J Appl Phycol (2017) 29:509–519
DOI 10.1007/s10811-016-0928-y

Production of guluronate oligosaccharide of alginate from brown algae *Stypocaulon scoparium* using an alginate lyase

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Received: 10 February 2016 / Revised and accepted: 4 August 2016 / Published online: 13 August 2016

Abstract The brown alga *Stypocaulon scoparium*, collected from the Mediterranean coast of Algeria in the Bejaia region, was chosen as a model to study its alginate. This polysaccharide was extracted and characterized by HPAEC-PAD, SEC-MALLS, ^{1}H-NMR spectroscopy and FT-IR. The ratio of D-mannuronate to L-guluronate (M/G ratio) in the alginate was 0.92 in FTIR spectra at bands of 815 and 900 cm^{-1} for mannuronic acid (ManA) and guluronic acid (GulA), respectively, which was comparable to alginate of other brown algae, and 0.73 with ^{1}H-NMR spectroscopy. Complete acid hydrolysis of alginate using the HPAEC technique showed the composition of the two main acid sugars as GulA (G) and ManA (M) with an M/G ratio of 0.6, and 50 % yield. The Mw value for *S. scoparium* was 2.236 × 105 g mol^{-1}, and polydispersity index Ip = Mw/Mn =1.714 ± 0.039, which was similar to that of other brown alga samples identified and cited. Partial acid hydrolysis of the alginate gave three fractions, which were characterized. Thus, the alginate sample from *S. scoparium* was very rich in guluronic block (G-blocks) structures (43.53 %). The guluronic blocks were degraded with polyguluronate lyase from *Flavobacterium multivorum* to oligoguluronate via a β-elimination reaction, with Mw = 3.742 × 10^{3} g mol^{-1}.

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Keywords *Stypocaulon* · Guluronic acid · Sec Malls · DIONEX · Ft-IR · ^{1}H-NMR

Introduction

Considerable attention has recently been directed to the anionic polysaccharides, which are found in most plants, animals, microorganisms and seaweed (Linhardt et al. 1986). Their anionic character gives them different biological and rheological proprieties, such as chelation or gelling in the presence of cations. An example of such a gelling agent is gulucoronan, which can form thermoreversible gels in the presence of monovalent cations such as Na + at high ionic strength. Thermally stable gels have been obtained with divalent cations (Ca^{2+}, Cu^{2+}, Ba^{2+}) and gel resistance was modulated by the acetylation degree of the polymers, which disturbs the ionic interaction (Elboutachfaiti et al. 2011). Anionic polysaccharides have antioxidant effects according to their specific groups, such as sulfate, amino, hydroxyl, and carboxyl, and also their molecular weight (Petit et al. 2005; Chen et al. 2009; Elboutachfaiti et al. 2011; Sun et al. 2011; Delattre et al. 2015).

Polysaccharide cleavage enzymes are widely exploited to degrade various polysaccharides. For example, polysaccharide hydrolases are used to degrade neutral polysaccharides, and polysaccharide hydrolases and lyases can degrade anionic polysaccharides (Delattre et al. 2011). Polysaccharide lyases are also used to reduce the molecular weight of polysaccharides to generate products with different degrees of polymerization (dp), ranging mainly from 2 to 5, and different biological activities (Østgaard and Larsen 1993; Hashimoto et al. 1997; Tavernier et al. 2008). Polysaccharide lyases are endolyases that usually degrade the glycoside chain, leading to a rapid decrease in viscosity of the polysaccharide solution (Hashimoto et al. 1996). Some polysaccharide lyases can be

used to fight against pathogenic bacteria by degrading the exopolysaccharide (EPS) protectors they excrete. Thus, administration of an alginate lyase to patients suffering from lung infections caused by *Pseudomonas aeruginosa*, producing a bacterium alginate, enhanced the effect of antibiotics and the activity of macrophages (Ramphal and Pier 1985).

Polysaccharide lyases are also used industrially to control the rheological properties of polysaccharides. Some polysaccharides such as gellan exhibit high viscosity in solution. The use of gellan lyase provides formulas of a low molecular weight and an increased spectrum of application of gellan (Hashimoto et al. 1996; Giavasis et al. 2000). Pectin lyase and rhamnogalacturonan lyases are used in food, usually in the form of an enzyme cocktail for the clarification of fruit juice (Demir et al. 2001).

Alginate is an anionic polysaccharide produced from marine brown algae (Phaeophyceae) (Vreeland 1972) and is partly responsible for the flexibility of seaweed (McHugh 2003). It is a linear polysaccharide with a sequence of two monosaccharides: the D-mannuronic acid (ManA) and L-guluronic acid (GulA) linked respectively by α (1.4) and β (1.4) bonds. The monomers are distributed in blocks of continuous β-ManA residues (M-blocks), α-GulA residues (G-blocks), or alternating residues (MG-blocks) (Haug et al. 1967; Gacesa 1988).

Sodium alginate has many industrial and biological applications. Its shear-thinning characteristics and viscosity are used in textile printing to ensure bright and even tones. It is also used for paper coating to obtain surface uniformity, and as a binding agent in the production of welding rods, cosmetic creams, and processed food (Onsøyen 1996). Alginate has been used in therapeutic angiogenesis for the systematic release of growth factors (Kawada et al. 1999). Alginate is also widely used in wound dressings, dental impressions, and formulations for preventing gastric reflux. Among the most advanced biotechnological and biomedical applications of this residue is as a hydrogel for cell immobilization in, for example, the production of ethanol, yeast cells and antibiotics or steroids (Smidsrød and Skjåk-Bræk 1990), as well as in transplantation and cell therapy (Joki et al. 2001; Rokstad et al. 2002). In the latter case, alginate gel is used as a selective immune barrier to protect transplanted cells from the host immune system.

