ELSEVIER

Carbohydrate Polymers 36 (1998) 239–249

Carbohydrate Polymers

NMR analysis of the chemical structure of ulvan and of ulvan-boron complex formation

M. Lahaye*, F. Inizan, J. Vigouroux

Laboratoire de Biochimie et Technologie des Glucides, Institut National de la Recherche Agronomique, BP 71627, 44316 Nantes Cedex 03, France

Received 27 May 1997; revised 30 October 1997; accepted 9 November 1997

Abstract

The NMR spectroscopic analysis of autohydrolysate fragments obtained from the gel-forming water-soluble cell-wall sulphated polysaccharides from the green seaweed *Ulva* sp. allowed the identification of two major repeating units in ulvan. These were the aldobiouronic acids → 4)-β-DGlc*p*A-(1 → 4)-α-L-Rha*p* 3-sulphate and → 4)-α-L-Ido*p*A-(1 → 4)-α-L-Rha*p* 3-sulphate that were given the name ulvanobiouronic acid 3-sulphate A and B, respectively. These structures failed to bind boric acid since no ulvan-boron complex was observed by ^{11}B or ^{13}C NMR spectroscopy at the optimum pH of ulvan gelation (7.5). Boron complexes were only observed at pH ≥ 9 with ulvanobiouronic acid A. These results indicate that the ring hydroxyl groups of the major repeating structures which contain the rarely encountered iduronic acid in plant polysaccharides are probably not involved in the boric acid fixation step during the gelation of ulvan.

Keywords: Ulvan; *Ulva* sp; Boric acid; Gel; Aldobiouronic acid; Sulphate; NMR

1. Introduction

The marine edible green seaweed *Ulva* sp (sea-lettuce) synthesizes a water-soluble polysaccharide composed of glucuronic acid, rhamnose, xylose, iduronic acid and sulphate (Quemener et al., 1997) referred to as ulvan. This polysaccharide corresponds to the water-soluble dietary fibers that resist both human digestive tract endogenous enzymes and degradation by the colonic flora (Bobin-Dubigeon et al., 1997a; Durand et al., 1997). Together with β-D-(1,4)-glucuronan, it limits the colonic bacterial degradation of the unusual amorphous α-cellulose in this algal cell wall (Bobin-Dubigeon et al., 1997b). It is able to form weak thermoreversible gels in the presence of boric acid and calcium at pH~7.5 (Haug, 1976; Lahaye & Axelos, 1993; Lahaye et al., 1996) and the gelling mechanism is thought to involve the formation of ulvan-boric acid complexes and their cross-linking is mediated by calcium. The chemical structure analysis of *U. 'rigida'* ulvan established that rhamnose, glucuronic acid and xylose are all 1,4-linked, rhamnose is sulphated on O-3 and can partially be branched by glucuronic acid on O-2 and xylose can partially be sulphated on O-2 (Ray & Lahaye, 1995; Lahaye & Ray, 1996). The aldobiouronic acid β-D-Glc*p*A-(1 → 4)-L-Rha*p*, referred to as ulvanobiouronic acid (Lahaye & Ray, 1996), is a major repeating disaccharide in this ulvan and was first recognised in ulvan from *U. lactuca* and other related seaweeds (O'Donnel & Percival, 1959; McKinnel & Percival, 1962a, b). Beside this disaccharide, other repeating fragments were isolated from the partial acid hydrolysate of *U. 'rigida'* ulvan among which was the ulvanobiouronic acid 3-sulphate: β-D-Glc*p*A-(1 → 4)-L-Rha*p* 3-sulphate (Lahaye & Ray, 1996). The purpose of the present work was to further the chemical structure characterisation of ulvan from 'sea-lettuce' and to establish the site of boron interaction on ulvan by NMR spectroscopy.

2. Materials and methods

2.1. Polysaccharide

Ulvan was extracted from *Ulva* sp. (Laitue de mer, Nature-Algue, Landerneau, France) as follows: *Ulva* flakes (5 g) were suspended in acidic deionized water (1 l containing 1.5 ml H_2SO_4, 96%) and stirred for 30 min at room temperature to convert ulvan to its acidic form and to allow for the removal of small contaminating compounds. The flakes were recovered after filtration of the slurry through nylon mesh (0.2 mm); the filtrate was discarded.

* Corresponding author. E-mail:lahaye@nantes.inra.fr

0144-8617/98/$19.00
PII S0144-8617(98)00026-5

Ulvan was neutralized and extracted from the residues by $NaHCO_3$ (1 l, 0.1 mol l^{-1}) with stirring for 30 min at room temperature before filtration as above. The filtrate was kept and the residues were extracted with deionized water (1 l) in a boiling water-bath for 1 h. The residues, recovered by filtration as above, were discarded and the filtrate was combined with the sodium carbonate extract. The pooled liquid fraction was centrifuged (15 000 *g*, 20 min), filtered through a 3 μm membrane filter, adjusted to pH 6.0 and concentrated to 10% of its initial volume by ultrafiltration through Amicon hollow fibers of 30 kD molecular weight cut-off (Millipore). Soluble starch in the extract was hydrolyzed by amyloglucosidase (0.5 ml, Sigma) for 30 min at room temperature with stirring. NaCl was then added to bring the solution to a 0.1 mol l^{-1} final concentration prior to precipitation of ulvan in 4 vol of 95% ethanol. The precipitate was recovered and washed twice with 95% ethanol and acetone before air drying (yield: 16.9%). In order to remove residual NaCl, this crude ulvan extract was redissolved in deionized water (500 ml) and extensively dialyzed against deionized water. The retentate was centrifuged (15 000 *g*, 20 min) and the supernatant was freeze-dried (ulvan yield: 14.4% from the starting dry algal weight.

2.2. Autohydrolysis

In order to generate ulvan oligosaccharides, the polysaccharide in its acidic form was depolymerized by autohydrolysis. The acidic ulvan was obtained as follows: ulvan (5 g) was dissolved in deionized-water (150 ml) to which was added IR 120 resin (H^+, BioRad) to obtain a stable pH reading (1.9). The slightly turbid solution was recovered by filtration (fritted-glass filter G1, porosity 90–150 μm) and the resin was washed with about 100 ml deionized-water. the filtrate and the wash were pooled in a screw-capped Erlenmeyer flask to which a small quantity of IR 120 (H^+) resin was added. The acidic ulvan was depolymerized at 80°C for 24 h and recovered after filtration (fritted-glass filter G1) and centrifugation (15 000 *g*, 20 min). The pellet was resuspended in deionized-water and centrifuged once again. The supernatant was pooled, the pH was brought to 7 by NH_4OH and then freeze-dried to yield SF24 (yield: 73.2%).

Ethanol fractionation of SF24. SF24 (2 g) was suspended in 85% ethanol (250 ml) with stirring at 40°C for 1 h. The slurry was filtered (fritted-glass filter G2, porosity 40–90 μm) and the residues treated twice more as above for 30 min each. The filtrates were combined, evaporated to dryness and redissolved in deionized-water (6 ml, SE24). The residues were redissolved in deionized-water, evaporated to dryness and redissolved again in water (7 ml, SR24).

