissolved in water and freeze-dried.

2.8. Gel permeation chromatography

Extract solutions (4 mg/mL) and fractions insoluble in ethanol from ulvan-lyase degradation were dissolved in 50 mM $NaNO_3$ containing 0.02% NaN_3, passed through 0.22 μm membrane filter and injected at 25 °C on a high-performance size exclusion chromatography system (HPSEC) constituted of two Shodex OH-pack SB HQ 804 and 805 columns (8 × 300 mm, Guard columns: OHpak SB-800P, 6 × 50 mm, Shodex, Showa Denko KK, Miniato, Japan) eluted at 0.7 mL/min with 50 mM $NaNO_3$ containing 0.02% NaN_3. A differential refractometer (ERC 7517 A) and UV detector were used to follow the elution profiles. Protein elution was followed by UV absorbance at 280 nm.

Oligosaccharides from ulvan enzymatic degradation were dissolved in 50 mM NaNO3 (0.02% NaN_3) and chromatographed on a column Shodex OH Pak SB-802.5 HQ (8 × 300 mm) eluted by NaNO3 (50 mM, 0.7 mL/min). Elution was followed by a differential refractometer (ERC 7517 A) and elution of chromatographic peaks (V_e) were expressed according to the void (V_0) and total volumes (V_t) of the column using the following relation: $K_{av} = (V_e - V_0)/(V_t - V_0)$.

3. Results

3.1. Sequential extraction

3.1.1. Yield and composition

The yield and chemical composition of the raw *U. rotundata*, its alcohol insoluble residue AIR, the various polysaccharides sequentially extracted and different insoluble extraction residues are given in Table 1. The sulfate and protein contents were determined only for the extracts available in sufficient quantity. The ulvan extraction efficiency is included in Table 1 and corresponded to the percentage recovery of the initial rhamnose content in AIR.

Glucose, rhamnose, uronic acids and xylose were the major sugars in the raw alga. Alcohol extraction increased the total sugar content in the material and decreased the content in minor sugars and glucose. The oxalate extract (E Ox) amounted to 27.5% of the starting AIR dry weight and included 45.6% of the initial AIR rhamnose content. Extraction of the residue after oxalate (Res-Ox) by sodium chlorite and by DMSO solubilized polymers that accounted for 2.0% and 8.0% of the dry seaweed weight, respectively, and contained 1.3% and 1.5% of the initial AIR rhamnose content. Compared to the oxalate extract (E Ox), the chlorite (E Chlo) and particularly the DMSO (E DMSO) extracts were enriched in glucose (17.3% ± 0.6 and 62.8% ± 0.1, respectively). The DMSO extract was also rich in protein (17.3 ± 1.4% dw) and poor in sulfate (3.3 ± 0.1% dw). Extraction of residue after chlorite and DMSO

Table 1
Yield, ulvan extraction efficiency and chemical composition of extracts from *U. rotundata*. Chemical composition of raw algae, AIR and extraction residues. Total sugars represent the sum of sugars content determined by GC and colorimetry. Total sugars, sulfate and protein contents were expressed in % dry weight, +/− standard deviation, $n = 3$. Sugar content was expressed in mol% +/− standard deviation, $n = 3$.

	Yield (%dw)	Ulvan extraction efficiency	Total sugars	Rha	Ara	Xyl	Man	Gal	Glc	UA[a]	Sulfate	Proteins
Raw algae			27.8	27.9 ± 0.2	0.0	15.5 ± 0.3	0.2 ± 0.3	1.2 ± 0.1	40.2 ± 0.4	15.1 ± 0.8	nd[b]	nd
AIR			41.1 ± 3.4	28.5 ± 1.4	0.0	16.3 ± 0.7	0.0 ± 0.0	0.3 ± 0.4	32.3 ± 0.2	22.6 ± 2.1	nd	nd
E Ox	27.5	45.6	49.3 ± 3.5	42.1 ± 2.4	0.0	14.3 ± 0.6	1.2 ± 0.0	0.8 ± 0.1	5.1 ± 0.1	36.4 ± 3.0	15.6 ± 1.0	6.6 ± 0.6
E Chlo	2.0	1.3	31.7 ± 3.3	33.2 ± 1.8	0.0	13.2 ± 0.5	5.4 ± 0.4	1.1 ± 0.0	17.3 ± 0.6	29.4 ± 2.5	nd	nd
E DMSO	8.0	1.5	23.6 ± 2.0	12.2 ± 0.7	0.5 ± 0.0	7.2 ± 0.3	5.4 ± 0.1	0.3 ± 0.4	62.8 ± 0.1	11.4 ± 1.0	3.3 ± 0.1	17.3 ± 1.4
E PAW	0.1	0.0	24.1 ± 2.5	7.2 ± 0.5	4.6 ± 0.1	4.4 ± 0.2	11.7 ± 0.2	0.0 ± 0.0	61.3 ± 0.2	10.8 ± 1.1	nd	nd
E HCl	21.5	20.3	51.7 ± 4.0	33.2 ± 1.9	0.0	15.2 ± 0.8	0.3 ± 0.1	0.5 ± 0.0	19.0 ± 1.2	31.7 ± 3.4	13.3 ± 0.3	2.9 ± 0.3
Res-Ox			32.5 ± 2.8	21.0 ± 1.1	0.0	20.4 ± 0.8	1.3 ± 0.1	0.0 ± 0.0	41.8 ± 0.7	15.6 ± 1.5	nd	nd
Res-Chlo/DMSO			29.4 ± 2.6	21.7 ± 1.0	0.0	21.8 ± 0.7	0.4 ± 0.5	0.0 ± 0.0	38.6 ± 0.2	17.6 ± 1.6	nd	nd
Res-PAW			31.2 ± 2.9	20.2 ± 1.3	0.0	21.5 ± 0.8	0.8 ± 0.1	0.5 ± 0.5	39.5 ± 0.7	17.5 ± 1.7	nd	nd
Res-HCl			26.6 ± 2.4	7.9 ± 0.4	0.0	27.5 ± 0.8	1.1 ± 0.0	0.3 ± 0.4	55.0 ± 0.5	8.0 ± 0.8	nd	26.9

[a] UA, uronic acids.
[b] nd, not determined.

(Res-Chlo/DMSO) with phenol-acetic acid-water (E PAW) yielded a minor fraction (0.1% dw of the AIR) composed essentially of glucose (61.3% ± 0.2), mannose (11.7% ± 0.2) and uronic acids (10.8% ± 1.1). The residue after PAW (Res-PAW) was then extracted with hot dilute hydrochloric acid. The HCl extract (E HCl) amounted to 21.5% dw of AIR and corresponded to an ulvan extraction efficiency of 20.3%. This extract contained 51.7% dw of total sugars and had a composition close to that of the oxalate extract but enriched in glucose (19.0% ± 1.2) and poorer in sulfate and protein (13.3% ± 0.3 and 2.9% ± 0.3, respectively).