Alginate can be depolymerized into alginate oligosaccharides using enzymatic degradation (Wong et al. 2000; Suzuki et al. 2006), or physicochemical treatments (Haug and Larsen 1962; Haug et al. 1966; Wasikiewicz et al. 2005). Enzymatic depolymerisation has certain advantages, including high production yields and the production of specific oligosaccharides (Delattre et al. 2005), compared to chemical and physical methods, which are generally difficult to carry out (Heyraud et al. 1993; Wasikiewicz et al. 2005).

Alginate lyase uses a β-elimination in which a non-reducing unsaturated bond is produced during cleavage of uronic acid, giving rise to a strong absorption at 235 nm in the UV region (Michaud et al. 2003; Song et al. 2003). These oligosaccharides are required for the development of biological functions. For example, they are used in signal transduction systems that regulate plant development and defensive processes (Ryan and Farmer 1991; Murphy et al. 2007). Furthermore, alginate oligosaccharides have been reported to inhibit the growth and differentiation of adipocytes and the absorption of saturated fatty acids (Choi et al. 1986; Kim et al. 2010). They also have anti-allergy properties, through the suppression of IgE (Uno et al. 2006). They have the ability to enhance the growth of human endothelial cells and keratinocytes (Kawada et al. 1997, 1999), and to induce cytokine production in a mouse macrophage cell line (Iwamoto et al. 2005).

Stypocaulon scoparium is a very abundant brown seaweed in the Mediterranean coast of Algeria, distributed in different regions, notably Mers El Hadjadj, Iles Habiba (Arzew), Sidi Ghiles (Cherchell), Ain Tagourait, Kouali, Bou-Ismail, Sidi Fredj, Tamentfoust, Surcouf, Bouharoum (Tipaza), Ain Benian (Alger), and the Gouraya National Park (Béjaia) (Seridi et al. 2007). We here describe for the first time the isolation and chemical characterization of alginate from *S. scoparium* collected in Béjaia. The characterization of the alginates obtained was performed using three methodologies (HPAEC-PAD, SEC-MALLS, 1H-NMR spectroscopy and FT-IR), and their advantages and disadvantages are discussed. Finally, the production of alginate oligosaccharides by enzymatic degradation from G-blocks using polyguluronate lyase is proposed.

Materials and methods

Extraction and purification of alginate

The brown seaweed *S. scoparium* was collected in August 2012 from the Mediterranean coast in Béjaia (eastern Algeria). The samples were cleaned, washed in distilled water, dried at 40 °C and crushed with an electric grinder. They were also thoroughly sifted with a 125 μm sieve.

Alginate was extracted according to Calumpong et al. (1999) (modified procedure). Dried seaweed was soaked in 2 % methanol/formaldehyde/water (80/2/18, 1:20 ratio) for 12 h at room temperature, and washed with water. After mixing with 0.2 M HCl, it was left to incubate for 24 h, and then the sample was washed again in distilled water before extraction with 4 % sodium carbonate for 2 h at 80 °C. The supernatant was recovered after centrifugation (10.000 ×*g*, 20 min) and acidified with 4 M HCl, pH .0. The insoluble fraction was collected by centrifugation (10,000×*g*, 20 min) and neutralized with NaOH 4 M. Polysaccharides were purified by ultrafiltration with membranes in polyethersulfone have a normal molecular weight cut-off (NMWCO) of

30 kDa, precipitated by three volumes of 95 % isopropanol and lyophilized.

Characterization of alginate

Determination of protein content The protein concentration was determined using the Bradford method (Bradford 1976) with Coomassie Brilliant Blue G-250. Calibration was performed by a range of standard bovine serum albumin (BSA).

Determination of monosaccharide content The concentrations of acid and neutral polysaccharides from *S. scoparium* were determined by colorimetric analysis. Uronic acids were measured by absorbance at 550 nm after reaction with carbazole (Bitter and Muir 1962), and neutral sugar at 490 nm after addition of phenol in the presence of sulfuric acid (Masuko et al. 2005).

The results are expressed as the equivalent of D-glucose for neutral sugars and D-glucuronic acid for acidic sugars.

Complete and partial acid hydrolysis of alginate Complete hydrolysis of alginate was performed according to Chandía et al. (2001). 10 mg alginate and 4.5 mL of 90 % formic acid were heated for 6 h at 100 °C in a sealed tube. The resulting solution was diluted with 20 mL of MilliQ water, heated at reflux for 2 h and concentrated in vacuum. The dried sample was dissolved in 300 μL of MilliQ water and analyzed by HPAEC-PAD. HPAEC-PAD analyses were carried out on a Dionex ICS-3000 system consisting of a SP gradient pump, an ED electrochemical detector with a gold working electrode, an Ag/AgCl reference electrode and Chromeleon version 6.5 (Dionex Corp., USA). All eluents were degassed by flushing with helium for 30 min. Separations were performed at 25 °C on a CarboPac PA-1 column (4 mm × 250 mm) connected to a CarboPac PA-1 guard column (4 mm × 50 mm Dionex). For eluent preparation, MilliQ water, 50 % (*w*/*v*) NaOH and NaOAc were used.

Two eluents were used for effective uronic acid separation: eluent A (100 mM NaOH) and eluent B (100 mM NaOH and 1 M NaOAc). The two eluents were mixed to form the following gradient (% of B in A): t = 0 min: 0 %; from 0 to 60 min: linear gradient of 0 to 100 %. After each run, the column was washed for 10 min with 100 % B and reequilibrated for 15 min with the starting conditions of the employed gradient. Samples (1 mg mL^{-1}) were injected through a 25 μL full loop and separations were performed at a rate of 1 mL min^{-1}.