2.3. Preparation of ulvanobiouronic acid

The ulvanobiouronic acid was prepared as described elsewhere (Bobin-Dubigeon et al., 1997a) by 2 N TFA hydrolysis of ulvan and BioGel P2 chromatography (85 × 3.5 cm, Bio Rad) of the hydrolysate. The disaccharide was further purified by anion exchange chromatography through AGIX8 (Bio Rad, 1.8 × 14 cm) eluted by deionized-water and an ammonium acetate gradient 0 → 1 mol l^{-1}. The ulvanobiouronic acid was the only fraction eluting in the gradient. It was desalted by repeated evaporation and by permeation through a Sephadex G-10 column (1.6 × 90 cm, Pharmacia) eluted with deionized-water and then freeze dried.

2.4. Anion-exchange chromatography

SE24 and SR24 were applied to DEAE-Sepharose CL-6B (1.6 × 20 cm, Pharmacia) and eluted first by deionized-water (200 ml) followed by a 0 → 0.8 mol l^{-1} NaCl gradient (600 ml), a 0.8 mol l^{-1} NaCl plateau (200 ml) and then a 1.0 mol l^{-1} NaCl plateau (200 ml). Fractions were collected and analysed for sugar content. Peaks were pooled and concentrated by rotary evaporation, desalted through a Sephadex G10 column (1.6 × 90 cm, Pharmacia) and concentrated as above.

2.5. Gel permeation chromatography

Desalted fractions collected from DEAE-Sepharose chromatography were applied to BioGel P2 or P4 (2.6 × 96 cm) and eluted by 0.05 mol l^{-1} $NaNO_3$ at 40°C. Elution was monitored by differential refractometry. Fractions were pooled, desalted as above and freeze-dried. The elution of oligosaccharides are expressed as *K*av where *K*av = $(V_e - V_0)/V_t - V_0)$ with V_t, V_0 being the total and void volume of the column, and V_e the elution volume of the sample.

2.6. NMR spectroscopy

^1H and ^{13}C 1D and 2D NMR spectra were recorded on a Bruker ARX 400 spectrometer using conventional pulse sequences provided by the manufacturer. Samples were exchanged once with 99.9% D_2O before resolubilization in 100% D_2O. Samples for ^{11}B NMR spectroscopy were dissolved in H_2O (80–50%) containing boric acid and D_2O (20–50%). The pH was adjusted in the NMR tube by addition of NaOH or HCl. Calibration of the pH meter was done with buffer solutions in water. Chemical shifts are referred to acetone (δ^1H: 2.225, $\delta^{13}C$: 31.45 ppm) and external boric acid ($\delta^{11}B$: 0 ppm).

2.7. Chemical analyses

The protein content of the fractions was determined by the Kjeldhal method ($N \times 6.25$). Neutral sugars and uronic acids in the chromatographic fractions were determined by the colorimetric method described by Thibault (1979) and Tollier & Robin (1979).

3. Results

3.1. Chemical structure of ulvan

The sugar composition on a dry weight basis of the purified ulvan was given by Quemener et al. (1997) as *U. lactuca* ulvan and contains rhamnose 20.8%, glucuronic acid 16.0%, iduronic acid 3.7%, xylose 3.5%, glucose 2.8%, galactose 0.7% and sulphate 21.2%. These values are within those reported for ulvan from different origins (DeReviers & Leproux, 1993; Percival & McDowell, 1967; Quemener et al., 1997; Ray & Lahaye, 1995). It also contained 11.6% proteins. In order to get to the repeating structures of ulvan, the polysaccharide was degraded under mild conditions to prevent as much as possible desulphation which can result from acid hydrolysis (Percival & McDowell, 1967). The fragments were recovered in 73.2% yield from the starting dry weight of the material and were fractionated by their differential solubility in 85% warm ethanol (40°C). Monomers and small oligomers soluble in ethanol (SE24) were fractionated by anion exchange chromatography (Fig. 1). Of the fractions recovered, only SEA was applied to gel permeation chromatography through BioGel P2. Two peaks eluting at *K*av 0.61 and 0.39 of the three obtained were collected and characterized by NMR spectroscopy. These were referred to as SEA-1 and SEA-2, respectively. The SEA-1 fraction was identified as rhamnose 3-sulphate as follows. The ^{1}H and ^{13}C NMR chemical shifts of this fraction (Fig. 2) were identified from COSY and HMQC experiments (Table 1) and the position of the sulphate on O-3 was deduced from the marked downfield shifts of H-3 and C-3 signals compared to those of rhamnose (Table 1). The overlapping H-2 and H-3 as well as H-4 and H-5 signals of the β-anomer resulted in complex coupling patterns and the definite attribution of these protons and carbons came from an HMQC-TOCSY experiment (Table 1). The ^{1}H NMR spectrum of SEA-2 corresponded to that of the aldobiouronic acid previously described (Lahaye et al., 1996).

Ethanol-insoluble oligosacharides (SR24) from the ulvan autohydrolysate were also fractionated by anion-exchange chromatography (Fig. 1) and only fractions SRA and SRB were further investigated. SRA was chromatographed on BioGel P4 into several peaks and that eluting at *K*av 0.57 was characterized by NMR spectroscopy. Its ^{1}H NMR spectrum showed anomeric signals attributed from their chemical shifts and coupling constants to rhamnose α and β, β-D-glucuronic acid and α-L-iduronic acid (Fig. 3A; Mulloy et al., 1994; Lahaye & Ray, 1996; Quemener et al., 1997). Most of the protons in these sugars were attributed from the COSY map and by comparison with data

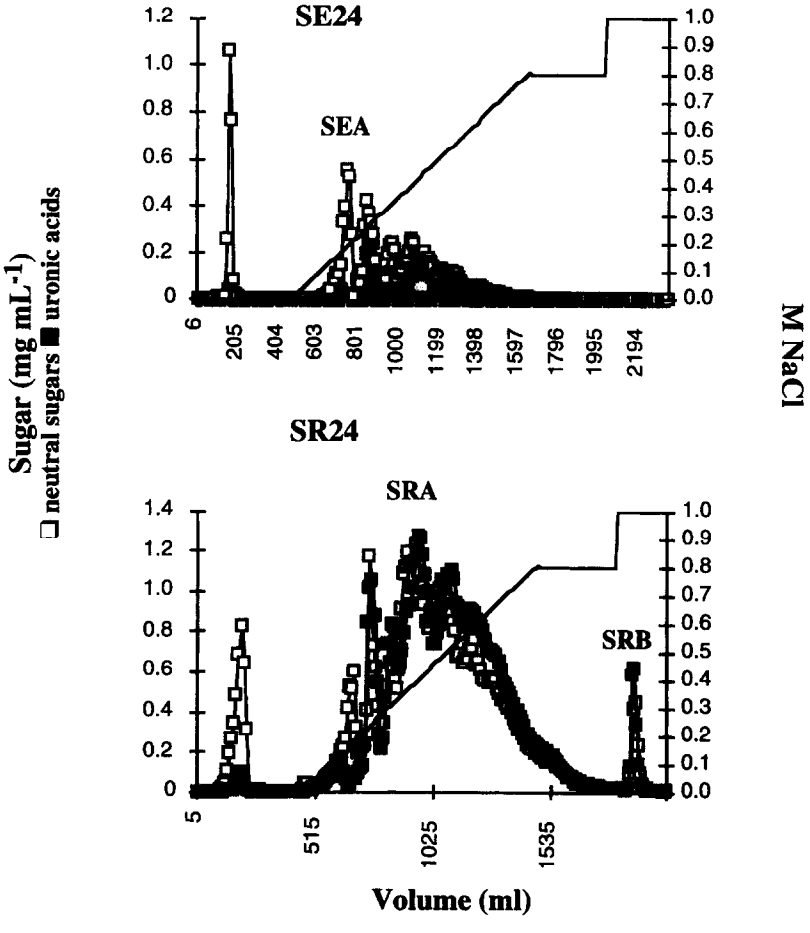

Fig. 1. DEAE-Sepharose chromatographic profiles of the ethanol-soluble (SE24) and -insoluble (SR24) ulvan fragments obtained after 24 h autohydrolysis.