All the residues contained about 30% dw of sugars that were mainly represented by glucose (38.6–55.0 mol%), xylose (20.4–27.5 mol%), rhamnose (7.9–21.7 mol%) and uronic acids (8.0–17.6 mol%) (Table 1). Oxalate, chlorite-DMSO and PAW residues still contained high amounts of rhamnose and uronic acids. HCl extraction markedly reduced these contents to the benefit of glucose and xylose.

3.1.2. Macromolecular distribution

The macromolecular distribution of the different extracts was determined by HPSEC (Fig. 2). Based on the RI profiles (Fig. 2A), oxalate extract was mainly composed by high molecular weight molecules. Chlorite extract contained a low proportion of high molecular weight macromolecules combined with a small proportion of low molecular weight molecules. DMSO extract profile showed several peaks in the low molecular weight region and HCl extract was mainly composed by a low molecular weight population. Based on the UV profiles (Fig. 2B), a small amount of protein was dispersed all over the fractionation domain of the oxalate extract, whereas chlorite extract showed a high peak in the high molecular weight region with another in the low molecular weight region. The DMSO extract showed a peak in the region of the low molecular weight molecules, whereas HCl extract was characterized by a peak in the region of the high molecular weight molecules.

3.1.3. Ulvan fine structure

^{13}C NMR spectra were recorded for the different extracts in solution (Fig. 3). Typical signals for ulvan structures were assigned by comparison with published data (Lahaye & Ray, 1996; Lahaye et al., 1997; Lahaye et al., 1998a, 1998b, 1999). Except for the DMSO extract, all spectra showed typical signals for the ulvanobiuronic acid 3-sulfate type A and B. The poor resolution of the chlorite extract spectrum did not allow the identification of minor structures. On the spectra of the oxalate and HCl extracts, several minor signals were observed and likely reflected other linkages between rhamnose, uronic acids and sulfate as well as the presence of other sugars residues like xylose, mannose and glucose. Signals were assigned to R1x(s) at around 98 ppm for C-1 in rhamnose 3-sulfate linked to xylose or xylose 2-sulfate, to X5 at 65 ppm for C-5 of xylose and to X5s for C-5 of xylose 2-sulfate. The resonance at 83 ppm for contiguous 1,4-linked β-D glucuronic acids (Gg4 for GlcA C-4) in ulvan or from contaminating glucuronan was observed in the spectrum of the oxalate extract but not on that of the HCl extract. The peaks at around 60 ppm (C-6) were more intense on the spectrum of the chlorite, HCl extracts and particularly in that of the DMSO extract than of the oxalate extract. For the DMSO extract, it was accompanied by other major resonances that were assigned to starch (Dais & Perlin, 1982) and agreed with the high glucose content of the extract (E DMSO) (Table 1). For the other extracts, these C-6 signals may be assigned to variable amounts of minor hexoses (mannose, glucose) beside trace of starch.

Typical ulvan ^{1}H NMR spectra were recorded from the oxalate and HCl extracts (data not shown). The contribution of peaks assigned to C-1 of rhamnose in A_{3S} disaccharide (R1, 4.81 ppm) and in B_{3S} disaccharide (R'1, 4.88 ppm) (Lahaye et al., 1998a, 1998b) were compared. The ratio of the area of R1/R'1 was higher for the oxalate extract than for the HCl extract. The ^{1}H NMR spectrum of DMSO extract showed characteristic signals for starch (notably H-1 at 5.34 ppm, data not shown). These were also observed in low intensity on the spectrum of the HCl extract but were absent in the spectrum of the oxalate extract.

All extracts were submitted to ulvan-lyase degradation and the degradation products as well as the enzymatic resistant fractions were analysed by size exclusion chromatography (Fig. 4). On the weight basis, 64% to 80% of the fractions were converted to alcohol soluble oligosaccharides with the highest degradation registered for the HCl extract (Table 2). Oligosaccharides were fractionated into five peaks with Kav 0.07, 0.15, 0.23, 0.35 and 0.56 (Fig. 4A), which proportion differed for the different extracts (Table 2). The HPSEC profiles obtained with the fractions insoluble in ethanol after ulvan-lyase degradation showed a marked shift of the elution toward the lower molecular weight population, with very little high molecular weight material remaining (Fig. 4B) compared with the crude extracts (Fig. 2).

3.1.4. Solid state NMR spectroscopy of ulvan and different algal insoluble fractions

^{13}C NMR CPMAS and SPEMAS spectra of the ulvan extract (E Ox) rehydrated to 50% and 66% humidity are shown on Fig. 5. These spectra were compared to that of an ulvan solution (oxalate extract). In the low hydrated conditions (50%), signals on the SPEMAS spectrum were broad and dominated by resonances corresponding

