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Sulfation of agarose from subantarctic *Ahnfeltia plicata* (Ahnfeltiales, Rhodophyta): studies of its antioxidant and anticoagulant properties in vitro and its copolymerization with acrylamide

Betty Matsuhiro · Tamara Barahona · María V. Encinas · Andrés Mansilla · Jonathan A. Ortiz

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Abstract Aqueous extraction of *Ahnfeltia plicata* collected in the Magellan ecoregion afforded agarose devoid of sulfate groups. This neutral agarose was subjected to sulfation with SO_3-pyridine complex, giving an aqueous soluble derivative with 35.5 % sulfate groups. Analysis by Fourier transform infrared spectroscopy (FT-IR) and by ^{1}H and ^{13}C NMR spectroscopy indicated that this derivative was sulfated at positions C-6 of the β-galactopyranosyl residue and C-2 of the α-3,6-anhydrogalactopyranosyl residue and partially sulfated at position C-2 of the β residue. The antioxidant capacity of sulfated agarose was evaluated by the oxygen radical absorbance capacity (ORAC) method, ABTS radical cation, hydroxyl radicals, and chelating assays. This capacity of sulfated agarose toward peroxyl radicals was higher than that of commercial λ-carrageenan, while native agarose presented good activity, with an ORAC value similar to that of commercial κ-carrageenan. Sulfated agarose presented good antioxidant capacity toward other radicals. Copolymerization of sulfated agarose with acrylamide was achieved using ceric ammonium nitrate as initiator. NMR spectroscopy indicated grafting of polyacrylamide at position C-4 of β-galactopyranosyl residues.

Keywords Agarose · Sulfation · Anticoagulant activity · Antioxidant capacity · Copolymerization

B. Matsuhiro (✉) · T. Barahona · M. V. Encinas · J. A. Ortiz
Departamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, Av. L. B. O'Higgins, 3363 Santiago, Chile
e-mail: betty.matsuhiro@usach.cl

A. Mansilla
Laboratorio de Macroalgas Antárticas y Subantárticas, Universidad de Magallanes, Av. Bulnes, 1465 Punta Arenas, Chile

Introduction

Agarans are a family of polysaccharides extracted with hot water from several species of red algae. They are composed of alternating 3-linked β-D-galactopyranosyl and 4-linked α-L-galactopyranosyl or 3,6-anhydro-α-L-galactopyranosyl residues, substituted with sulfate, methyl, and/or pyruvate groups (Duckworth and Yaphe 1971; Izumi 1971; Usov 2011). Agarose, a →3-β-D-galactopyranosy-1→4-3,6-anhydro-α-L-galactopyranosyl-1→ polymer is the most important member of this family due to its application in many biochemical separation processes (Guiseley 1987; Matsuhashi 1998). Agarans contain a small amount of hemiester sulfate (~3.0 %) compared with carrageenans (22–38 %), (Duckworth and Yaphe 1971; Campo et al. 2009). The biological properties of sulfated polysaccharides from marine algae are known (Usov 2011; Wijesekara et al. 2011). Studies on the antioxidant capacity of sulfated polysaccharides from brown and red seaweeds indicated the influence of sulfate content, molecular weight, and structure of the glycosyl residues (Ruperez et al. 2002; Zhang et al. 2003; Zhao et al. 2004; 2008; Rocha de Souza et al. 2007; Barahona et al. 2011, 2012). On the other hand, it was found that sulfation of polysaccharides increased their biological properties, such as antioxidant, antitumor, and anticoagulant activity (Sun et al. 2009; Wang et al. 2009a; 2009b; 2010; Zhang et al. 2010; Telles et al. 2011). Also, over the last two decades, there has been increasing interest in the copolymerization of polysaccharides with vinyl monomers, a modification that allowed the preparation of graft copolymers with new properties for industrial applications (Jenkins and Hudson 2001; Rana et al. 2011). Various vinyl monomers such as acrylamide, acrylic acid, acrylonitrile, *N*-vinyl-2-pyrrolidone, 2-acrylamidoglycolic acid, and vinylsulfonic acid were grafted onto κ-carrageenan via free radical polymerization (Meena et al. 2006; Pourjavadi

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et al. 2008; Hosseinzadeh 2009; Mishra et al. 2010; Sand et al. 2012; Yadav et al. 2012).

Recently, it was found that *Ahnfeltia plicata* (Hudson) E.M. Fries (Ahnfeltiales, Rhodophyta) forms extensive natural beds with high biomass production in the Magellan ecoregion of southernmost Chile (Mansilla et al. 2012, 2013). Its phycocolloid would be a very interesting starting material for the preparation of derivatives with potential biological applications. The aim of this work is the structural characterization of the aqueous extract from *A. plicata*, the preparation, characterization, and studies of the biological properties of a sulfated derivative and its conjugation with acrylamide.