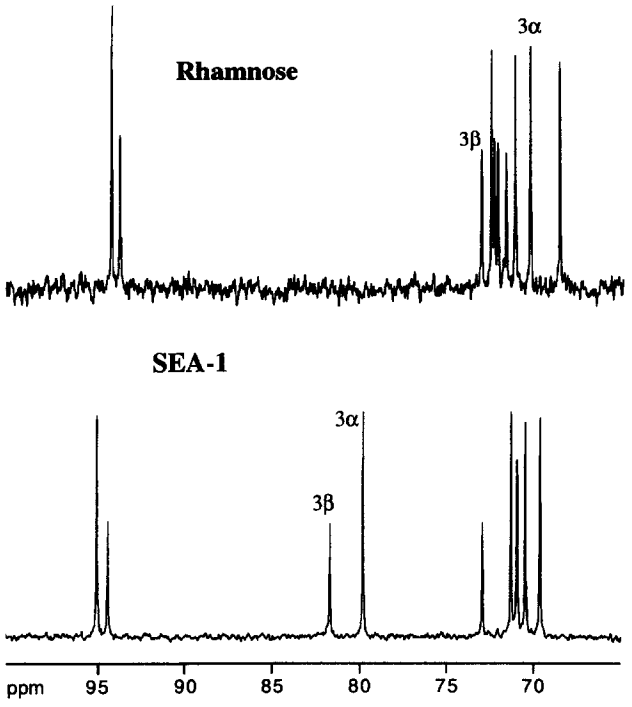

Fig. 2. ^{13}C NMR spectra at 27°C of rhamnose and fraction SEA-1 corresponding to rhamnose 3-sulphate recovered after DEAE-Sepharose anion exchange and BioGel P2 gel permeation chromatography of the ethanol soluble ulvan autohydrolysate fragments; α3 and β3 refer to C-3 of rhamnose and rhamnose 3-sulphate in configuration α and β, respectively.

previously obtained on ulvan oligosaccharides (Table 1, Lahaye & Ray, 1996). Rhamnose 3-sulphate occupied both the reducing and non-reducing ends of the oligosaccharides. The protons chemical shifts of the reducing-end rhamnose 3-sulphate were in agreement with those of the ulvanobiouronic acid 3-sulphate (Lahaye & Ray, 1996) but some of them were split (Table 1). The proton chemical shifts of the non-reducing end rhamnose 3-sulphate were deduced from the COSY map and by comparison with those of H-3, H-4, H-5 and H-6 of the free α-rhamnose 3-sulphate (SEA-1, Table 1). Most of the carbon chemical shifts (Fig. 3) were determined from HMQC and HMQC-TOCSY experiments and, as for proton NMR, two series of anomeric signals were observed (Fig. 3, Table 1). The small splitting observed for C-1 of the glucuronic acid (Fig. 4) was attributed to the α and β anomeric effect of the reducing end rhamnose 3-sulphate. The linkages of the different non-reducing end rhamnose 3-sulphate and that of the iduronic and glucuronic acids were deduced from HMBC and ROESY experiments (Figs. 4 and 5). Correlations between the anomeric proton and carbon of one of the non-reducing end rhamnose 3-sulphate (R1') and H-4 and C-4 of iduronic acid (I4) were clearly seen on the HMBC and ROESY maps. The correlations of the anomeric proton and carbon of iduronic acid (I1) with H/C-4 of α/β-rhamnose 3-sulphate at the reducing end established the linkage of this uronic acid to C-4 of one reducing end

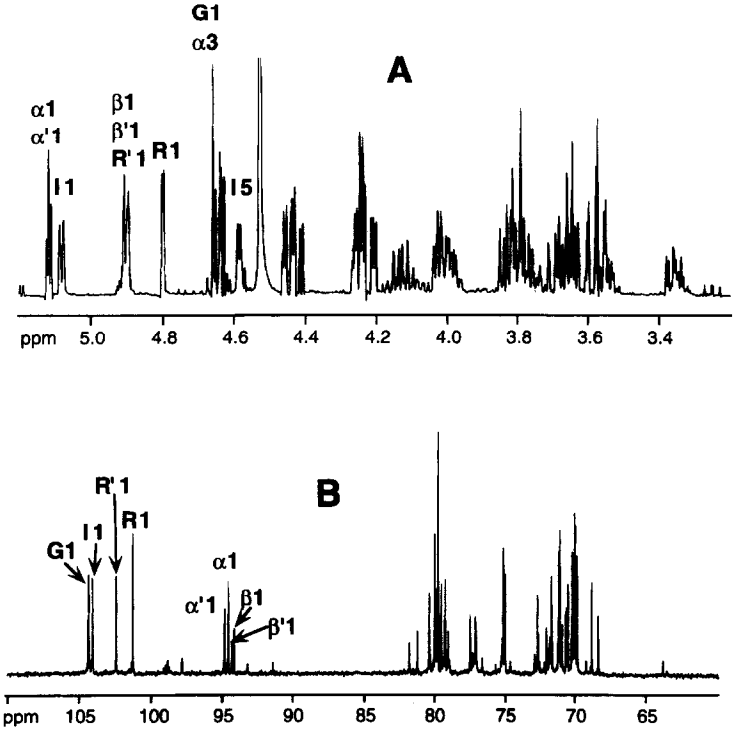

Fig. 3. ^{1}H (A) and ^{13}C NMR spectra (B) of fraction SRA at 47°C recovered after DEAE-Sepharose anion exchange and BioGel P4 gel permeation chromatography of the ethanol insoluble ulvan autohydrolysate fragments; α1, β1, α'1, β'1 refer to the anomeric proton and carbon of the different α- and β-L-rhamnose 3 sulphate at the reducing end, respectively; I1, R1, R'1, G1 refer to the anomeric proton and carbon of the iduronic acid, the non-reducing end rhamnose 3-sulphate linked to glucuronic acid, the non-reducing end rhamnose 3-sulphate linked to iduronic acid, and of the glucuronic acid, respectively; α3 and I5 refer to H-3 and H-5 of reducing end α-rhamnose 3-sulphate and iduronic acid, respectively.