The neutral monosaccharides were eluted isocratically with 160 mM NaOH at a flow rate of 1 mL min^{-1}. Each neutral monosaccharide concentration was determined after integration of the respective areas and compared with standard curves obtained with rhamnose, arabinose, mannose, galactose, glucose and fucose (Sigma Aldrich).

Sodium alginate from *S. scoparium* was partially hydrolyzed according to the modified procedure of Leal et al. (2008). One gram of purified polysaccharide in 100 mL of MilliQ water was heated at reflux with 3 mL of 3 M HCl for 30 min. After cooling, the suspension was centrifuged (3000×*g*, 20 min) and the supernatant was neutralized (1 M NaOH) and supplemented with 100 mL of ethanol. The precipitate collected by centrifugation (10,000×*g*, 20 min) was then dissolved in MilliQ water (25 mL) and freeze-dried (Fraction 1, MG-block). The insoluble fraction from the first centrifugation was heated at reflux with 100 mL of 0.3 M HCl for 2 h. After centrifugation (10,000×*g*, 20 min), the insoluble material was neutralized (1 M NaOH) and the pH was adjusted to 2.85 with 1 M HCl. The soluble fraction was neutralized, dialyzed with 10-kDa dialysis membranes against MilliQ water and freeze-dried (fraction 2, M-block). The precipitate was dissolved by neutralization, dialyzed with 10-kDa dialysis membranes and freeze-dried (fraction 3, G-blocks).

SEC-MALLS analyses Average molecular weights and molecular weight distributions for alginate from *S. scoparium* were determined by high pressure size exclusion chromatography (HPSEC) with on-line multi-angle laser light scattering (MALLS) filled with a K5 cell (50 μL) and two detectors: a He–Ne laser (λ = 690 nm) and a differential refractive index (DRI). Columns [OHPAK SB-G guard column, OHPAK SB806, 804 and 803 HQ columns (Shodex)] were eluted with NaNO3 0.1 M at 0.7 mL min^{-1}. The solvent was filtered through a 0.1 μm filter, degassed and filtered through a 0.45 μm filter upstream column. The sample was injected through a 100 μm full loop. The collected data were analyzed using the Astra 4.90 software package.

Fourier transform infrared (FT-IR) spectroscopy Dried polysaccharide samples (2 mg) were dispersed in 0.08 g of anhydrous KBr and pressed. The IR spectra were recorded at room temperature in the wave number range of 400–4000 cm^{-1} and referenced against air with an IRAFFinity-1 instrument (SHIMADZU). A total of 10 scans were averaged for each sample at 4 cm^{-1} resolution.

Nuclear magnetic resonance spectroscopy The freeze dried samples was dissolved in D_2O at 10 g L^{-1}. ^1H NMR spectrum was recorded at 70 °C on a Bruker Avance 500 MHz spectrometer operating at 500.08 MHz for 1H, using a multinuclear probe BBI 5 mm. 1D proton with a water suppression pulse sequence (NOESY 1D) was acquired. The sequence repeat was –D1-t-90°-t-90°-tm-90°-AQ, where D1 (10 s) is the relaxation delay, 90° is the already determined 90° radio-frequency pulse length, t (9.49 μs) is a very short delay, tm (0,15 s) is a mixing time delay and AQ (5.45 s) is the data acquisition time. Low power rf

irradiation was applied at the water frequency during D1 and tm to presaturate the water signal. The spectrum was acquired using 256 scans of 64 K data points, using spectral widths of 6009.615 Hz. The resulting ^{1}H spectrum was manually phased, baseline-corrected, and calibrated to TMSP (TriMethyl Silyl propionate) at 0 ppm, all using TopSpin 3.2 (BRUKER BioSpin, Germany).

Hydrolysis of polyguluronic acid (G-blocks) by alginate lyase Alginate lyase from *Flavobacterium multivorum* (Sigma Aldrich) activity was assayed at 30 °C in 100 mL of reaction mixture containing 1.0 % (*w*/*v*) polyguluronic acid, 0.15 M NaCl, 10 mM sodium phosphate (pH .0), and 0.5 units of enzyme. The enzyme kinetics were followed on thin layer chromatography (TLC-plates 60) at different intervals of time (0 h, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h), and the reaction was stopped by heating in boiling water for 2 min at 100 °C. The depolymerization products were analyzed by TLC using a solvent system of 1-butanol: acetic acid: water (2:1:1, *v*/v), and the distance between the spotting line and the solvent front was 5 cm. The products were visualized by heating the TLC plate at 110 °C for 5 min after spraying with 10 % (v/v) sulfuric acid in ethanol. Glucuronic acid (Sigma Aldrich) was used as a control to detect sugars dp1.

Results

Extraction and biochemical analysis

The obtained extraction yield of sodium alginate, based on dried algae, was 5 %. The same yield has been reported for *Padina perindusiata*, while higher yields of 17.4 and 24.6 % were obtained from *Sargassum filipendula* and *Turbinaria turbinate*, respectively, collected in Mexico (García-Ríos et al. 2012). These differences in yield may be due to the type of algae studied and variable seasonal conditions (Chandía et al. 2004).

Levels of protein and neutral and acidic sugars were determined in order to characterize the polysaccharides in the extracted sample. The protein content of *S. scoparium* polysaccharides was very low (5 %) compared to other brown seaweeds, for example, 8.72 and 9.86 % reported for the alginate of *Sargassum filipendula* and *Padina gymnospora*, respectively (Robledo and Freile-Pelegrin 1997) and 15.76 % for *Sargassum vulgare* from Brazil (Marinho-Soriano et al. 2006). According to Dhargalkar et al. (1980), protein content varies among genera and species of the same genus. The variability is largely attributed to the nature of the surrounding water (Dave and Parekh 1975).