Materials and methods

Analytical grade solvents were purchased from Merck (Germany); reagent grade chemicals were purchased from Sigma (USA). Absorbance was measured in a Genesys 5 Thermospectronic spectrophotometer (Thermo Fisher Scientific, USA). Fourier transform infrared (FT-IR) spectra in KBr pellets of seaweeds and polysaccharides were recorded in the 4,000–400 cm^{-1} region in a Bruker IFS 66*v* instrument (Bruker, UK) according to Leal et al. (2008). All the samples were previously milled under liquid nitrogen and dried for 8 h in vacuo at 56 °C. ^{1}H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra were recorded in D_2O, after isotopic exchange (3×0.75 mL) at 70ºC in a Bruker Avance DRX 400 spectrometer (Bruker, UK) using the sodium salt of 3-(trimethylsilyl)-1-propionic-2,2,3,3-d_4 acid as internal reference. The two-dimensional experiments were performed using a pulse field gradient incorporated into an NMR pulse sequence. The number of scans in each experiment was dependent on sample concentration. Gas–liquid chromatography (GC) was performed on a Shimadzu GC-14B chromatograph (Shimadzu, Japan) equipped with a flame ionization detector using an SP 2330 column (0.25 mm×30 m), and it was carried out with an initial 5-min hold at 150ºC and then at 5ºC min^{-1} to 210ºC for 10 min. The helium flow was 20 mL min^{-1}. *A. plicata* (Ahnfeltiales, Rhodophyta) was collected at Riesco Island, Magellan ecoregion (Mansilla et al. 2013).

Extraction The dried ground seaweed (100 g) was heated with distilled water (2 L) at 95 °C for 3 h, cooled to 45 °C and centrifuged at 3,500 *x g*. The supernatant was allowed to gel for 12 h and frozen overnight. The frozen gel was allowed to thaw on a piece of muslin and was washed with distilled water until the filtrate became colorless. Purification of the gel was performed by three freeze-thaw cycles and the gel was dissolved in distilled water and freeze-dried. The residue of the extraction was re-extracted twice with water under the same conditions.

Chemical analysis Reducing sugars were determined by the phenol-sulfuric acid method using D-galactose as standard (Chaplin 1986). The 3,6-anhydrogalactose content was determined according to Matsuhiro and Zanlungo (1983) using D-fructose as standard. The amount of sulfate was determined by a modification of the Dodgson and Price method, using glycerin instead of gelatin (Barahona et al. 2012). The molecular weight of the polysaccharide was determined by the reducing end method (Cáceres et al. 2000). Sulfur content was determined by microanalysis at the Facultad de Química, Universidad Católica de Chile.

Acid hydrolysis The purified extract (0.005 g) was heated with 0.75 mL of 2 M trifluoroacetic acid for 2 h at 120 °C and the acid was removed by co-evaporations with distilled water. The product of hydrolysis was dissolved in 2 mL of distilled water, treated with sodium borohydride during 2 h and concentrated in vacuo. The resulting syrup was acetylated with acetic anhydride in dry pyridine and analyzed by GLC. Per-*O*-acetates of D-galactitol, D-glucitol, D-mannitol, L-rhamnitol, L-fucitol and D-xylitol were used as standards.

Sulfation Sulfation was performed with SO_3-pyridine complex (Vogl et al. 2000). Freeze-dried agarose (0.200 g) was dissolved in 5 mL of formamide and 2 mL of dry pyridine, and 2.0 g of the SO_3-pyridine complex were added with stirring. The resulting mixture was heated at 90 °C under a nitrogen atmosphere for 4 h, cooled to room temperature, and poured over 20 mL of ice water. The resultant solution was neutralized with 0.1 N NaOH and precipitated over 150 mL of ethanol and centrifuged. The pellet was dissolved in distilled water, dialyzed using Spectra/Por membranes (MWCO 3500) (Spectrum Laboratories, USA) against 0.1 M sodium acetate for 3 days, followed by distilled water, concentrated in vacuo, and freeze-dried.

Antioxidant capacity assays

Oxygen radical absorbance capacity assay

The consumption of fluorescein due to incubation with AAPH [2,2'-azo-bis(2-amidinopropane) dihydrochloride] at different times was estimated from fluorescence measurements (Alarcón et al. 2008; Barahona et al. 2011). A reaction mixture containing 10 mM AAPH with and without the native agarose or the sulfated agarose at different concentrations (0.1–1.0 mg mL^{-1}) in distilled water was incubated in a phosphate buffer (10 mM, pH 7.0) at 37 °C. Fluorescein (1.5 μM) consumption was evaluated from the decrease of the

fluorescence intensity (exCitation 491 nm, emission 512 nm) in the presence of polysaccharide. Fluorescence measurements were made in a Fluorolog-Spex 1681/0.22 spectrofluorometer (Spex, USA). Bandwidths of 1.25 nm were used for the excitation and emission slits. The fluorescence intensity values, F, were plotted with respect to the initial value F/F^o as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (F/F^o) was close to zero. Oxygen radical absorbance capacity assay (ORAC) values were obtained from the ratio of the slope of the (AUC–AUC^o) plots versus the concentrations of polysaccharide and expressed taking vitamin C as reference.

Hydroxyl radical scavenging activity assay

Hydroxyl radicals were generated from $FeSO_4$ and H_2O_2 (Fenton reaction) at 20 °C, and their antioxidant capacity was studied using salicylate as probe (Smirnoff and Cumbes 1989). The absorbance of aqueous solutions of 20 mM sodium salicylate, 1.5 mM $FeSO_4$, and varying concentrations of polysaccharide (0–1.5 mg mL^{-1}) was measured at 510 nm as a function of time immediately after the addition of H_2O_2.