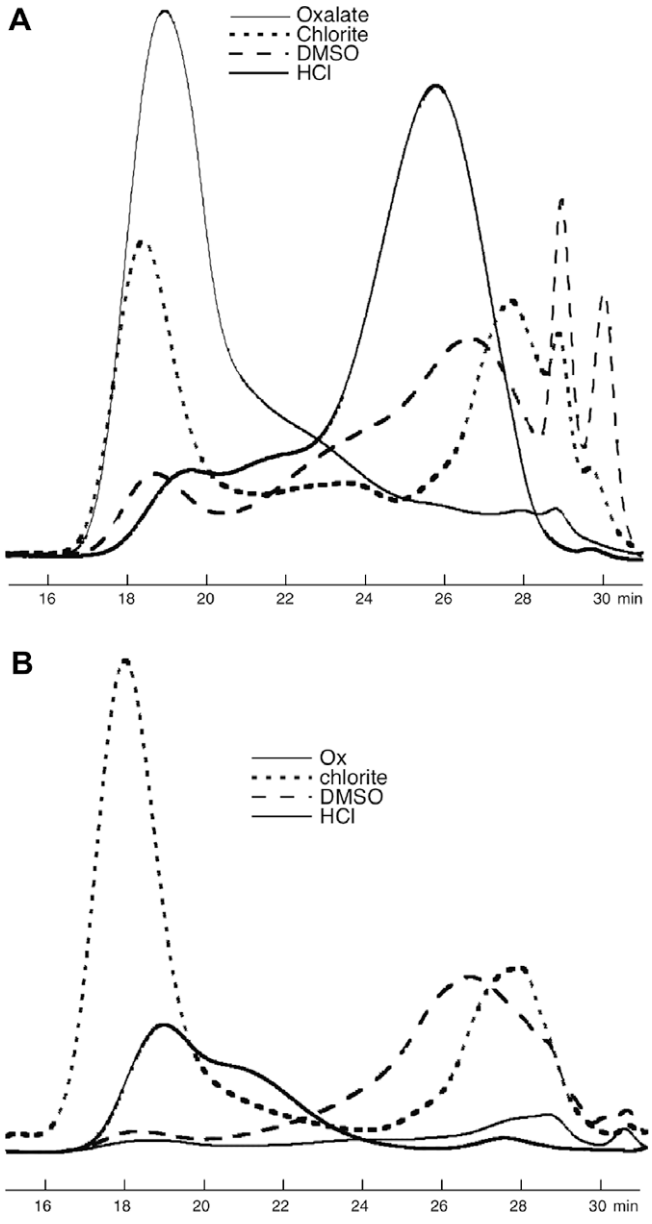

Fig. 2. High pressure size exclusion chromatographic profiles of extracts from *U. rotundata* detected by (A) refractive index (RI) detector and (B) UV detector.

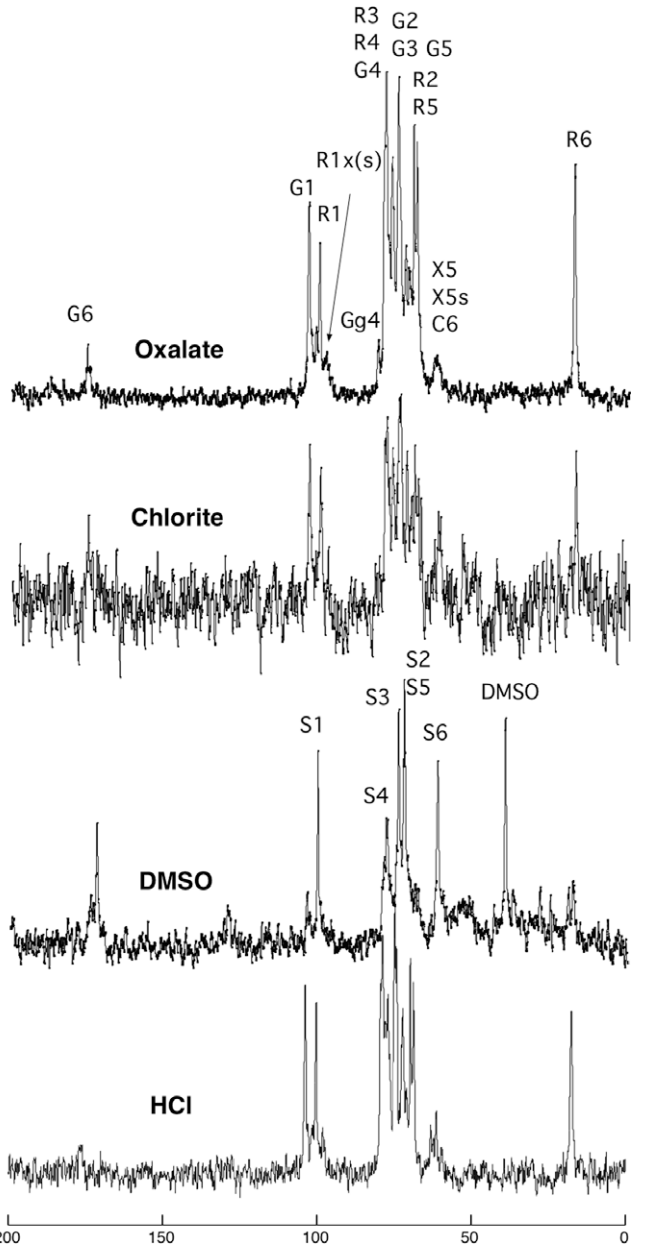

Fig. 3. ^{13}C NMR spectra of extracts from *U. rotundata*. R refers to rhamnose 3-sulfate linked to glucuronic or iduronic acids, G refers to uronic acids, X to xylose and S to starch.

to carboxylate carbon of uronic acids (170–180 ppm), to carbons in sugars (120–50 ppm) and to the methyl carbon of rhamnose (20 ppm). The resolution and the sugar signals increased with the hydration of the sample to 66%. The CPMAS spectrum of ulvan rehydrated at 50% was poorly resolved but better than the corresponding SPEMAS spectrum. The resolution was improved at 66% hydration while the carbonyl signal intensity decreased. Signals assigned to methyl of rhamnose, carboxylate of uronic acids and designed as R3-R4-G4 (79 ppm), G2-G3-G5 (75 ppm) and R2-R5 (69 ppm) on the liquid state ^{13}C NMR spectrum were observed in these spectra.

The SPEMAS and CPMAS ^{13}C NMR spectra of *Ulva*, AIR and different extraction residues are shown in Figs. 6 and 7, respectively. For comparison purpose, the ^{13}C NMR spectrum of ulvan in solution is shown with the SPEMAS spectra (Fig. 6) and the CPMAS spectrum of rehydrated ulvan powder is shown with the CPMAS spectra of the different algal and residues (Fig. 7). Both SPEMAS and CPMAS spectra of fresh *Ulva* showed a high noise level probably linked to a high water content of the fresh algae and to the presence of other constituents such as minerals, which may have affected signals relaxation. The SPEMAS spectrum of fresh *Ulva* revealed mobile compounds at chemical shifts close to those of extracted ulvan in solution (Fig. 6). Additional small and more or less resolved resonances in the 0–30 ppm and 120–140 ppm regions were observed and likely corresponded to small metabolites (free sugars, amino acids, short peptides, phenolic compounds...). The CPMAS spectrum of fresh *Ulva* (Fig. 7) showed poorly resolved signals assigned to the algal cell wall polysaccharides (ulvan and cellulose). Drying of the algal material resulted in a lower signal resolution but improved signal to noise ratio and particularly in the spectral region corresponding to signals attributed to small metabolites and proteins (0–30 ppm; Fig. 7).