The content of uronic acid found in this study is 43.11 %, it is similar to that reported by García-Ríos et al. (2012) in *Turbinaria turbinata* (56.9 %), *Sargassum filipendula* (51.1 %), *Dictyota caribaea* (55.1 %) and *Padina perindusiata* (52.7 %).

FT-IR analyses and ^{1}H NMR analysis of alginate samples

The FT-IR spectrum of sodium alginate isolated from *S. scoparium* is presented in Fig. 1. A broad band at 3432 cm^{-1} was assigned to hydrogen-bonded O-H stretching vibrations. The weak signal at 2929 cm^{-1} is due to C-H stretching vibrations, and the asymmetric stretching vibration of O-C-O is centered at 1612 cm^{-1}. The band at 1415 cm^{-1} may be due to the C-OH bending vibration with a contribution of the carboxylate group O-C-O (Mathlouthi and Koenig 1986; Silverstein et al. 1991). The weak bands at 1322 cm^{-1}, 1125 cm^{-1}, and 1091cm^{-1} may be assigned to C-C-H and O-C-H deformation and C-O stretching vibrations of pyrannose rings, respectively; the band at 1029 cm^{-1} may be also due to C-O stretching vibration. Moreover, the anomeric region between 950 and 750 cm^{-1} is the most controversial in carbohydrates (Mathlouthi and Koenig 1986). The spectrum showed a band at 945 cm^{-1}, which was assigned to the C-O stretching vibration of uronic acid residues. More signals at 900 and 815 cm^{-1} were assigned to the α-L-guluronic asymmetric ring vibration and to the mannuronic acid residues, respectively (Mathlouthi and Koenig 1986; Chandía et al. 2001).

IR spectroscopy has proven useful for the quantitative estimation of the mannuronic to guluronic acid (M/G) ratio in alginate (Mackie 1971). In the present study, the ratio of band intensities in FTIR spectra, approximately at 900 and 815 cm^{-1}, was used to estimate the M/G ratio of brown seaweeds. Accordingly, the M/G ratio of sodium alginate from *S. scoparium* was 0.92. Effectively, the M/G ratio can vary among brown seaweed alginates from 0.5 to 2.5 due to the heterogeneity between chain length and distribution of G and M blocks (Miller 1996).

^{1}H NMR spectra of sodium alginate extracted from *S. scoparium* are shown in Fig. 2. We assigned the characteristic signals of the guluronic acid anomeric proton H_1-**G** at 5.07 ppm; the guluronic acid H_5-G**G**M at 4.78 ppm, H_5-M**G**M at 4.74 ppm and H_5-**G**G at 4.46 ppm; and the mannuronic acid anomeric protons H_1-**M**G at 4.71 ppm and H_1-**M**M at 4.65 ppm. The detailed block structure of the alginate, giving the two monad values (FM and FG), the four diad frequencies (FGG, FMM, FMG, FGM) and the M/G ratio were obtained by comparison of signal areas using the equations proposed by Gradsalen et al. (1979).

$$FG = IA/(IB + IC)$$
$$FM = 1 - FG$$
$$M/G = (1-FG)/FG$$

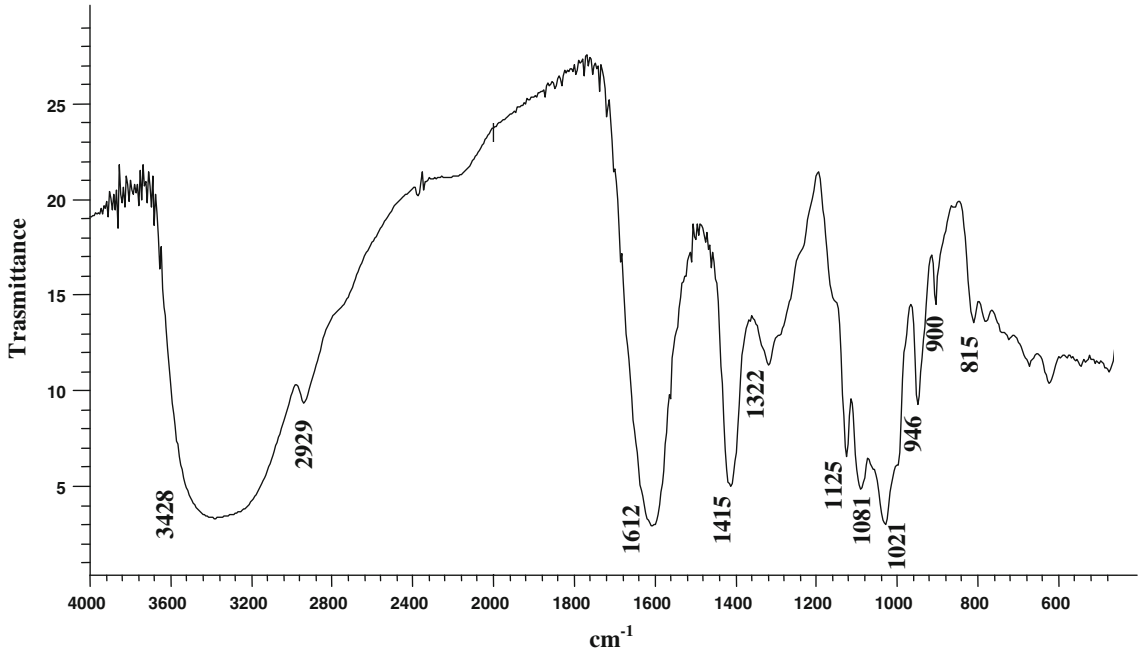

Fig. 1 FT-IR spectra of sodium alginate from *Stypocaulon scoparium*

The following relations were then used to establish the proportions of the different diad sequences:

$$FGG = IC/(IB + IC)$$
$$FGG + FGM = FG$$
$$FMM + FMG = FM$$

IA corresponds to the proton area of H_1-**G**; IB to the sum of H_5-G**G**M, H_5-M**G**M, H_1-**M**G and H_1-**M**M; and IC to H_5-**G**G.