Ferrous ion-chelating ability

The ferrous ion-chelating ability was studied according to Decker and Welch (1990). Samples of native agarose or sulfated agarose in the concentration range of 0.1–2.0 mg mL^{-1}were mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. Formation of the ferrozine-Fe^{2+} complex was followed spectrophotometrically as a function of time at 562 nm. All absorbance measurements were made using an HP8453 diode array spectrophotometer (Hewlett-Packard, Germany).

ABTS assay

The ABTS radical cation ($ABTS^{\bullet+}$) was obtained by reaction of ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) with 2.45 mM potassium persulfate according to Re et al. (1999). The $ABTS^{\bullet+}$ solution was diluted with pH 7.0 PBS to an absorbance of 0.70 at 734 nm, added to polysaccharide aqueous solutions (0.90–2.40 mg mL^{-1}), and the absorbance was recorded at 734 nm.

Anticoagulant activity

Anticoagulant activity by the activated partial thromboplastin time assay

Human plasma (0.4 mL) from healthy donors was mixed with 0.1 mL of sulfated agarose (1–7×10^{-3} mg mL^{-1}), and heparin (0.2–6.0×10^{-3} mg mL^{-1}) (Claris Lifescience, India). All samples were incubated with 0.005 mL of STA-Cephascreen solution (Diagnostica Stago Inc., USA) at 37ºC for 4 min. Then, 0.005 mL of 0.025 M $CaCl_2$ solution was added and the clotting time was measured in triplicate on STA Compact (Diagnostica Stago Inc.) equipment and repeated on three different days. Distilled water was used as negative control. The anticoagulant activity was expressed as sample activated partial thromboplastin time (APTT)/negative control APTT (APTT/APTT^0).

Copolymerization with acrylamide

Sulfated agarose (0.200 g) was dissolved in 5 mL of distilled water and 0.250 g of acrylamide, 0.5 mL of 0.1 N HNO_3, and 0.0022 g of ceric ammonium nitrate were added, and the resulting solution was heated at 60 °C for 90 min under N_2 atmosphere. Then, it was dialyzed against distilled water for 72 h, precipitated into 20 mL of acetone and centrifuged. The precipitate was stirred with 50 mL of formamide/acetic acid 1:1 *v/v* solution and centrifuged. Finally, the supernatant was dialyzed for 48 h against distilled water, concentrated in vacuo and freeze-dried.

Results

Milled and dried samples of *A. plicata* were analyzed by FT-IR spectroscopy (Fig. 1a). It was found that the normal and second-derivative spectra present bands at 931.8, 893.6, 790.9, 771.3, 741.0, 717.7, and 693.9 cm^{-1} which may be ascribed to the presence of agaran-type polysaccharides. Aqueous extraction of *A. plicata* at 95 °C followed by freeze-thaw purification cycles yielded an agaran in 16.0 % yield. Figure 1b presents the normal and second-derivative FT-IR spectra of the purified aqueous extract; the spectra are very similar to those of the algal material and clearly showed the two characteristic "clusters" of agarans. Chemical analysis showed that the extract contained 96.5±0.8 % of reducing sugars and 48.3±0.8 % of 3,6-anhydrogalactose. The molecular weight of the agaran determined by the reducing end method was 550,000±9,000. Total acid hydrolysis of the agaran and GC analysis of the alditol acetates indicated the presence of galactose as the only acid-stable monosaccharide. Analysis of the agaran by the turbidimetric method could not detect the presence of hemiester sulfate group; furthermore, microanalysis showed 0.00 % of sulfur in the extract. Taken together, these results indicate the presence of neutral agarose. Proton and ^{13}C NMR spectroscopy confirmed the proposed structure for the polysaccharide extracted from *A. plicata*. Figure 2a shows the 2D $^{13}C/^{1}H$ HSQC NMR spectrum of the agaran, some of the correlations between ^{13}C and ^{1}H nuclei are drawn, this spectrum and 2D $^{1}H/^{1}H$ COSY NMR