The preparation of AIR permitted to markedly improve the signal to noise ratio of both CPMAS and SPEMAS spectra. The SPEMAS spectrum clearly showed the disappearance of the small reso-

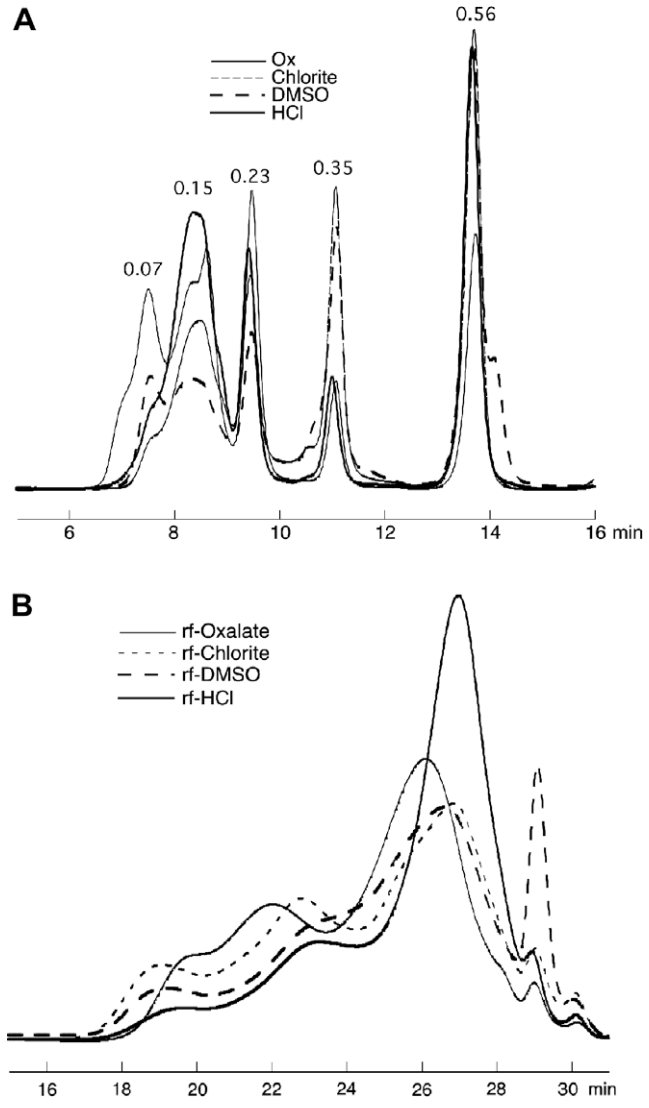

Fig. 4. High pressure size exclusion chromatographic profiles of oligosaccharides produced by ulvan-lyase degradation of extracts from *U. rotundata* on Shodex OH Pak SB-802.5 HQ column and of fractions insoluble in ethanol on Shodex OH-pack SB HQ 804 and 805 columns.

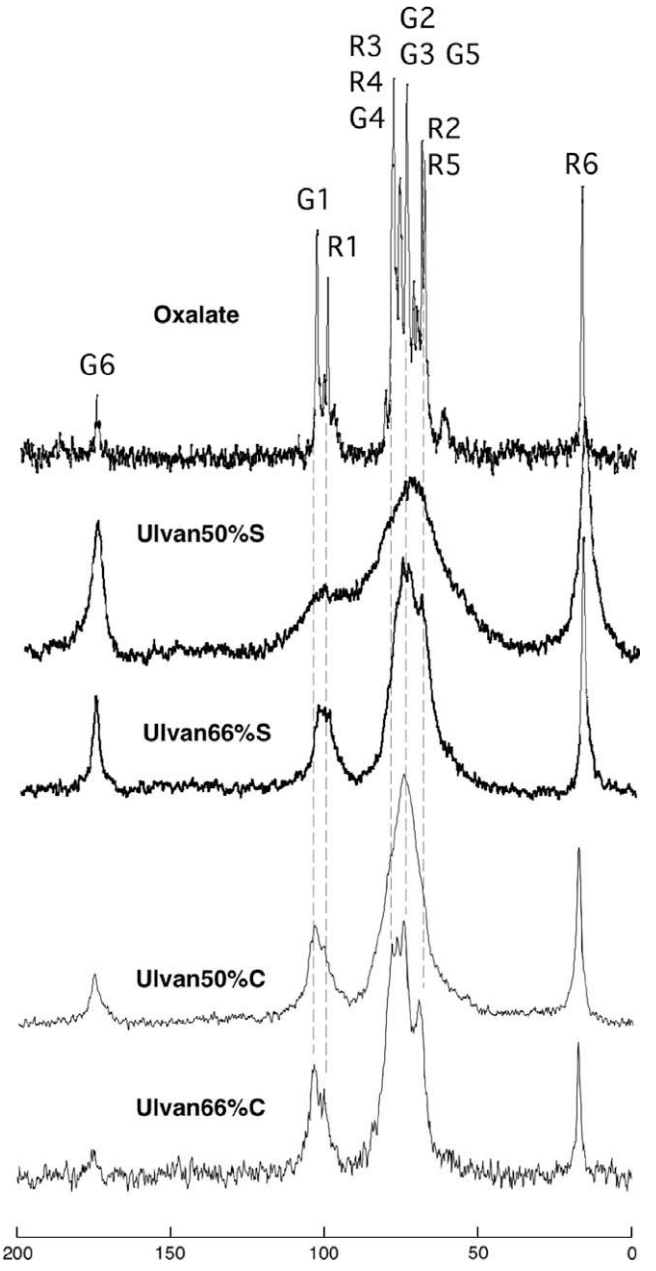

Fig. 5. ^{13}C NMR spectrum of oxalate extract (Oxalate), ^{13}C CPMAS (C) and ^{13}C SPEMAS (S) spectra of ulvan rehydrated to 50% and 66% moisture content.

Table 2
Proportion of total weight fraction recovered in alcohol soluble oligosaccharides and of each oligosaccharide.

Extract	Oligo recovery[a]	Oligosaccharides proportion[b]				
		0.07[c]	0.15	0.23	0.35	0.56
Oxalate	69.7	26.0	32.8	18.0	6.5	16.7
Chlorite	64.3	5.2	27.3	15.2	21.0	31.2
DMSO	68.1	11.8	14.0	12.3	22.0	40.0
HCl	80.1	6.1	44.6	14.0	6.6	28.7

[a] Percentage of total weight fraction recovered in alcohol soluble oligosaccharides.
[b] As % of total peak area.
[c] Peak identity as Kav.

nances assigned to small metabolites. The main signals were assigned to ulvan with minor resonances around 60 ppm, which corresponded to the contribution of other hexoses containing polysaccharides. The CPMAS spectrum of AIR showed the presence of ulvan with a small shoulder at about 80–90 ppm and signals around 60–65 ppm corresponding respectively to C4 and C6 of cellulose and hemicelluloses.