^{1}H NMR spectroscopy is a reliable method for the determination of the composition and also the block structures of alginate molecules (Panikkar and Brasch 1996; Larsen et al. 2003). The values obtained were compared with data in the literature for other species of alginate-producing brown algae (Table 1). The alginate extracted from *S. scoparium* had an M/G ratio of 0.73. It was richer in guluronic acid (FG = 0.58) than in mannuronic acid (FM = 0.42). The way in which these M and G units are arranged in the chain and the overall M/G ratio of the two units in a chain can vary from one species of seaweed to another and its geographical location. The M/G ratio also varies according to the extraction methodology (Davis et al. 2003).

Chemical composition of alginate after complete acid hydrolysis

The total hydrolysis of alginate from *S. scoparium* was performed with formic acid, as described in the "Material and methods" section, and HPAEC was used to separate the uronic acids and determine the M/G ratio of alginate (Aida et al.

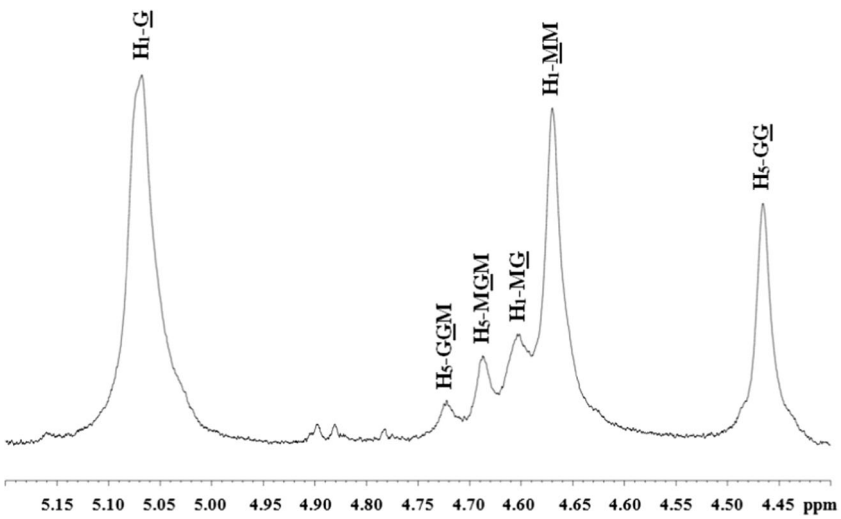

Fig. 2 ^{1}H NMR spectrum (70 °C) of alginate from *Stypocaulon scoparium*

Table 1 Composition and sequence parameters of algal alginates

Species	Localisation	M/G	FM	FG	FMM	FMG = F_{GM}	FGG	References
Eisenia arborea	Mexico	1.08	0.52	0.48	0.37	0.15	0.33	Murillo and Hernández-Carmona 2007
S. vulgare	Brazil	1.27	0.56	0.44	0.55	0.02	0.43	Torres et al. 2007
Sargassum turbinarioides	Madagascar	0.94	0.48	0.52	0.36	0.25	0.39	Fenoradosoa et al. 2010
Laminaria degitata	Norway	1.44	0.59	0.41	0.43	0.16	0.25	Smidsrød and Draget 1996
Laminaria japonica	China	1.86	0.65	0.35	0.48	0.17	0.18	Nai-yu et al. 1994
S. scoparium	Algeria	0.73	0.42	0.58	0.34	0.08	0.49	this study

2010, 2012; Zhang et al. 2012). HPAEC is known to be an accurate method to determine the M/G ratio (Jiaojiao et al. 2015), and in our study, it gave the neutral monosaccharide content as 10.48 %, a very similar result to that obtained with the colorimetric assay (8,96 %). The predominant monosaccharide was xylose (3.95 %), followed by galactose (1.95 %), rhamnose (1.85 %), arabinose (1.64 %), fucose (0.7 %) and glucose (0.39 %). This content is higher than that found by Fenoradosoa et al. (2010) in *Sargassum turbinarioides* (0.06 %) and Patankar et al. (1993) in *Petalonia fascia* (5 %).

The chromatogram obtained for uronic acid shows two peaks: GulA (G) and ManA (M), detected at 15.60 and 16.05 min, respectively. These polysaccharides have a peak area (G) greater than the peak area (M), indicating they are rich in GluA with a surface of 230.2 nC * min, when compared with ManA, with a surface of only 138.503 nC * min. Consequently, the yield of GluA is 31.3 %, while the yield of ManA is 18.85 % (Fig. 3a). The molar fractions of GulA and ManA were determined in absence of calibration, assuming the same response coefficient for both uronic acids; it also supposes a complete hydrolysis of alginates (but the different osidic linkage does not have the same sensitivity in acid medium) and absence of degradation of the monosaccharides produced. The M/G ratio calculated was 0.6, confirming the previous evaluation by ^{1}H NMR. No relative data are described in the literature for alginate from *S. scoparium* but ratios obtained in other algae using the same method are 0.70 in alginate of *Sargassum fusiforme*, 1.40 in *Macrocystis pyrifera* and 1.8 in *Saccharina japonica* extracted in China (Lu et al. 2015).