Table 1
Chemical shifts (coupling constant) of SEA-1 (27°C), SRA (47°C) and SRB (60°C) (acetone δ ^{1}H: 2.225, ^{13}C: 31.45 ppm). α, β refer to the protons and carbons of the α and β anomers of the free rhamnose, rhamnose 3-sulphate and rhamnose 3-sulphate at reducing-ends, the ' refers to 4-linked rhamnose 3-sulphate by iduronic acid; R refers to non-reducing end rhamnose 3-sulphate linked to glucuronic acid, R' refers to non-reducing end rhamnose 3-sulphate linked to iduronic acid, G refers to the glucuronic acid, and I refers to the iduronic acid. (nd not determined)

		1	2	3	4	5	6
Rhamnose							
α	^{13}C	95.18	72.01	71.15	13.38	69.48	17.98
β	^{13}C	94.70	72.53	73.95	73.02	73.23	17.98
SEA-1							
α	^{1}H	5.13	4.27	4.49	3.58	3.96	1.30
	^{13}C	95.02	70.45	79.78	71.28	69.62	18.07
β	^{1}H	4.92	4.13	4.29	3.50	~3.50	1.32
	^{13}C	94.38	70.93	81.69	72.93	70.93	18.07
SRA							
α	^{1}H	5.11 (2.3)	4.23	4.64	3.78	4.00	1.33
	^{13}C	94.62	70.60	79.30	79.55	68.91	17.96
α'	^{1}H	5.11 (2.3)	4.25	nd	~3.78	nd	nd
	^{13}C	94.88	70.70	79.95	77.50	~68.4	nd
β	^{1}H	4.91 (1.2)	4.25	4.45	3.71	3.54	1.34
	^{13}C	94.20	70.99	81.24	79.07	nd	17.73
β'	^{1}H	~4.90	~4.27	~4.45	~3.80	nd	nd
	^{13}C	94.35	71.03	81.80	77.14	nd	nd
R	^{1}H	4.80 (1.9)	4.24	4.44	3.57	4.13	1.27
	^{13}C	101.3	70.07	79.79	71.16	70.13	17.68
R'	^{1}H	4.89 (1.6)	4.20	4.42	3.57	3.99	1.30
	^{13}C	102.40	69.96	79.79	79.23	70.29	17.91
G	^{1}H	4.65	3.35	3.65	3.63	3.80	
	^{13}C	104.36	75.04	75.17	80.02	77.05	175.93
I	^{1}H	5.08 (4.1)	3.67	3.83	4.02	4.58	
	^{13}C	104.09	71.74	72.74	80.41	72.09	175.12
SRB							
R	^{1}H	4.81	4.22	4.60	3.77	4.15	1.30
	^{13}C	100.9	70.2	79.4	79.4	69.3	18.1
G	^{1}H	4.62	3.35	3.63	~3.64	3.74	
	^{13}C	104.4	75.1	75.3	80.1	77.7	176.1

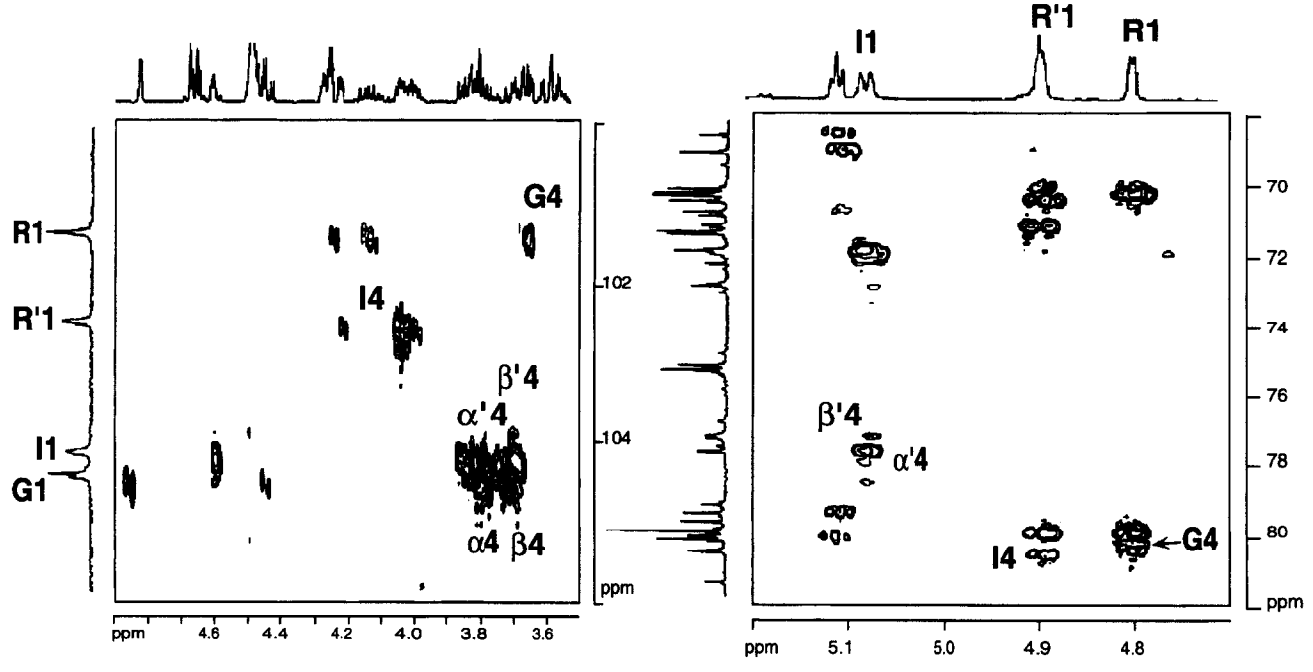

Fig. 4. Partial HMBC maps of oligosaccharide SRA; α4, α'4, β4, β'4, I4 and G4 correspond to cross peaks between H-4 and C-1 of the different sugars in the fragment; R, R', I, G, α, α', β, β' refer respectively to rhamnose 3-sulphate linked to glucuronic acid, rhamnose 3-sulphate linked to iduronic acid, iduronic acid, glucuronic acid and the different α and β rhamnose 3-sulphate reducing ends. The experiment was carried out at 47°C, 1024 experiments of 2 K data points and 32 transients each with a recycling time of 1.4 s were recorded and transformed on 1 × 2 K data points (F1 × F2 dimensions) with an unshifted sine bell multiplication in the F2 dimension and an exponential multiplication (line broadening of 0.1 Hz) in the F1 dimension. The delay for the long range coupling evolution was 75 ms.