The oxalate extraction residue (Res-Ox) yielded a SPEMAS spectrum with better resolved signals assigned to ulvan and to C6 of hexoses at about 60 ppm. An increase of the signal at 100 ppm indicated an enrichment of the residual material with mobile domains of starch and/or hemicelluloses. The CPMAS spectrum of Res-Ox showed a significant decrease of signals assigned to ulvan (R6, R2-R5, R4-R3, R1; Fig. 7) and an increase of signals at about 80 and 60–65 ppm, assigned to C4 and C6 of cellulose and hemicelluloses.

The material remaining after sodium chlorite and DMSO (Res-Chlo/DMSO) and PAW extractions (Res-PAW) yielded SPEMAS spectra of a mobile ulvan fraction with better resolved signals compared to the oxalate residues and with the contribution of polysaccharides rich in hexoses. The CPMAS spectra showed the

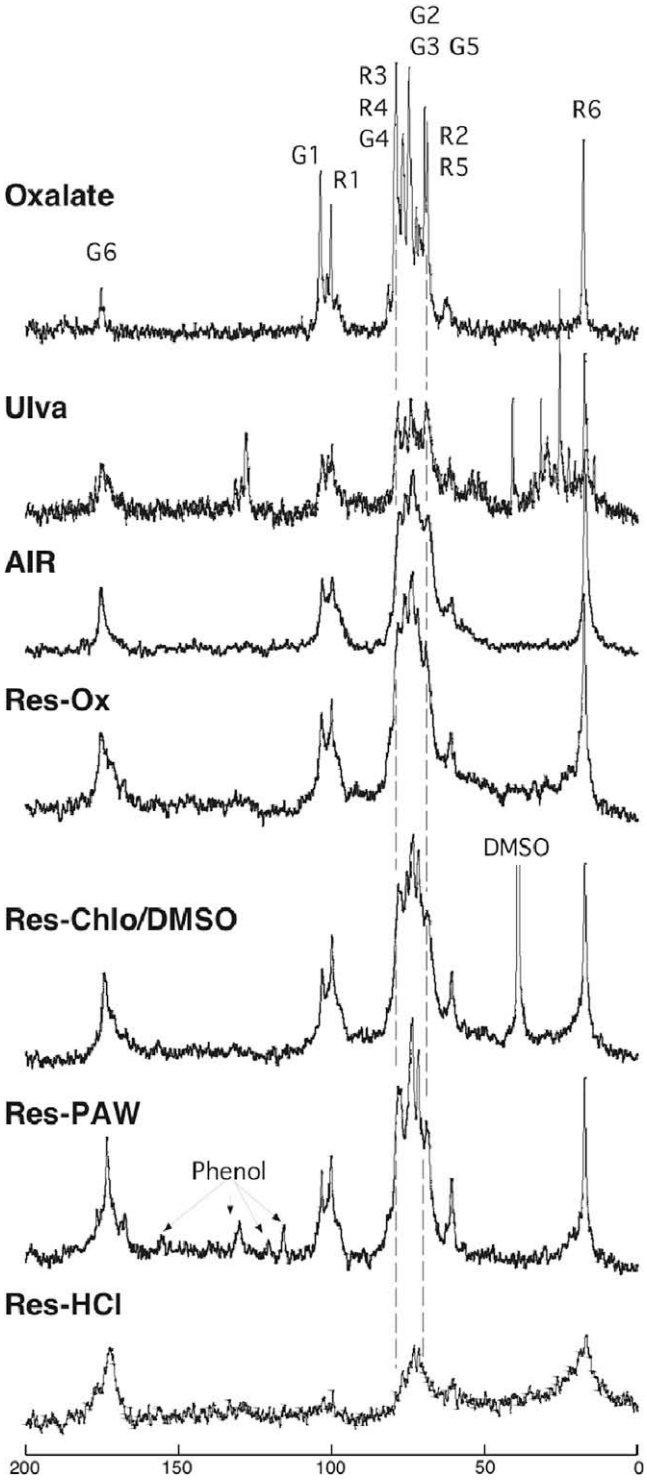

Fig. 6. ^{13}C NMR spectrum of oxalate extract (Oxalate) and ^{13}C SPEMAS spectra of *Ulva*, AIR and extraction residues rehydrated to 66% moisture content.

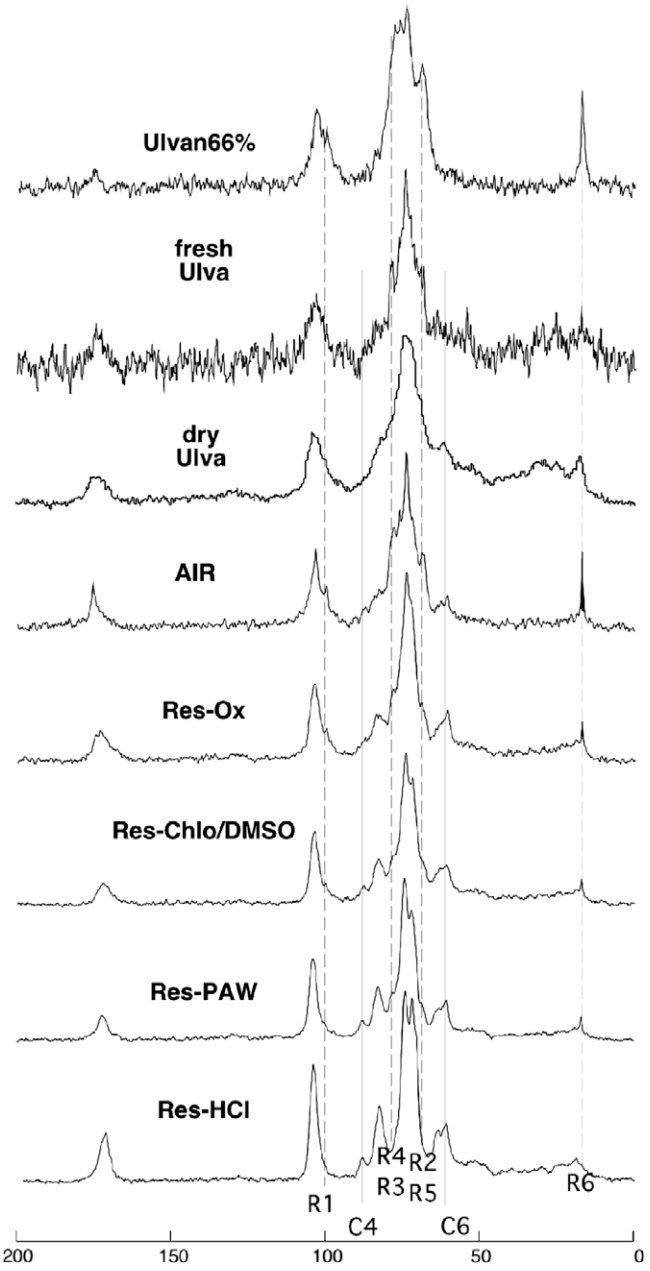

Fig. 7. ^{13}C CPMAS spectra of ulvan (oxalate extract), *Ulva* AIR and extraction residues rehydrated to 66% moisture content.

presence of residual ulvan and better resolved signals of cellulose/hemicelluloses.