Molecular weight distribution

The molar mass and mass distribution for the polysaccharides of *S. scoparium* are shown in Table 2. The alginate was found to have a molecular weight (Mw) of 2.236×10^5 g mol^{-1}. These results are supported by Torres et al. (2007), who report the molar mass of alginate to be between 1.94 and 7.34×10^5 g mol^{-1}. This compound is polydispersed, as revealed by the polydispersity index (Mw / Mn) of 1.714 ± 0.039. Polydispersity index values between 1.4 and 6.0 have been previously reported for alginates and the differences are related to varying types of preparation and purification processes (Mackie et al. 1980; Martinsen et al. 1991).

Partial acid hydrolysis of alginate

The partial hydrolysis of the alginate from *S. scoparium* was carried out as described in "Materials and methods" section, and three fractions were generated with 57.39 % recovery yields (Fig. 3b, c, and d). The first fraction, obtained at the first step of hydrolyzing with HCl 0.3 M, was composed mainly of heteropolymeric blocks (MG-blocks). The second fraction, which was soluble at pH .85, was enriched by blocks of polymannuronic acid (M-blocks). The third fraction, insoluble at pH .85, was composed principally of blocks of polyguluronic acid (G-blocks). The yields of the various fractions showed that the studied alginate is richer in polyguluronic acid (43.53 %) than in polymannuronic acid (12.48 %) and heteropolymeric blocks (MG-blocks) (1.39 %).

The blocks of *S. scoparium* showed a lower molecular weight and polydispersity index compared to the native alginate (Table 2, Fig. 4). The M/G ratios of polymannuronic and polyguluronic acid blocks were evaluated by HPAEC at 15.60 and 16.05 min respectively, showing enrichment in homopolymeric fractions.

Oligoguluronates obtained by enzymatic degradation of G-blocks

The depolymerization of polyguluronic acid blocks by alginate lyase of *F. multivorum* is shown in Fig. 5. The monosaccharide products were formed at an early stage of the reaction, indicating that the enzyme expressed its function in an exolytic manner. After a prolonged reaction, the spots were distinct. The lyase cleavage progressed over time. At the end, the mixture contained only mondispersed oligosaccharides, with 98 % recovery yields and a Mw = 3.742×10^3 g mol^{-1} close to Mn = 3219×10^3 g mol^{-1}, the dispersity index being lower compared to the native alginate and G-Blocks (Fig. 6).

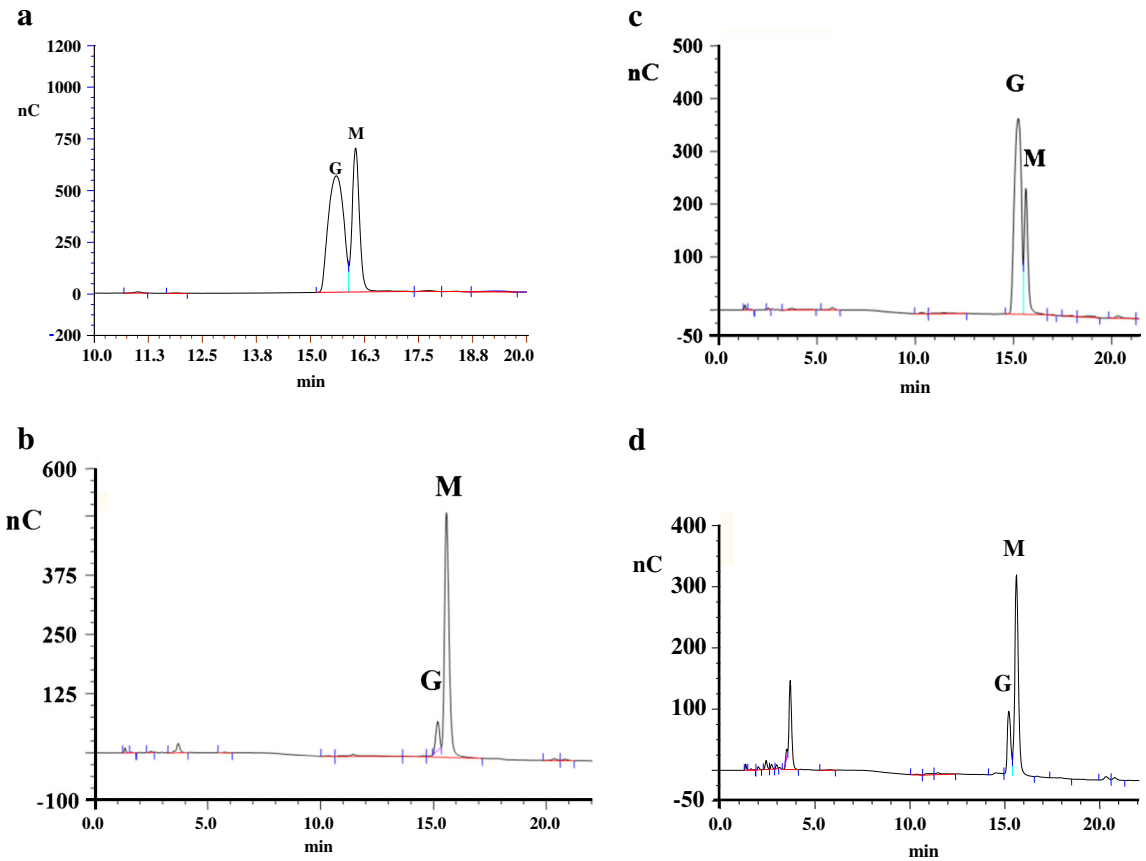

Fig. 3 The chromatograms HPAEC of alginate (a), M-blocks (b), G-blocks (c) and MG-blocks(d) from *Stypocaulon scoparium,* with G: α -L-guluronic acid*;* M:β -D-mannuronic acid

Discussion

The characterization of alginate isolated from brown seaweed (*S. scoparium*) from the Mediterranean coast in Algeria indicated that this polysaccharide has a low molecular weight (Mw) (2.236×10^5 g mol^{-1}) in comparison with other species such as *L. japonica* ($M_W = 7.44x10^5$ g mol^{-1}), but it is higher compared to *F. vesiculosus and A. nodosum* (Mw = $1.17x10^5$ g mol^{-1}, and $1.32x10^5$ g mol^{-1}, respectively) (Fourest and Volesky 1997). Additionally, it has a low polydispersity index (Pi =1.714).