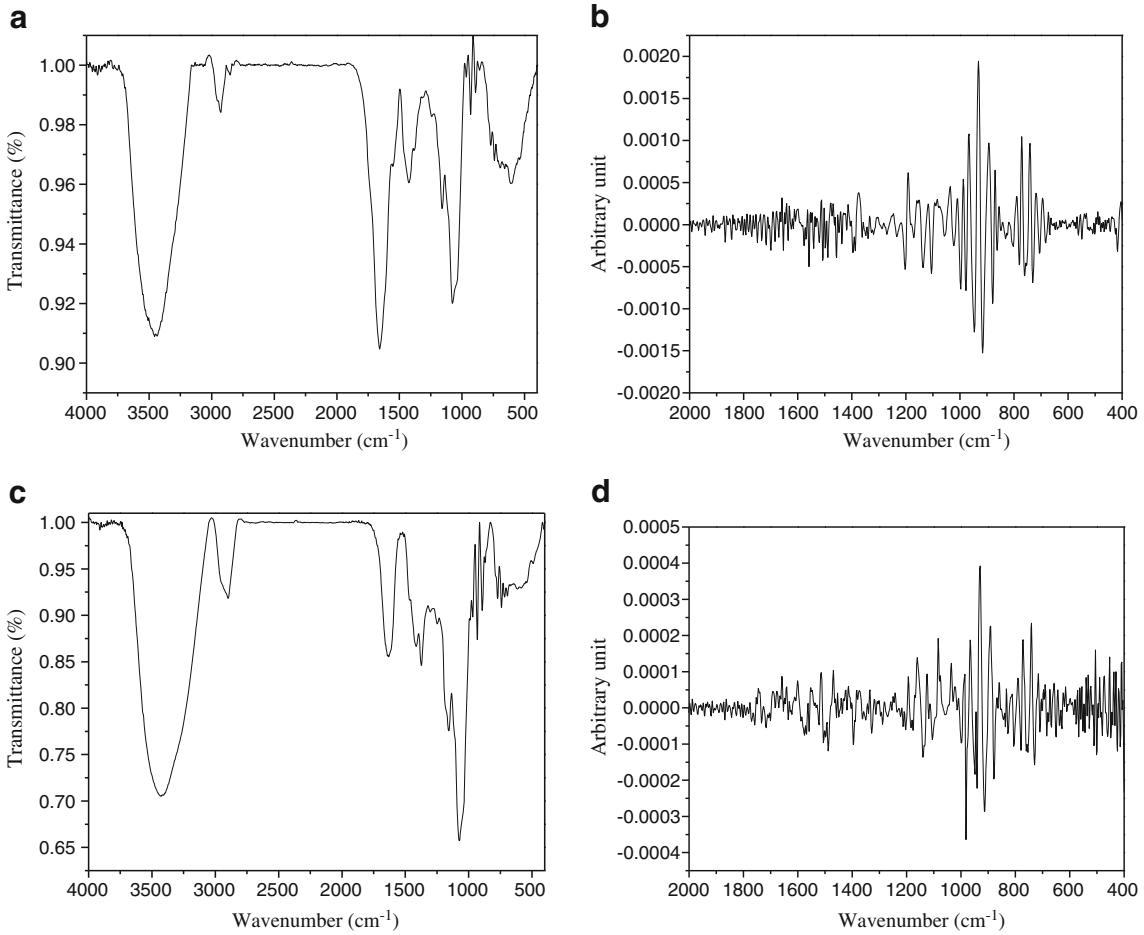

Fig. 1 FT-IR spectra in the region 4,000–400 cm^{-1} of **a** normal spectrum of *Ahnfeltia plicata*, **b** second-derivative spectrum of *A. plicata*, **c** Normal spectrum of aqueous extract from *A. plicata*, and **d** second-derivative spectrum of aqueous extract from *A. plicata*

experiment allowed the full assignments of the 1D ^{1}H and ^{13}C NMR spectra as shown in Table 1.

Sulfation of agarose with an SO_3-pyridine complex yielded an aqueous soluble polysaccharide containing 35.5 % of hemiester sulfate group as determined by the turbidimetric method and microanalysis. Its IR spectrum (not shown) presented characteristic bands associated with the presence of sulfate groups (1,250 and 582 cm^{-1}), the second-derivative spectrum showed two bands at 830 and 808 cm^{-1} which were assigned to a hemiester sulfate group linked to primary

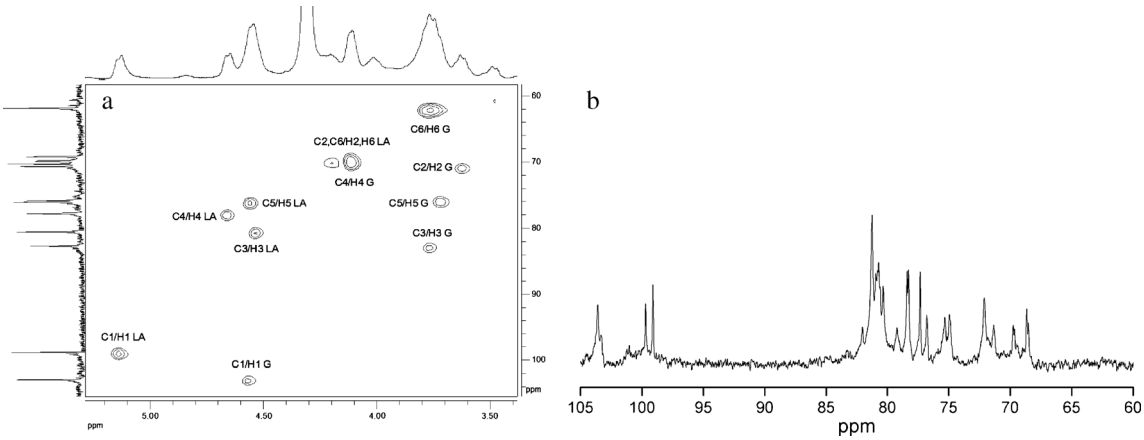

Fig. 2 **a** $^{13}C/^{1}H$ HSQC 2D NMR spectrum of *Ahnfeltia plicata* agarose. **b** ^{13}C NMR spectrum (100 MHz) of sulfated agarose

Table 1 Assignment of chemical shifts in the ^{1}H and ^{13}C NMR spectra (400 and 100 MHz, respectively) in D_2O at 70 °C of agarose from *Ahnfeltia plicata*