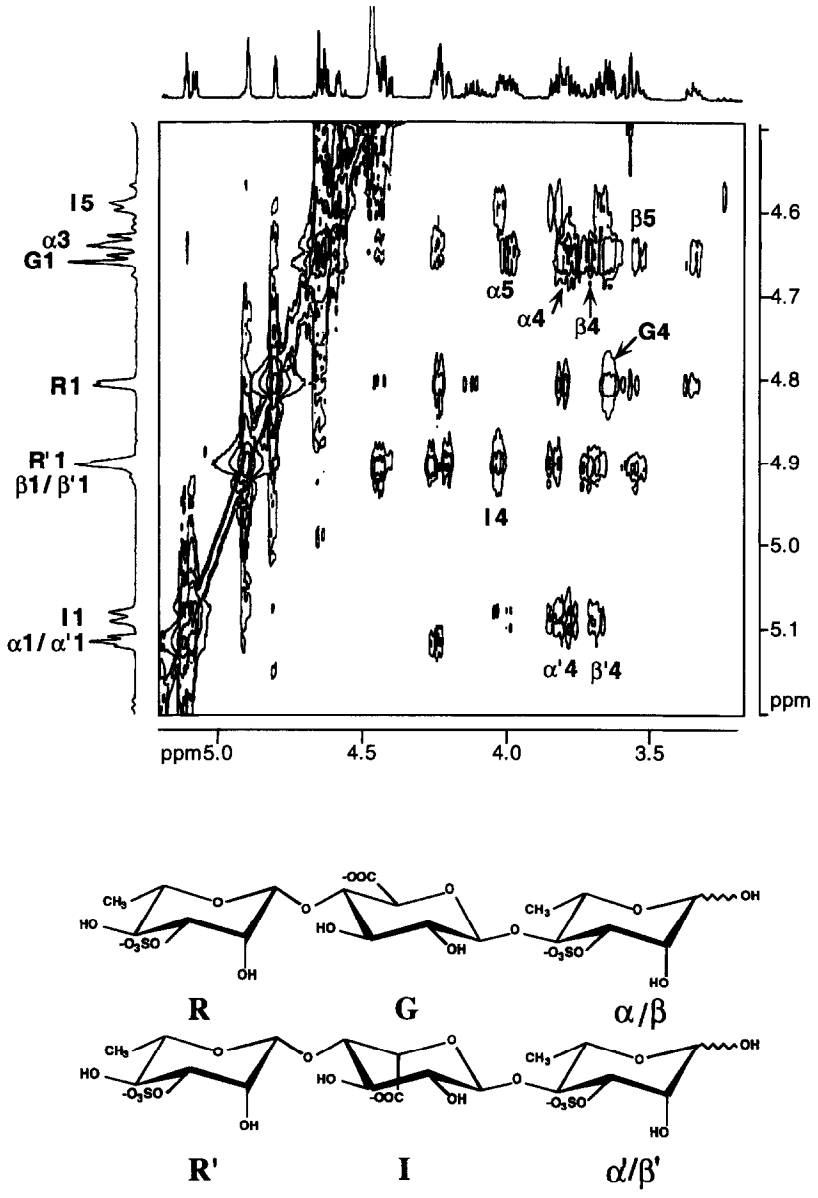

Fig. 5. Chemical structure and partial ROESY map of oligosaccharide in SRA; letters, numbers and symbols are described in the legend of Fig. 4. The experiment was carried out at 47°C, 1024 experiments of 2 K data points and 16 transients each with a recycling time of 1 s were recorded and transformed on 2 × 2 K data points with unshifted sine bell multiplication in both dimensions. The ROESY spinlock pulse duration was 500 ms.

rhamnose 3-sulphate. Although the signal of H-1 of glucuronic acid and H-3 of the reducing α-rhamnose 3-sulphate overlap, the linkage of glucuronic acid to C-4 of α- and β-rhamnose 3-sulphate at the reducing end was established from the ROESY experiment. Cross-peaks between H-1 of glucuronic acid and H-4 and H-5 of α- and β-rhamnose 3-sulphate were observed (Fig. 5). This linkage was confirmed by the carbon chemical shifts observed for G1, α4 and β4 which corresponded to those of the ulvanobiouronic acid 3-sulphate (Lahaye & Ray, 1996). The linkage of rhamnose 3-sulphate at the non-reducing end to C-4 of glucuronic acid (G4) was established by the cross-peaks observed on both HMBC and ROESY maps. Based on the intensities of the different anomeric protons, this fraction appears to be composed of two major trisaccharides [α-L-Rha*p* 3-sulphate-(1 → 4)-α-L-Ido*p*A-(1 → 4)-L-Rha*p* 3-sulphate] and [α-L-Rha*p* 3-sulphate-(1 → 4)-β-D-Glc*p*A-(1 → 4)-L-Rha*p* 3-sulphate] in the proportion 48.6/51.4. The nature of the uronic acid in these trisaccharides was likely to be the source of the reducing rhamnose 3-sulphate proton and carbon splittings.

The ^{1}H and ^{13}C NMR chemical shifts of the main sugar residues in SRB (Table 1) were determined by COSY, HMQC and HMQC-TOCSY experiments and demonstrated similarities with those of the ulvanobiouronic acid 3-sulphate [β-D-Glc*p*A-(1 → 4)-LRha*p* 3-sulphate] previously identified (Lahaye & Ray, 1996). The linkages of this disaccharide repeating unit were obtained from a HMBC

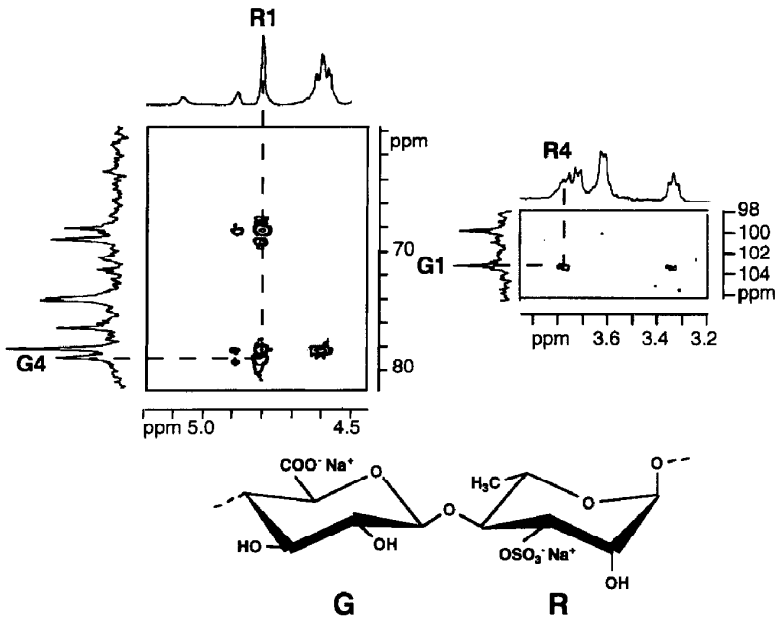

Fig. 6. Partial HMBC maps of fraction SRB recovered after DEAE-Sepharose anion-exchange chromatography of the ethanol-insoluble ulvan autohydrolysate and chemical structure of the ulvanobiouronic acid 3-sulphate A. The letter and numbers are as described in the legend of Fig. 4. The experiment was carried out at 57°C, 512 experiments of 2 K data points and 56 transients each with a recycling time of 0.7 s were recorded and transformed on 1 × 2 K data points (F1 × F2 dimensions) with an unshifted sine bell multiplication in the F2 dimension and an exponential multiplication (line broadening of 0.1 Hz) in the F1 dimension. The delay for the long range coupling evolution was 50 ms.

experiment (Fig. 6) and that on C-4 of the glucuronic acid residue was also in good agreement with the marked downfield shift observed for this carbon signal (80.1 ppm) compared with that of the ulvanobiouronic acid 3-sulphate (72.7 ppm; Lahaye & Ray, 1996). Thus, SRB was identified as large fragments of repeating ulvanobiouronic acid 3-sulphate.

Combining the information obtained from SRA and SRB, major ^{13}C and ^{1}H NMR resonances in ulvan were attributed (Figs. 7 and 8). The proton and carbon chemical shifts of the ulvanobiouronic acid 3-sulphate are those of SRB (Table 1). The anomeric proton chemical shifts of iduronic acid and rhamnose 3-sulphate linked to iduronic acid determined at 80°C are 5.11 and 4.92 ppm, respectively. The carbon chemical shifts of the iduronic acid at 80°C in ulvan are I1:103.9, I2:71.8, I3:72.8, I4:80.0, I5:71.8 ppm. The

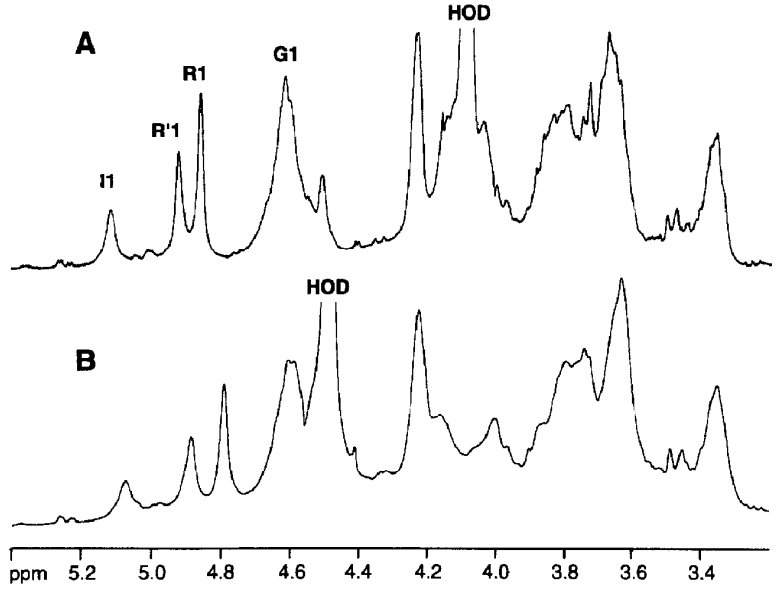

Fig. 7. ^{1}H NMR spectra of ulvan at 80 (A) and 47°C (B). Letters and numbers are as described in the legend of Fig. 4.