In addition to the Mw, the mannuronic to guluronic acid ratio (M/G) is important for the selection of appropriate applications for an alginate (Gacesa 1988; Sen 2011): the resulting gels are brittle when the M/G ratio is low, and elastic when high (Penman and Sanderson 1972).

The M/G ratio of alginate from Algerian *S. scoparium* was found to be 0.93 using FT-IR, a qualitative method that can be used to calculate the M/G ratio of any alginate. Infrared spectroscopy has been effectively employed to obtain rapid qualitative information on the composition of alginates (Szejtli 1966). When ^{1}H NMR spectroscopy was used, the M/G ratio was found to be 0.73. In agreement with data reported for various other alginates of brown algae previously cited, this acidic polysaccharide is richer in guluronic acid ($F_G = 0.58$) than mannuronic acid ($F_M = 0.42$). ^{1}H NMR spectroscopy is the main technique used in the investigation of alginate composition and structural patterns (Gradsalen et al. 1979; Larsen et al. 2003), but it requires substantial amounts of sample, and spectra often need to be acquired at a high temperature to decrease the viscosity of the alginate solution. Thus, the

Table 2 Determination by SEC-MALLS of molar mass (g mol^{-1}), the polydispersity index (Pi = Mw/Mn) of alginate and his different blocks

	Blocks			
	Alginate	G	M	MG
Mw (g mol^{-1})	2.236×10^5	2.168e x 10^4	2.365×10^4	2.139×10^5
Yield (%)	5	43.53	12.48	1.39
Pi (Mw/Mn)	1.714 ± 0.039	1.408 ± 0.066	2.071 ± 0.205	4.392 ± 0.154

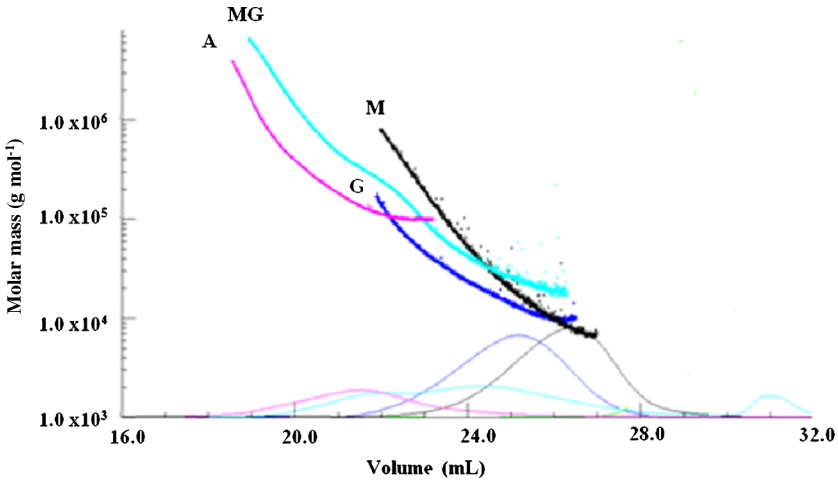

Fig. 4 SEC-MALLS profile of alginate and different blocks from *Stypocaulon scoparium*. A: Native alginate, MG: MG-blocks, G: G-blocks, M: M-blocks

application of NMR to measure the M/G ratio of alginate and its derivatives can be challenging for small scale samples or the screening of many alginate samples (Zhang et al. 2004; Wang et al. 2005).

The M/G ratio determined with ^{1}H NMR spectroscopy was confirmed by comparing manuronic acid and guluronic acid areas by HPAEC-PAD (M/G = 0.6), a vigorous recently developed method (Voragen et al. 1982; Guttman 1997; Rumpel and Dignac 2006). The released ManA and GulA were well separated and sensitively detected without any derivatization after hydrolysis. The complete hydrolysis of alginate is another critical strategy for the accurate determination of M/G values (Jiaojiao et al. 2015). HPAEC-PAD is an efficient technique to estimate the composition of different blocks after partial hydrolysis. We found that the polysaccharide of *S. scoparium* is rich in polyguluronate blocks, indicating that this alginate is resistant. The polyguluronic acid block content of alginates is crucial for their gel-forming capacity (Fenoradosoa et al. 2010).

From all these data, HPAEC-PAD may be considered as the best tools for the determination of the M/G ratio of alginate polysaccharides.

The present study also reports the production of alginate oligosaccharides by degradation of polyguluronic acid blocks from *S. scoparium* using polyguluronate lyase of *Flavobacterium multivorlum* to generate a oligosaccharide mixture.

The alginate lyases have been classified into two groups according to their substrate specificities: one is a G block-specific lyase (polyguluronate lyase, E C4.2.2.11), and the other is an M block-specific lyase (polymanuronate lyase, E C4.2.2.3) (Preiss and Ashwell 1962). Polymannuronate lyase presents a high activity upon sequences corresponding to mannuronate pentamers and is also able to cleave alginate heteropolymers; consequently GM linkages are cleaved but not GG and MG, which are cleaved by polyguluronate lyase (Ochi et al. 1995; Heyraud et al. 1996).