HCl extraction residues were nearly free of ulvan as indicated by both SPEMAS and CPMAS spectra. The SPEMAS spectrum was characterized by compounds rich in carbonyl (170–180 ppm) and in aliphatic carbons (10–20 ppm), which were assigned primarily to proteins and to a lesser extend to polyesters of the cuticle (Pacchiano, Sohn, Chlanda, Garbow, & Stark 1993) if we consider the protein content of the residues (Table 1). The CPMAS spectrum showed signals assigned to partially crystalline cellulose and a strong signal for carbonyls (170–180 ppm). Small broad resonances observed between 20 and 50 ppm can correspond to proteins contributions.

3.1.5. Evaluation of conditions to improve ulvan extraction efficiency

Based on the ulvan recovery in the sequential extraction, acidic conditions were tested directly on AIR as a mean to improve the ulvan extraction. The ulvan extraction efficiency, the chemical composition and the molecular distribution of extracts treated with 50 mM HCl at 85 °C for 3 times 30 min (30A, 30B, and 30C), once for 60 min (60A) and with 20 mM acidified ammonium oxalate (A-Ox) were compared to that obtained by sodium oxalate (50 mM, pH 6.5, 2 h, 85 °C) used as a reference (Table 3) Ulvan extraction efficiency varied from 2.9% to 87.4% depending on the

extraction process with the highest value obtained by HCl for 60 min (60A). Very low proportions of ulvan were extracted with subsequent re-extraction for 30 min of the 30 min HCl residues. Except for extracts 30B and 30C, the sugar composition of extracts was close. Compared to the reference extract, an increase in the glucose and protein was observed on the weight basis with the sequential HCl extraction while sulfate content decreased. All acidic extractions induced a decrease in the molecular weight of ulvan in comparaison with the reference extract (Fig. 8). A lower decrease was noted with acidic ammonium oxalate whereas a high degradation was observed with HCl. The degradation increased with the time of extraction.

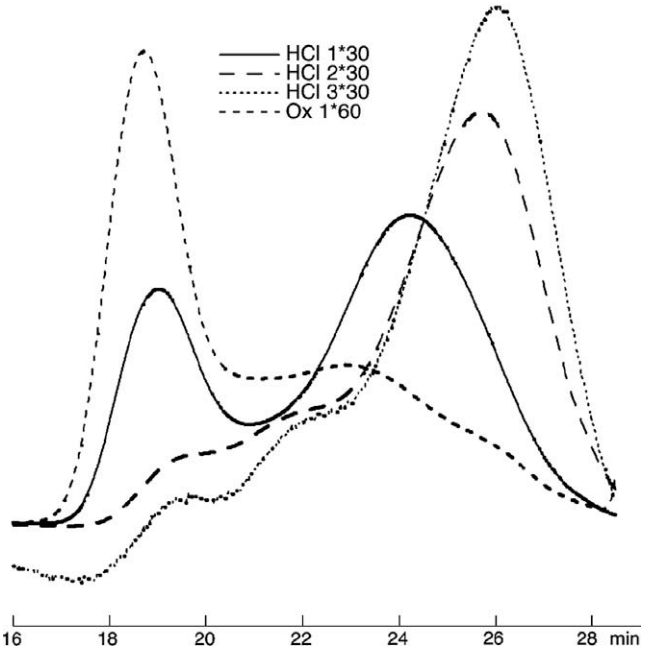

Fig. 8. High pressure size exclusion chromatographic profiles of alternative extracts from *U. rotundata* detected by refractive index.

4. Discussion

Ulvan extraction yields by hot water or chelator solutions vary according to species, to the period of collect and to the stabilization treatment (Abdel-Fattah & Edrees, 1973; Lahaye et al., 1999; Medcalf, Lionel, Brannon, & Scott, 1975; Siddhanta, Goswami, Ramavat, Mody, & Mairh, 2001; Robic et al., 2009). High extraction yields were obtained during the active growth period of *U. rotundata* and at the end of the proliferation period of *U. armoricana* (Robic et al., 2009). These yields were interpreted as the incomplete ulvan setting in newly formed walls or as the beginning of wall breakdown in degenerating algae. Storage of algae in high saline conditions resulted in low ulvan extraction likely because of the aggregative properties of ulvan in the presence of high salt concentrations (Paradossi, Cavalieri, & Chiessi, 2002; Robic et al., unpublished). In contrast, algae in low acid brine yielded very high amounts of ulvan probably due to the beginning of algal degradation by microorganisms and/or to the acidic environment (Robic et al., 2008). Previous work also showed that residual ulvans after extraction by hot sodium oxalate solution were only partially extracted by alkaline solutions (Ray & Lahaye, 1995a, 1995b).

In order to understand what limits ulvan accessibility in *Ulva*, a sequential extraction with different solvent was realized. The extracts and residues were characterized chemically and by ^{13}C NMR spectroscopy. Solid-state ^{13}C NMR spectra of insoluble residues were recorded in an attempt to further evaluate the effect of the sequential extractions on the structure and molecular mobility of ulvan in the cell wall. Single pulse excitation (SPEMAS) of carbons with proton decoupling resulted in the characterization of the most mobile structural domains, whereas the CPMAS technique was used to over-express the rigid segments of cell wall polysaccharides.

To ease interpretation of solid-state ^{13}C NMR spectra of *Ulva* and those of the extraction residues, SPEMAS and CPMAS spectra of a partially rehydrated ulvan extract were recorded (Fig. 5). Ulvan yielded poorly resolved NMR spectra similar to other already published (Laza et al., 2007 CPMAS without rehydration) but resolution was improved with hydration and was better observed on the CPMAS spectra. It has been shown that hydration can induce some conformational reorganization of polysaccharides resulting in a decrease in the chemical shift anisotropy and generally accompanied with chemical shifts variations (Cheetham & Tao, 1998; Jarvis & McCann, 2000; Lahaye, Rondeau-Mouro, Deniaud, & Buléon, 2003; Saito, Yokoi, & Yamada, 1990; Saito, Yokoi, & Yoshioka, 1989). Molecular modeling studies proposed different helical structures for ulvan based on the two major repeating sequences (Paradossi et al., 2002). If such structures are present, no water dependent conformational transition of ulvan was observed on chemical shifts as sometimes observed in helix forming polysaccharides (Cheetham & Tao, 1998; Lahaye et al., 2003). On the CPMAS spectra, the observed line narrowing can also be associated with the disappearance of structural domains that show increased segmental mobility with the water mobilization effect (Morgan, Furneaux, & Larsen, 1995). As pointed out by Morgan et al. (1995), cross-polarisation depends on $^{1}H/^{13}C$ heteronuclear dipolar coupling. If these couplings are averaged because of fast molecular motion, cross-polarisation does not occur preventing any signal observation in CPMAS conditions. This can particularly be noticed with the uronic acids carbonyl signal and the rhamnose methyl signal that are also well observed using the SPEMAS technique. The poor signal resolution and the low intensity of sugar carbon signals on SPEMAS spectra compared to that of CPMAS indicated that ulvan is made of rather rigid structural domains. This statement is in agreement with other data showing that water is a poor solvent for ulvan. The polysaccharide in water is essentially in the form of a