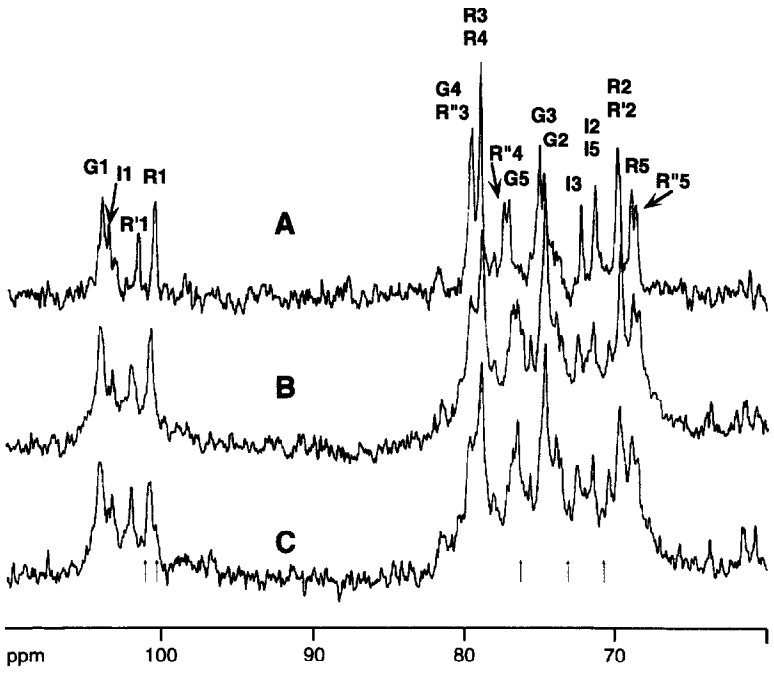

Fig. 8. ^{13}C NMR spectra of ulvan at 80°C with 0.050 mol l^{-1} H_3BO_3 at pH 7.5 (A), 27°C without H_3BO_3 (B) and at 27°C with 0.050 mol l^{-1} H_3BO_3 at pH 7.5. Letters and numbers are as described in the legend of Fig. 4; arrows are discussed in the text.

chemical shift at 80°C of the anomeric carbon of rhamnose 3-sulphate linked to iduronic acid is R'1 102.0 and those of rhamnose 3-sulphate to which iduronic acid is linked are R''3:80.0, R''4:77.9, R''5:69.1. Small variations in proton and carbon chemical shifts were induced by temperature changes and were particularly observed for the anomeric carbons and protons of the rhamnose residues (Figs. 7 and 8). Several minor signals remain unattributed and may reflect the presence of other distributions of these sugars and the presence of xylose and glucose residues. According to the chemical composition given by the chemical-enzymatic method (Quemener et al., 1997), and assuming a regular distribution of one rhamnose per uronic acid or xylose residue, we should expect about 15.1% of disaccharides containing iduronic acid. Taking the integral of the iduronic anomeric proton measured at 27 and 80°C (Fig. 8, 5.08–5.11 ppm) and the two different rhamnose anomeric protons (4.88–4.92, 4.79–4.86 ppm), the amount of iduronic acid containing disaccharide is 29.1%. Taking only the integral of the two anomeric protons for the different rhamnose residues, the proportion of the iduronic acid containing disaccharide is 40.5%. A similar value is obtained using the height of the two different rhamnose anomeric carbons (40%, 102.0–100.9 ppm). Although it is likely that other signals are present under the proton and carbon anomeric signals attributed to the rhamnose linked to iduronic acid leading to an overestimation of iduronic acid containing disaccharides, it appears that the chemical-enzymatic method for ulvan sugar determination underestimates the concentration of iduronic acid. The latter was determined using the HPAEC-PAD weight response factor for glucuronic acid which may not be appropriate for iduronic acid (Quemener et al., 1997).

3.2. Boron-ulvan association

In order to determine the site of boric acid fixation on ulvan, the ^{11}B NMR spectrum of 15, 25 and 90 g l^{-1} boric acid at pH 7.5 was recorded at 27 and 80°C. The highest ulvan concentration yielded a turbid viscous solution and a thermoreversible gel when boric acid was present. In the latter case, the gel melted at high temperatures (80°C) leading to the formation of small flocs in a clear liquid phase which may be related to the proteins contained in the ulvan extract. Such flocs were not observed with lower ulvan concentrations which only formed viscous solutions. The only boron signal observed on the different spectra was that of boric acid even in the gelled ulvan. No signal was observed for boric acid complex nor borate ion. The ^{11}B NMR spectrum of the desulphated ulvanobiouronic acid in presence of boric acid also failed to demonstrate boron complex at pH 7.5 although at pH 9 and 12, complexes were observed and most likely corresponded to borate interactions with the disaccharide (Fig. 9). The ^{13}C NMR spectrum of the 90 g l^{-1} ulvan solution with and without boric acid was also recorded at both 27 and 80°C (Fig. 8). Beside the temperature effect, no chemical shift variation was observed for the major carbon signals of samples with and without boric acid at pH 7.5. A difference in the chemical shifts of the C-6 of the uronic acids could not be observed due to the relatively low resolution of the spectra and the other small shifts observed (arrows Fig. 8) remain to be confirmed.