Residue	^{1}H chemical shift (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
β-D-galactose (G)	4.57	3.63	3.76	4.12	3.75	3.77
3,6-anhydrogal (LA)	5.14	4.12	4.55	4.66	4.56	4.11
	^{13}C chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-galactose (G)	102.88	70.69	82.68	69.20	76.07	61.86
3,6-anhydrogal (LA)	98.80	70.29	80.55	77.83	76.84	69.85

alcoholic and secondary axial alcoholic groups, respectively. The ^{13}C NMR spectrum (Fig. 2b) of sulfated agarose is quite different from that of the starting material (Fig. 2a); the signals assigned to C6 of the β residue and to C2 of the α residue are not present, and new signals at 99.71, 81.70, and 75.36 ppm can be seen. The ^{1}H NMR spectrum of the sulfated agarose (not shown) showed news signals in the anomeric region at 5.34 and 5.39 ppm which were assigned to H1 of the non-sulfated and sulfated α residues, while the signals of H2 and H3 of the same residue were shifted to lower field (4.74 and 4.63 ppm) due to the presence of the sulfate group in *O*-2 axial position. No signal in the 3.70–3.80 ppm region was seen.

The peroxyl radical scavenging capacity of these polysaccharides was measured by the ORAC method using fluorescein as probe. Figure 3a depicts the decrease of the fluorescent emission mediated by peroxyl radicals in the presence of different concentrations of polysaccharide sample. A good linear relation can be seen between the net area under the curve (AUC) and the concentration of sulfated agarose (insert in Fig. 3a). The ORAC values, taking ascorbic acid as reference, were $6.5\pm1.4\times10^{-3}$ and $9.3\pm1.5\times10^{-3}$ for native agarose and sulfated agarose, respectively. Figure 3b shows absorbance versus time plots for the scavenging of the $ABTS^{\bullet+}$ radical cation by agarose and sulfated agarose. Both polysaccharides had a behavior linearly related with concentrations.

The antioxidant capacity of sulfated agarose against hydroxyl radicals produced by the Fenton reaction was measured by the competitive salicylate hydroxylation reaction. Figure 4a presents the decay of salicylate absorption at 510 nm. In addition, the chelating activity of sulfated agarose was followed by the competitive formation of the ferrozine-Fe^{2+} complex, which shows an absorption maximum at 562 nm (Fig. 4b). It is seen that native agarose did not inhibit the formation of the ferrozine-Fe^{2+} complex at 1 mg mL^{-1} concentration, while sulfated agarose showed good chelating ability with ferrous ion at lower concentrations (0.1–0.75 mg mL^{-1}).

The anticoagulant activity of sulfated agarose was measured using the activated partial thromboplastin time (APTT) test (Table 2). It is noticeable that at low concentration (2.0×10^{-3} mg mL^{-1}), sulfated agarose showed an APTT value of 1.36±0.04, close to the value presented by heparin at similar concentration.

The copolymerization of acrylamide with sulfated agarose was carried out using Ce^{4+} ion as initiator. The yield of the polymeric material obtained, under the polymerization conditions given in the experimental section, showed a 58 % of conversion with respect to the initial materials. The nitrogen content, determined by microanalysis, was 2.4 %. The second-derivative FT-IR spectrum of the reaction product (figure not shown) presented besides the sulfated agarose vibration bands, absorptions at 1,666, 1,610, and 1,411 cm^{-1} assigned