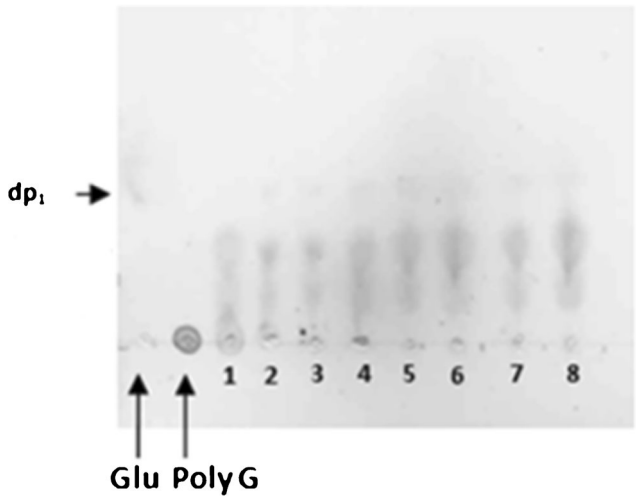

Fig. 5 Degradation of G-blocks of alginate from *Stypocaulon scoparium* by alginate lyase. The reaction was performed at 30 °C in a mixture consisting of 0.1 % polyguluronate and 0.5 U enzyme (6 h). The reaction products were periodically analyzed by TLC-60 with Glu: Glucuronic acid and Poly G: polugulurononate, Reaction times: lane 1, 0 min; lane 2, 30 min; lane 3, 1 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h; lane 7, 5 h; lane 8, 6 h, dp1 represent monosaccharide

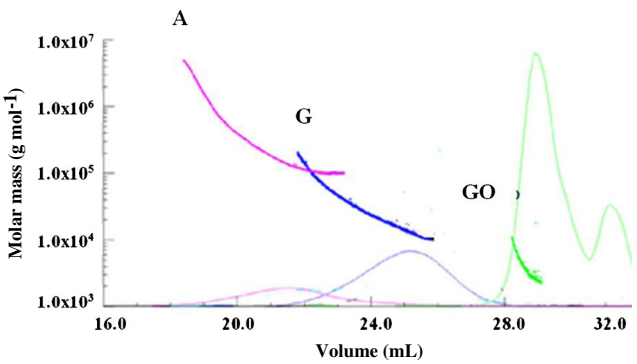

Fig. 6 Degradation profile of G-blocks of alginate from *Stypocaulon scoparium* in oligoguluronate. A: native alginate, G: G-block and GO: oligoguluronate

The alginate lyase of *F. multivorum* used in this study is a poly-guluronate lyase, which acts specifically only on polyguluronic blocks. Indeed, the poly-guluronate lyase eliminates the cleavage of polysaccharides containing a terminal α-L-guluronate group, to give oligosaccharides with 4-deoxy-α-L-erythro-hex-4-enuronosyl groups at their non-reducing ends (Davidson et al. 1976; Boyd and Turvey 1977). The oligoguluronate obtained with alginate depolymerases of G-blocks of sodium alginate can be used for testing biological properties.

In conclusion, this is the first report on the characterization of a polysaccharide such as alginate and several oligosaccharides from *S. scoparium*. The alginate produced by *S. scoparium* has a higher content of uronic acids and a low quantity of proteins. The M/G ratio was determined with different analytical tools including HPAEC (0.60), FT-IR (0.92) and ^{1}H NMR (0.73) methods. HPAEC may be considered as the best and most reliable methodology for the determination of the M/G ratio of alginate polysaccharides. The molecular weight of the alginate was determined as 2.236×10^5 g mol^{-1}, and the molecular weight of G-, M- and MG-blocks was determined as 2.168×10^4 g mol^{-1}, 2.365×10^4 g mol^{-1} and 2.139×10^5 g mol^{-1}, respectively.

The different block compositions estimated by HPAEC show that this polysaccharide is rich in polyguluronate blocks (G-blocks), which gives it rigidity and high viscosity, favorable for obtaining more resistant gels.

The process of production and purification of the oligoguluronates by poly-guluronate lyase of *F. multivorum*, made it possible to determine the duration of degradation necessary to generate these monodispersed oligosaccharide mixtures with 98 % recovery yields and a molecular weight of 3.742×10^3 g mol^{-1}. While the polyguluronate lyase from *F. multivolum* is applied principally for the preparation of polyG blocks, in our work, pure oligoguluronates were produced by using this enzyme to degrade alginate G-blocks, with the control of various influencing parameters.

Plants usually respond to pathogen attack by producing secondary compounds that limit the infection by interfering with the metabolism of pathogens. This response begins with the recognition by the plant cell membrane of an elicitor formed by the pathogen action. Alginate oligosaccharides, due to their chemical structure, could act as elicitors, constituting a stimulus that would be transferred mainly from the membrane to the cell by a signal transduction system, and producing changes that would ultimately lead to the formation of endogenous defense compounds such as phytoalexins. Many of these compounds are secondary metabolites of interest for the chemical-pharmaceutical industry. For these reasons, the alginate oligosaccharides obtained in this study could be assayed as compounds able to switch on the defense responses to pathogens of economically important crops, increasing their resistance, or to increase the production of target secondary compounds in selected plants. Exploring the potential of these compounds as elicitors for increasing the biotechnological production of high-added value compounds would be another very interesting study.

Acknowledgments This work has been carried out in France at the laboratory of Plant Biology and Innovation (Amiens). We wish to thank Dr. E. Petit for generously providing the lab facility and for sharing his knowledge in carbohydrate biochemistry, and also Dr. R. Elboutachfaiti for his help and Dr. R. Molinié for the RMN analysis, as well as Dr. Rosa M. Cusidó of the Plant Physiology laboratory at the University of Barcelona for valuable suggestions and corrections, and S. Amari, Prof. R. Yahaoui Zaidi, and Miss Smiel Amel (Translator).

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