Table 3
Ulvan extraction efficiency and chemical composition of ulvan alternative extractions from *U. rotundata*. Total sugars represent the sum of sugars content determined by GC and colorimetry. Total sugars, sulfate and protein contents were expressed in % dry weight, +/− standard deviation, $n = 3$. Sugar content was expressed in mol% +/− standard deviation, $n = 3$.

	Ulvan extraction efficiency	Total sugars	Rha	Xyl	Gal	Glc	UA[a]	Sulfates	Proteins
30A	59.1	28.1	39.0 ± 1.0	15.6 ± 0.0	0.6 ± 0.0	6.1 ± 0.1	38.7 ± 1.0	10.6 ± 0.4	1.9 ± 0.3
30B	16.3	15.4	33.3 ± 0.8	12.8 ± 0.4	0.3 ± 0.3	23.3 ± 0.4	30.3 ± 1.0	5.5 ± 0.0	2.9 ± 0.2
30C	2.9	7.9	25.9 ± 1.0	11.5 ± 0.4	0.0 ± 0.0	34.6 ± 2.7	27.9 ± 1.7	0.3 ± 0.4	3.7 ± 0.0
60A	87.4	28.7	42.3 ± 1.1	16.0 ± 0.2	0.7 ± 0.1	5.8 ± 0.1	35.1 ± 1.2	11.1 ± 0.5	1.8 ± 0.0
A-Ox	50.7	19.3	38.8 ± 1.6	15.5 ± 0.2	0.9 ± 0.1	5.7 ± 0.1	38.6 ± 1.6	11.0 ± 0.2	2.4 ± 0.0

[a] UA: uronic acids.

dispersion of condensed nano-sized bead-like structures with some fiber-like structures (Robic et al., unpublished). The core of the beads would be observed by CPMAS spectroscopy while their surface chains showing a higher mobility would be observed by the SPEMAS technique. The relative low resolution of ulvan signals on the SPEMAS spectra would support large chemical shift anisotropy and dipolar interactions affecting signal width in relation with different chain environments consecutive to beads aggregation.

Ulvan structure and mobility in the raw alga were first characterized by solid-state ^{13}C NMR spectroscopy. The SPEMAS spectrum revealed low molecular weight metabolites and mobile ulvan structural domains proving that part of polysaccharide was loosely held in the cell wall. The alcohol extraction markedly improved the NMR signals intensity in enriching the material in cell wall polysaccharides. The AIR SPEMAS and CPMAS spectra were characteristic of ulvan with the contribution of other resonances likely coming from hemicelluloses and starch (SPEMAS spectrum) and cellulose (CPMAS spectrum). The AIR SPEMAS spectrum was close to that of the ulvan SPEMAS spectrum with a resolution lower than that of the ulvan liquid-state ^{13}C NMR spectrum. This result contrasted with that of a red algal xylan, which showed close signals resolution and chemical shifts between the SPEMAS spectrum of the rehydrated algal AIR and the solution spectrum of the extracted xylan (Lahaye et al., 2003). The mean conformation and environment of ulvan is likely to be similar in the cell wall and in partially rehydrated extracted polysaccharides. This suggested that ulvan might also have a bead-like organization in the cell wall.

Ulvan gel formation requires the presence of both a divalent cation (such as calcium) and borate (Lahaye & Axelos, 1993). These interactions are likely to occur in the algal cell wall and thus, their destabilization by a chelating agent is often employed to ease extraction of ulvan. In this study, the hot sodium oxalate solution allowed the highest recovery of ulvan with the sequential extraction scheme used (Table 1). The remaining ulvan was assumed to be covalently linked, retained by strong physical bonds and/or entrapped within the fibrillar wall. In a previous work, an extraction yield of 9.0% on the basis of dry algae weight with an ulvan extraction efficiency of 29.8% on the rhamnose weight in the raw algae was obtained with hot sodium oxalate from seaweeds ground to pieces of about 0.5 cm (Robic et al., 2009). Using the same algal sample in this study, but ground to particles of $\leqslant$100 μm and depigmented, ulvan yield was increased to 27.5% on the basis of AIR weight with an extraction efficiency of 45.6% on the rhamnose weight in the AIR. The ratio of ulvan extraction efficiency on extraction yield was better with the large fragments of algae compared to the particles of AIR. This can arise from the loss of low molecular weight molecules rich in rhamnose during the depigmentation. However, ulvan accessibility is also improved with a thorough disintegration of the algal material. As most of the ground material passed through the 100 μm mesh, the small particles were representative of the all algal tissue. The small dimensions of the particles allowed exposure of a large specific surface of cell wall to the solvent and thus, facilitated ulvan diffusion in the extracting solution. The resulting algal residues showed a marked decrease in the intensity of ulvan signals on the oxalate residues (Res-Ox) CPMAS spectrum. However, as revealed on the Res-Ox SPEMAS spectrum (Fig. 6), the residual ulvan still showed thin signals characteristic of high molecular mobility. The 54.4% of the initial rhamnose content remaining in the seaweed after extraction were likely retained by punctual covalent cross-links and/or strong physical bonds in the wall in association with mobile pending chains segments.

To cleave these structures several treatments were tried on the residues. In plant cell walls, $NaClO_2$ solutions are known to extract proteins and glycoproteins by peptides bonds cleavages particularly affecting the structural protein extensin (Fry, 1988). Extensin-like proteins rich in proline and hydroxyproline are present in *Ulva* cell walls (Gotelli & Cleland, 1968). These proteins cross-link cell walls by peroxidases through isodityrosin or lysine-tyrosine linkages (Kieliszewski & Lamport, 1994; Passardi, Penel, & Dunand, 2004) and are thought to be partly linked to pectins in higher plant (Qi, Behrens, West, & Mort, 1995). Extraction of Res-Ox by $NaClO_2$ released a small amount of ulvan with proteins as already reported (Ray & Lahaye, 1995a, 1995b). Elution on HPSEC of high molecular weight polymers (Fig. 2) giving both an UV and RI response from the chlorite extract would support ulvan-protein linkages or tight physical interactions. However, if present, these are not solely responsible for the retention of residual ulvan in the wall.