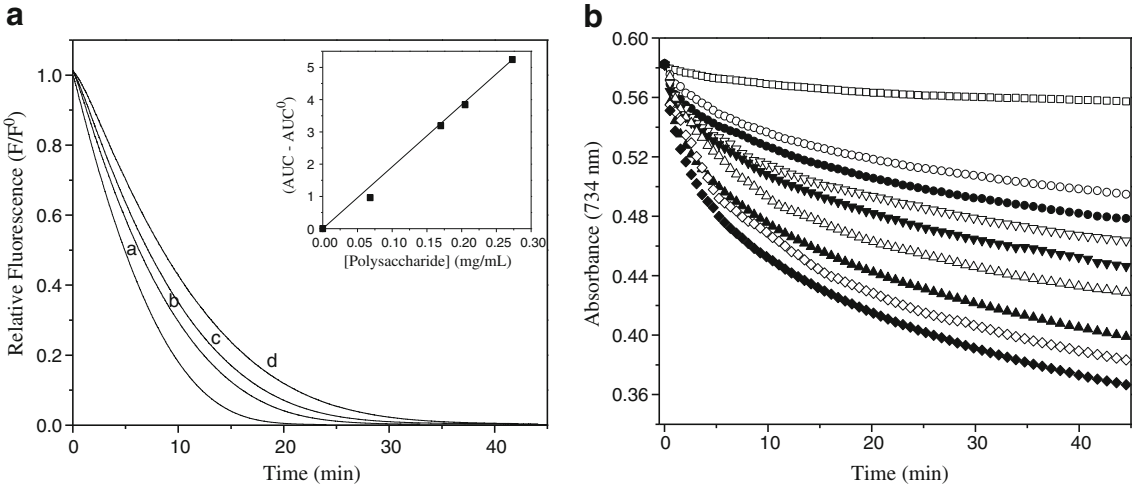

Fig. 3 **a** Decrease of fluorescein fluorescence in the presence of AAPH at different concentrations of sulfated agarose at 37 °C and pH 7.0. (a) In the absence and in the presence of (b) 0.032 mg mL^{-1}, (c) 0.065 mg mL^{-1}, and (d) 0.13 mg mL^{-1} of polysaccharide. *Insert*: dependence of net area under plot vs concentrations of sulfated agarose. **b** Decrease of $ABTS^{\bullet+}$ absorbance at 734 nm in the presence of different concentrations of native agarose from *Ahnfeltia plicata* and sulfated agarose. In the absence of polysaccharides (*open box*), and in the presence of agarose (*open points*) and sulfated agarose (*solid points*): (*solid points*) 0.90 mg mL^{-1}, (*inverted solid triangle*) 1.20 mg mL^{-1}, (*solid triangle*) 1.80 mg mL^{-1}, and (*solid diamond*) 2.40 mg mL^{-1}

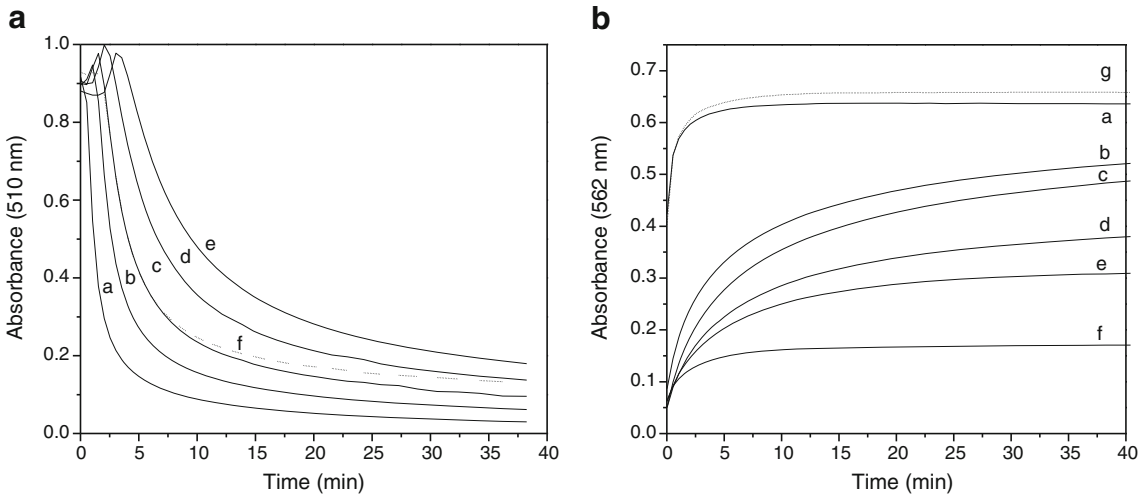

Fig. 4 **a** Decay of sodium salicylate absorbance in the presence of HO˙ radicals at different concentrations of sulfated agarose: (a) in the absence of polysaccharides, and in the presence of (b) 0.25 mg mL^{-1}, (c) 0.50 mg mL^{-1}, (d) 0.75 mg mL^{-1}, (e) 1.0 mg mL^{-1}, and (f) 1.0 mg mL^{-1} of native agarose. **b** ferrozine-Fe^{2+} complex formation in the (a) absence of polysaccharides, in the presence of sulfated agarose (b) 0.10 mg mL^{-1}, (c) 0.25 mg mL^{-1}, (d) 0.50 mg mL^{-1}, (e) 0.65 mg mL^{-1}, (f) 0.75 mg mL^{-1}, and in the presence of 1.0 mg mL^{-1} of native agarose from *Ahnfeltia plicata*

to amide groups. Furthermore, the 2D $^{13}C/^{1}H$ HMBC NMR spectrum of the copolymer (Fig. 5) shows a correlation between carbon C-4 of β-galactopyranosyl residue and the methylene protons of polyacrylamide.

Discussion

Truus et al. (2006) reported that *A. plicata* produces agaran- or carrageenan-type polysaccharides depending on environmental conditions. In this work, analysis of normal and second-derivative FT-IR spectra of dried and milled *A. plicata* collected in the Magellan ecoregion allowed the assignments of signals ascribed to agaran, indicating the presence of an agarophyte. As previously found, the second-derivative spectrum constitutes a good tool to differentiate agaran-producing from carrageenan-producing red seaweeds (Matsuhiro and Rivas 1993; Matsuhiro 1996). The FT-IR spectra of purified aqueous extracts of *A. plicata* clearly presented the two characteristic clusters of agaran. The presence of a neutral agaran was deduced from the absence of vibrations ascribed to sulfate groups in the FT-IR spectra, by determination of the sulfate group using the turbidimetric assay, and from the sulfur content by microanalysis. In addition, the content of 3,6-anhydrogalactose determined spectrophotometrically and of galactose as the only acid-stable monosaccharide suggested the presence of neutral agarose. Furthermore, the full assignments of ^{1}H and ^{13}C NMR spectra of agarose were achieved with the aid of 2D NMR spectra as depicted in Table 2. The chemical shifts are very similar to those published in the literature for agarans and commercial agarose and confirm that the agaran obtained from *A. plicata* is a neutral agarose (Usov, et al. 1980; Truus et al. 2006; Maciel et al. 2008). This neutral agarose constitutes a very special polysaccharide for the preparation of new products with valuable biological properties.