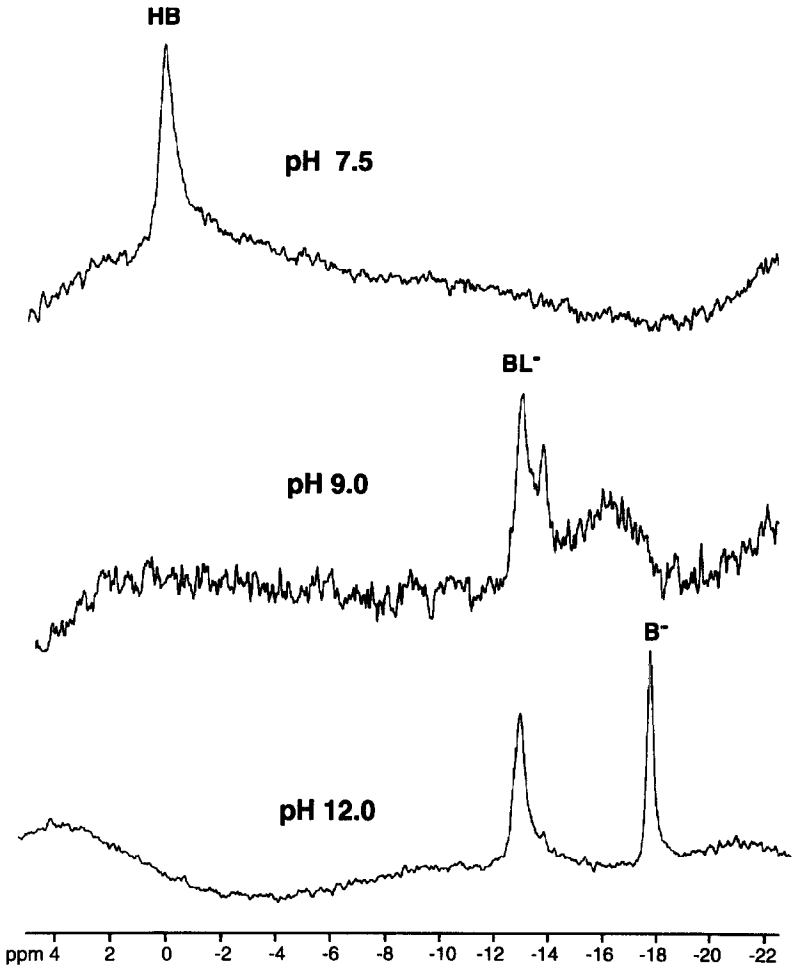

Fig. 9. ^{11}B NMR spectra of 0.03×10^{-3} mol l^{-1} ulvanobiouronic acid A (β-D-Glc*p*A-(1- > 4)-L-Rha*p*) with 0.015×10^{-3} mol l^{-1} H_3BO_3 at different pH. HB, BL^- and B^- refer to the signals of boric acid, borate-ulvanobiouronic acid complexes and free borate, respectively.

4. Discussion

The thermoreversible gelation of ulvan was proposed to involve as a first step formation of borate diesters with free 2,3-*vic* diol in rhamnose residues, followed by association of these ulvan-boron complexes by calcium ions (Haug, 1976; Lahaye et al., 1996). In order to verify this hypothesis, we first re-investigated in more detail the chemical structure of the polysaccharide. Percival & McDowell (1967) proposed that ulvan from *U. lactuca* was built on the following basic sequence: GlcA-(1 → 4)Rha 2 sulphate-(1 → 4)-GlcA-(1 → 3)-Xyl-(1 → 4)-Rha 2-sulphate-(1 → 3)-Glc-(1 → 4)-Xyl or a related sequence in term of sugars distribution but with a majority of GlcA-(1 → 4)-Rha 2-sulphate repeating structure. From studies on the chemical structure of the ulvan of *U.* '*rigida*', we found that almost all the rhamnose residues were sulphated on O-3 and that some of the xylose residues were sulphated on O-2 but neither 1,3-linked xylose were observed nor O-2 sulphation on rhamnose (Ray & Lahaye, 1995; Lahaye & Ray, 1996) despite the observation of a major infrared absorbance at 850 cm^{-1} as also reported by Percival & Wold (1963). The extent of sulphation on O-3 of rhamnose excludes this sugar as being a major site for boron fixation. In an attempt to investigate further the chemical structure of ulvan and to look for other possible sites of boric acid fixation, several oligosaccharide repeating sequences were identified from a partial acid hydrolysate of *U.* '*rigida*' ulvan but most of them were partially desulphated as a side reaction of the degradation method (Lahaye & Ray, 1996). In this present work, autohydrolysis was used as a milder hydrolytic condition to generate sulphated fragments that were taken as model compounds for the NMR structural analysis of ulvan. Such a degradation method allowed Bourne et al. (1970) to obtain sulphated monomers and short oligosaccharides in their structural analysis for the complex sulphated arabinogalactan from the green alga *Cladophora rupestris*. Applied to ulvan, this method yielded several fragment fractions after anion exchange and gel permeation chromatographies that were analysed by NMR spectroscopy. The complexity of the spectra precluded the identification of all of oligosaccharide fractions but the three of them that were characterized clearly demonstrated that the major repeating structure of ulvan from edible *Ulva* sp ('sea lettuce') is

the ulvanobiouronic acid 3-sulphate [→ 4)-β-D-Glc*p*A-(1 → 4)-α-L-Rha*p* 3-sulphate-(1 →]. In agreement with the recent identification of iduronic acid in the chemical-enzymatic hydrolysate of several different ulvans (Quemener et al., 1997), the NMR data of the oligosaccharide in fraction SRA established that this uronic acid occurs in the following structure, α-L-Rha*p* 3-sulphate-(1 → 4)-α-L-Ido*p*A-(1 → 4)-L-Rha*p* 3-sulphate, and thus form another aldobiouronic acid sequence in ulvan that can be recognized by typical ^{1}H and ^{13}C NMR resonances. The presence of iduronic acid is rare among plant polysaccharides and may play a role in the *Ulva* sp. cell-wall cohesion as in the mammalian glycosaminoglycans heparin, heparan sulphate and dermatan sulphate self-associations (Fransson, 1985). In the light of these structural results and NMR data, the formation of boric acid complex with the polysaccharide which is thought to be the first step involved in ulvan gel formation was investigated by ^{11}B and ^{13}C NMR spectroscopy of the polymer and by ^{11}B NMR spectroscopy of the desulphated ulvanobiouronic acid repeating unit. ^{11}B NMR spectroscopy is a sensitive technique for detection and quantification of the different boron species in complexes (Henderson et al., 1973; Van Duin et al., 1984) and was recently used in the study of particular land plant type II rhamnogalacturonan interactions with boric acids (Matoh et al., 1993; Ishii & Matsunaga, 1996; Kaneko et al., 1997). ^{13}C NMR spectra of ulvan in the presence and absence of boric acid were also recorded to determine the sites of boric acid fixation as has already been done for borate-galactomannan (Noble & Taravel, 1987) or borate-sugars interactions (Van den Berg et al., 1994). However, from the absence of a borate complex signal and a boric acid-induced shift of the major carbon signals on the ^{11}B and ^{13}C NMR spectra of ulvan and desulphated ulvanobiouronic acid at pH 7.5, it is unlikely that the hydroxyls in the pyranose ring of rhamnose, glucuronic acid and iduronic acid of the major repeating disaccharides are involved in boric acid fixation. The absence of borate complex signal even in the gelled sample suggest that either the boron-ulvan complex is very weak and the exchanges between free boric acid and complexed boron are very rapid, the amount of boron complexes are very low and/or the complex involves the planar boric acid. The latter complexes are known to induce only small boron chemical shifts that may not be distinguished from the chemical shift of free boric acid (Van Duin et al., 1984). However, at the pH of optimum ulvan gelation (pH ≈ 7.5, Lahaye & Axelos, 1993) which is between the p*K*a of uronic acids (≈ 3.0–3.7; Kohn & Kovac, 1978) and that of boric acid (≈ 9.0), a borate ester signal in the range of − 14 to − 12 ppm was expected but not observed (Van Duin et al., 1984). The presence of borate ester signals with the ulvanobiouronic acid at pH ≥ 9.0 involves most likely 2,3-*vic* diols of rhamnopyranose in agreement with the known complex formed with 2,3-*vic* diol of α-D-mannopyranose (Van den Berg et al., 1994) and probably also between 1,2-diol in β-rhamnose because of adequate hydroxyl configurations.