Dimethyl sulfoxide (DMSO) is known to extract weakly hydrogen bounded hemicelluloses and starch (Fry, 1988; Ring & Selvendran, 1978). The major components obtained in the DMSO extract from chlorite treated residues were starch and proteins that distributed essentially in medium to low molecular weight range (Fig. 2). The amyloglucosidase treatment used before oxalate extraction was clearly not sufficient to free the algal material from starch. Resistant starch may have been encapsulated in cells and thus inaccessible to amylase. *Ulva* hemicelluloses were likely too tightly hydrogen bonded to be extracted by DMSO and linkages of residual ulvan in the wall remained unaffected by the treatment. Treatment of plant cell walls with phenol-acetic acid-water (PAW) is generally used to remove non-covalently bound proteins and glycoproteins (Selvendran, Stevens, & O'Neill, 1985). It is a very poor solvent of polysaccharides and is used to prepare clean cell walls (Fry, 1988). Applied to the algal residues after DMSO extraction, the PAW extract yield was very low and poor in sugars (Table 1). These three treatments failed to improve ulvan extraction as very little of the polysaccharides was recovered even with the water washes of residues. None of the treatment markedly affected the CPMAS ^{13}C NMR spectra on the basis of the ulvan signal intensity. However, they contributed to increase the molecular mobility of ulvan as seen on the SPEMAS ^{13}C NMR spectra and to favor conformational rearrangements as judged from the increased signal resolution on the CPMAS spectra. Proteins being mainly affected by these treatments, they appear to be responsible in part for entrapping ulvan and other wall polysaccharides.

At this step, 51.6% of ulvan remained in the cell wall of *Ulva*. The major holding mechanism was mainly attributed to covalent bonds as strong alkali used to extract hydrogen bonded hemicelluloses had a limited effect on ulvan extractions (Ray & Lahaye, 1995a, 1995b). In fact, alkaline pH were shown to promote ulvan aggregation (Robic et al., unpublished). Treatment with hot dilute chlorhydric acid as used in plant cell wall pectin extraction (Fry, 1988), extracted the totality of residual ulvan together with residual starch and proteins (Table 1). Acid pH below the pK_a of glucuronic acid was shown to disperse aggregates of ulvan nano-sized bead-like structure in solution (Robic et al., unpublished). Acidic extraction would thus favor ulvan dissociation together with cleavages of linkages. The low molecular weight of the acid extracted ulvan likely resulted from the degradation of the polysaccharide, probably between the uronic acids and rhamnose (Be Miller, 1967). The co-elution of a small amount of high molecular weight polymers followed by refractometric and UV detection would suggest, as for the chlorite extract, a co-occurrence of ulvan and protein, either covalently linked or in tight physical interactions.

Although the mean chemical structure of ulvan by liquid-state ^{13}C NMR spectroscopy did not markedly differ between extracts (Fig. 3), the fine chemical structure of the polysaccharides, assessed by the oligosaccharides profile produced by an ulvan-lyase degradation, showed clear differences (Fig. 4). Thus, ulvan forms a wide continuum of related polysaccharides differing in the extent and

type of fine structural characteristics that remain to be fully identified. The nature and distribution of these structures may be characteristic of populations of ulvan more or less cross-linked in the wall. Sequential extraction by hot oxalate and hot dilute acid may represent a mean to extract different pools of ulvan.

Based on the chemical analysis (Table 1) and the CPMAS ^{13}C NMR spectrum (Fig. 7), the insoluble material after hot dilute HCl extraction was mainly composed of cellulose and hemicelluloses with residual proteins (Dennis & Preston, 1961; Lahaye et al., 1994; Pizzoferrato, 1995). Lahaye et al. (1994) and Pizzoferrato (1995) showed that *Ulva* cellulose is rather amorphous and Pizzoferrato (1995) demonstrated that acidic conditions can partly crystallize it. It is thus likely that the crystalline part of *Ulva* cellulose observed after acidic treatment arose from the degradation of amorphous cellulose that led to partial rearrangement of the fibrils. However, the total sugar content accounting to only a part of the material weight and beside ashes, cuticle material and proteins were expected. The small resonances observed for aliphatic carbons by NMR spectroscopy and the major carbonyl signal that could not be totally assigned to a polyuronan due to the low uronic acid content in the residues imply the presence of a peculiar carboxylated polymeric structure that remains to be identified. This carbonyl signal was also observed on the CPMAS spectrum of residues after the hot dilute HCl extraction in the alternative trials. This observation excluded its origin from the oxidation of cellulose by the chlorite treatment or due to the presence of residual sodium oxalate.

In an attempt to improve ulvan extraction yield, hot dilute HCl conditions were shown to markedly improve the extraction efficiency. Repeated 30 min extractions were less efficient than a single 1 h extraction and yielded extracts likely contaminated by starch. The decrease in molecular weight of ulvan is a drawback of these treatments if texturing ability of ulvan is looked for. Ammonium oxalate extraction at pH 4.6 allowed a more modest increase in ulvan extraction efficiency (5%) but limited polysaccharide degradation. The desaggregation of ulvan bead-like structures were shown to occur below the pK_a of uronic acid (3.28, Kohn & Kovac, 1978; Robic et al., unpublished). It thus appears that a solution with a pH close to this pK_a would favor ulvan extraction. Other extraction conditions with hydrophobic co-solute should also be considered taking into account that water is a poor solvent for ulvan.

5. Conclusion

Only part of the total ulvan present in *Ulva* cell wall is extracted using a chelating agent solution. Beside algal particle size, limitations in extraction appear to be associated with the peculiar chemical structure of the polysaccharide, which may alter water solubility of the polyelectrolyte. Additionally, they also likely result from covalent linkages, which bond types and partners remain to be established. Among them, cell-wall proteins appear important contributors as they were shown to entrap *Ulva* cell wall polysaccharides. Considering their roles in higher plant cell wall adaptation to developmental or environmental conditions (Passardi et al., 2004), they may be determinant in ulvan extraction. However, oxidative and alkali treatments that cleave them, are not sufficient to free wall-bonded ulvan. Further studies should consider other solvents, co-solutes and physicochemical conditions to break strong *Ulva* cell wall macromolecular complexes.

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