It has been known that sulfation of polysaccharides enhances biological properties such as antioxidant, anticoagulant, and antitumor activities compared to native polysaccharides (Tao et al. 2006; Wang et al. 2009a; 2009b; 2010; Zhang et al. 2010; Telles et al. 2011). In this work, the treatment of neutral agarose with the SO_3-pyridine complex produced a soluble derivative in 91 % yield. Analysis by FT-IR and NMR spectroscopies indicated sulfation at positions C-2 of the α residue and C-6 of the β residue; moreover, taking into account the sulfate group content, it can be proposed that the sulfated agarose was also partially sulfated at position C-2 of the β residue.

Table 2 Anticoagulant activity of sulfated agarose

Polysaccharide	APTT[a]				
Sulfated agarose	2.0 μg mL^{-1} 1.36±0.04	4.0 μg mL^{-1} 1.80±0.14	6.0 μg mL^{-1} 2.48±0.33	8.0 μg mL^{-1} 3.32±0.30	
Heparin	0.2 μg mL^{-1} 1.02±0.01	0.4 μg mL^{-1} 1.11±0.05	0.7 μg mL^{-1} 1.30±0.08	2.1 μg mL^{-1} 1.92±0.12	5.0 μg mL^{-1} 4.0±0.58

APTT activated partial thromboplastin time

[a] APTT is expressed as the quotient between sample APTT and negative control APTT°

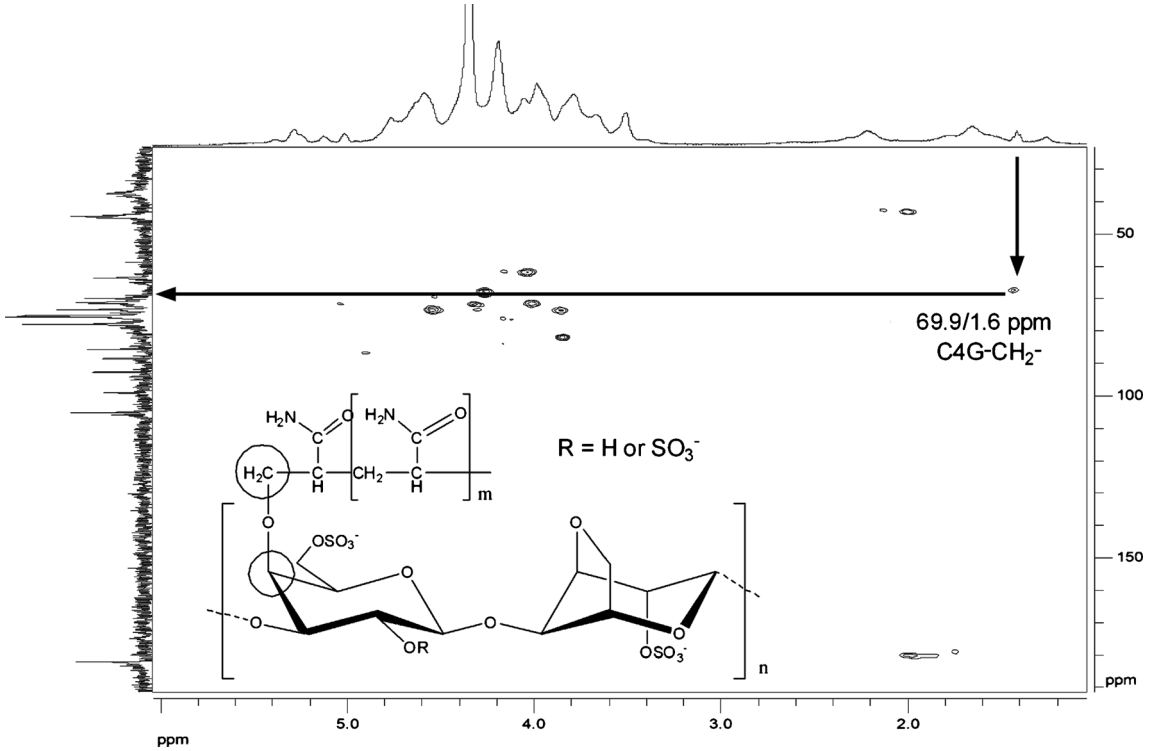

Fig. 5 $^{13}C/^{1}H$ HMBC 2D NMR spectrum in D_2O of sulfated agarose-polyacrylamide graft copolymer

The antioxidant properties of the sulfated agarose as well as the native agarose from *A. plicata* towards peroxyl and hydroxyl radicals and the $ABTS^{\bullet+}$ radical cation presented interesting characteristics. The ORAC values showed that the antioxidant capacity of these polysaccharides towards peroxyl radicals (Fig. 3a) is close to that reported for partially depolymerized fucoidan from the brown seaweed *Lessonia vadosa*, which contained similar amounts of sulfate groups and much higher than the ORAC value found for commercial λ-carrageenan with 33.4 % of sulfate groups (Barahona et al. 2011). Moreover, native agarose, even though it does not contain sulfate groups, presented good activity with an ORAC value similar to that of commercial κ-carrageenan (Barahona et al. 2011). This result indicates that not only the presence of sulfate groups, but other factors such as the position of the sulfate and the structure and molecular weight of the polysaccharide are important features involved in the antioxidant activity.