5. Conclusion

The structure of two major repeating structures in ulvan have been characterized by NMR spectroscopy as a result of which a new sulphated aldobiouronic acid [→ 4)-α-L-Ido*p*A-(1 → 4)-α-L-Rha*p* 3-sulphate (1 →] was identified. In order to differentiate between the two major disaccharide repeating units, we propose to refer to them as ulvanobiouronic acid 3-sulphate A and B for the glucuronic acid and the iduronic acid-containing aldobiouronic acids, respectively. Further work should determine whether ulvanobiouronic acid 3-sulphate B is the biological precursor of ulvanobiouronic acid 3-sulphate A (or the other way around) controlled by the action of a C5-epimerase as for the conversion of mannuronic to guluronic acid in alginates (Gacesa, 1988) and glucuronic to iduronic acid in mammalian glycosaminoglycans (Fransson, 1985). The ^{13}C and ^{1}H NMR chemical shifts of the major signals in ulvan have been attributed but those of xylose and glucose present in the polysaccharide remain to be identified. According to ^{11}B and ^{13}C NMR spectroscopy results, the ring hydroxyls of the rhamnose, glucuronic and iduronic acids in the major disaccharide repeating units are unlikely candidates for the formation of boron complexes at pH 7.5. The implication of diols from minor sugars or other compounds will be the focus of future studies.

Acknowledgements

This work was supported in part by the programme VANAM from the Région Pays de la Loire.

References

Bobin-Dubigeon, C., Lahaye, M., & Barry, J.-L. (1997a). Human colonic bacterial degradability of dietary fibres from sea-lettuce (*Ulva* sp). *J. Sci. Food Agric., 73*, 160–168.

Bobin-Dubigeon, C., Lahaye, M., Guillon, F., Barry, J.-L., & Gallant, D.J. (1997). Factors limiting the biodegradation of *Ulva* sp. cell-wall polysaccharides. *J. Sci. Food Agric., 75*, 341–351.

Bourne, E.J., Johnson, P.G., & Percival, E. (1970). The water-soluble polysaccharides of *Cladophora rupestris*. Part IV. Autohydrolysis, methylation of the partly desulphated material, and correlation with the results from Smith degradation. *J. Chem. Soc. Sec. C*, 1561–1569.

DeReviers, B., & Leproux, A. (1993). Characterization of polysaccharides from *Eneromorpha intestinalis* (L.) Link. *Chlorophyta. Carbohydr. Polym., 22*, 253–259.

Durand, M., Beaumatin, P., Bulman, B., Bernalier, A., Grivet, J.P., Serezat, M., Gramet, G., & Lahaye, M. (1997). Fermentation of the green alga sea-lettuce (*Ulva* sp.) and metabolism of its sulphate by human colonic microbiota in a semi-continuous culture system. *Reprod. Nutr. Dev., 37*, 267–283.

Fransson, L.-A. (1985). Mammalian glycosaminoglycans. In G.O. Aspinall (Ed.), *The polysaccharides* (Vol. 3, pp. 337–415). New York: Academic Press.

Gacesa, P.(1988). Alginates. Carbohydr. Polym., *8*, 161–182.

Haug, A. (1976). The influence of borate and calcium on the gel formation of a sulfated polysaccharide from *Ulva lactuca*. *Acta Chem. Scand., B30*, 562–566.

Henderson, W.G., How, M.J., Kennedy, G.R., & Mooney, E.F. (1973). The interconversion of aqueous boron species and the interaction of borate with diols: A ^{11}B NMR study. *Carbohydr. Res., 28*, 1–12.

Ishii, T., & Matsunaga, T. (1996). Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydr. Res., 284*, 1–9.

Kaneko, S., Ishii, T., & Matsunaga, T. (1997). A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochem., 44*, 243–248.

Kohn, R., & Kovac, P. (1978). Dissociation constants of D-galacturonic and D-glucuronic acid and their O-methyl derivatives. *Chem. Zvesti., 32*, 478–485.

Lahaye, M., & Axelos, M.A.V. (1993). Gelling properties of water-soluble polysaccharides from proliferating marine green seaweeds (*Ulva* spp.). *Carbohydr. Polym., 22*, 261–265.

Lahaye, M., & Ray, B. (1996). Cell-wall polysaccharides from the marine green alga *Ulva 'rigida'* (Ulvales Chlorphyta)-NMR analysis of ulvan oligosaccharide. *Carbohydr. Res., 283*, 161–173.

Lahaye, M., Ray, B., Baumberger, S., Quemener, B., & Axelos, M.A.V. (1996). Chemical characterisation and gelling properties of cell wall polysaccharides from species of *Ulva* (Ulvales, Chlorophyta). *Hydrobiologia, 326/327*, 473–480.

Matoh, T., Ishigaki, K.-I., Ohno, K., & Azuma, J.-I. (1993). Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol., 34*, 639–642.

McKinnel, J.P., & Percival, E. (1962a). The acid polysaccharide from the green seaweed, *Ulva lactuca. J. Chem. Soc.*, 2082–2083.

Mckinnel, J.P., & Percival, E. (1962b). Structural investigations on the water-soluble polysaccharide of the green seaweed *Enteromorpha compressa. J. Chem. Soc.*, 3141–3148.

Mulloy, B., Forster, M.J., Jones, C., Drake, A.F., Johnson, E.A., & Davies, D.B. (1994). The effect of variation of substitution on the solution conformation of heparin: a spectroscopic and molecular modelling study. *Carbohydr. Res., 255*, 1–26.

Noble, O., & Taravel, J.-F. (1987). Nuclear magnetic resonance evidence of the guar-borate interaction. *Carbohydr. Res., 166*, 1–11.

O'Donnel, J.J., & Percival, E. (1959). Structural investigations on the water-soluble polysaccharides from the green seaweed *Acrosiphonia centralis* (*Spongomorpha arcta*). *J. Chem. Soc.*, 2168–2178.

Percival, E. & McDowell, R.H. (1967). *Chemistry and enzymology of marine algal polysaccharides*, 219 pp. London: Academic Press.

Percival, E. & Wold, J.K. (1963). The acid polysaccharide from the green seaweed *Ulva lactuca*. Part II. The site of the ester sulphate. *J. Chem. Soc.*, 5459–5468.

Quemener, B., Lahaye, M., & Bobin-Dubigeon, C. (1997). Sugar determination in ulvans by a chemical-enzymatic method coupled to high performance anion exchange chromatography. *J. Appl. Phycol., 9*, 179–188.

Ray, B., & Lahaye, M. (1995). Cell-wall polysaccharides from the marine green alga *Ulva 'rigida'* (Ulvales Chlorophyta)-Chemical structure of ulvan. *Carbohydr. Res., 274*, 313–318.

Thibault, J.F. (1979). Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényl. *Lebensm.-Wiss. u.-Technol., 12*, 247–251.

Tollier, M.T., & Robin, J.P. (1979). Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucidea neutres totaux. *Annu. Technol. Agric., 28*, 1–15.

Van den Berg, R., Peters, J.A., & Van Bekkum, H. (1994). The structure and (local) stability constants of borate esters of mono- and di-saccharides as studied by ^{11}B and ^{13}C NMR spectroscopy. *Carbohyr. Res., 253*, 1–12.

Van Duin, M., Peters, J.A., Kieboom, A.P.G., & Van Bekkum, H. (1984). Studies on borate esters. I. The pH dependence of the stability of esters of boric acid and borate in aqueous medium as studied by ^{11}B NMR. *Tetrahedron, 40*, 2901–2911.