The antioxidant capacity of sulfated agarose and native agarose with respect to an electron transfer reaction, tested with the $ABTS^{\bullet+}$ radical cation, showed that the decay of the radical cation absorption presented a complex decay (Fig 3b). A two-phase decay can be seen: the fast reaction with the radical cation was followed by a slow reaction to form secondary products (Barahona et al. 2011). Both polysaccharides showed a linear dependence on concentration. These results are similar to those previously found for sulfated galactans from *Schizymenia binderi* and from tetrasporic *Gigartina skottsbergii* (Barahona et al. 2011, 2012); it is noteworthy that neutral agarose showed a moderate antioxidant capacity.

Additionally, the antioxidant capacity of sulfated agarose and native agarose towards hydroxyl radicals was studied using the competitive hydroxylation of sodium salicylate. The latter forms a complex with Fe^{2+} which strongly absorbs at 510 nm, and this absorbance decreases by reaction of salicylate with hydroxyl radicals. In Fig. 4a, it is seen that in the absence of polysaccharides, the complex was formed immediately after addition of H_2O_2, decreasing its absorbance with time, reaching a constant value. In the presence of sulfated agarose, the decay of absorbance was slower and it was related to its concentration. The insert of Fig. 4a shows that native agarose also inhibited the hydroxylation of salicylate, but the decay of the absorbance of the complex is faster compared with that of sulfated agarose at the same concentration. These results indicate that the antioxidant capacity of polysaccharides towards hydroxyl radicals follows complex mechanisms; but for sulfated polysaccharides, the formation of the Fe^{2+} complex may be related to its antioxidant ability. The chelating capacity of native agarose and sulfated agarose was studied by the formation of the ferrozine-Fe^{2+} complex (Fig. 4b), which in the presence of neutral agarose is fast, reaching a constant absorbance in 5 minutes, while for sulfated agarose, the formation of the complex was much slower and decreased as its concentration increased. On the other

hand, the presence of Fe^{2+} in the Fenton reaction may also play a role in the antioxidant activity of sulfated polysaccharides due to the formation of the Fe^{2+} complex. The good chelating activity of sulfated agarose was close to that reported for commercial λ-carrageenan containing a similar amount of sulfate groups (Barahona et al. 2011, 2012). These results suggest that the antioxidant capacity of sulfated agarose in the hydroxyl radical assay may be partially due to the interaction of sulfate groups with Fe^{2+}.

The anticoagulant activity of sulfated agarose was studied with the APTT test. It is interesting to note that sulfated agarose presented good anticoagulant activity at low concentration similar to that for heparin. This activity may be due not only to the high sulfate content but to sulfation at positions *O*-6 in the β-galactopyranosyl residue and *O*-2 of the α-3,6-anhydrogalactopyranosyl residue (Pereira et al. 2002; De Araujo et al. 2013). Mourão (2004) studied the anticoagulant activity of sulfated regular α-L-galactans and of fucoidans from invertebrates and concluded that the sulfation pattern and monosaccharide composition exert an important effect, more important than the sulfate content in this biological property.

The spectroscopic analysis of the obtained material in the polymerization of acrylamide in the presence of the sulfate agarose using Ce^{4+} as initiator clearly showed the grafting of acrylamide into the sulfated agarose backbone. FT-IR spectra of the isolated copolymer show the amide bands ascribed to polyacrylamide residues. Most interesting is the connectivity of C-4 of β-galactopyranosyl residue with the protons of the methylene group of acrylamide in the $^{13}C/^{1}H$ HMBC 2D NMR spectrum. This correlation clearly indicates that the graft copolymerization occurs at the C-4 of the sulfated agarose units. This is in agreement with the deduced structure of sulfated agarose that presented a free OH group at position C-4 of β-galactopyranosyl residue, constituting a good target for the grafting of acrylamide. Mishra et al. (2010) and Rana et al. (2011) synthesized polyacrylamide grafted agar and proposed a mechanism of graft copolymerization; however, these authors employed commercial agar of unknown sulfate or other substituent contents, and they did not propose the structure for the graft copolymer. On the other hand, it is interesting to point, that the conversion of initial monomers to the polymeric material based on the N content of the copolymer (51 %) was similar to that estimated from the gravimetric measurements (58 %). This similarity of conversion yields also indicates that the obtained copolymer is mainly the graft copolymer.

In conclusion, *A. plicata* collected in southernmost Chile is a very special seaweed in the sense that it synthesizes a neutral agarose; it is unusual to find agarophytes that produce a unique phycocolloid. This polysaccharide constitutes a very interesting starting material for the preparation of a soluble derivative with known structure. The results obtained in this work confirm that the antioxidant and anticoagulant properties of agarose were improved by sulfation. However, neutral agarose also presented significant antioxidant activity towards the radicals studied here, indicating that complex mechanisms are involved in the antioxidant assays. A graft copolymer of sulfated agarose and acrylamide has been synthesized. NMR spectroscopic data confirm that the grafting of acrylamide has taken place on carbon 4 of the β-galactopyranosyl residue. Modification of agarose by sulfation and graft copolymerization produced derivatives with potential biotechnological applications.

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