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A sulfated galactan with antioxidant capacity from the green variant of tetrasporic *Gigartina skottsbergii* (Gigartinales, Rhodophyta)

Tamara Barahona [a], María V. Encinas [a], Andrés Mansilla [b,c], Betty Matsuhiro [a,*], Elisa A. Zúñiga [d]

[a] Departamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, Av. B. O'Higgins 3363, Santiago, Chile
[b] Departamento de Ciencias y Recursos Naturales, Facultad de Ciencias, Universidad de Magallanes, Casilla 113-D, Punta Arenas, Chile
[c] Instituto de Ecología y Biodiversidad, Casilla 653, Santiago, Chile
[d] Departamento de Química, Facultad de Ciencias Básicas, Universidad Metropolitana de Ciencias de la Educación, Av. José Pedro Alessandri 774, Santiago, Chile

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ABSTRACT

The water soluble polysaccharide produced by the green variant of tetrasporic *Gigartina skottsbergii* was found to be composed of D-galactose and sulfate groups in a molar ratio of 1.0:0.65. ^{1}H and ^{13}C NMR spectroscopy studies of the desulfated polysaccharide showed a major backbone structure of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl units, and minor signals ascribed to 3-*O*-methyl-substitution on the latter unit. Ethylation analysis of the polysaccharide indicated that the sulfate groups are mainly located at position *O*-2 of 4-linked α-D-galactopyranosyl residue and partially located at positions *O*-6 of the same unit and at position *O*-2 of 3-linked β-D-galactopyranosyl residue, and confirmed the presence of 3-*O*-methyl-galactose in minor amounts (4.4%). The sulfated D-galactan presents a similar structure to λ carrageenan but with much lower sulfation at position *O*-6 of the α-residue and at position *O*-2 of β-residue. The antioxidant capacity of the sulfated D-galactan was evaluated by the peroxyl radicals (ORAC method), hydroxyl radicals, chelating activity, and $ABTS^{\cdot+}$ assays. Kinetic results obtained in these assays were compared with those obtained for the commercial λ carrageenan. The antioxidant activity toward peroxyl radicals was higher for commercial λ carrageenan, this agrees with its higher content of sulfate group. The kinetics of the reaction of both polysaccharides with hydroxyl and $ABTS^{\cdot+}$ radicals showed a complex mechanism, but the antioxidant activity was higher for the polysaccharide from the green variant of tetrasporic *Gigartina skottsbergii*.

1. Introduction

Recently, a green variant of *Gigartina skottsbergii* (Gigartinales, Rhodophyta) was found in a natural population, growing next to red individuals on the south coast of Chile.[1] Color mutants are not uncommon, they have been reported in *Mazzaella laminarioides* and species of the genus *Gracilaria*, most of them are green.[2–6]

The sulfated polysaccharides produced by the red algae *G. skottsbergii* (Gigartinales, Rhodophyta), have been thoroughly studied. Cystocarpic individuals produce κ/ι carrageenans and their precursos μ/ν carrageenans, and tetrasporophytes synthesize λ type carrageenans.[7–12] Carrageenans are sulfated galactans of alternating 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranose or 4-linked 3,6-anhydrogalactopyranose.[13] The aim of this work was the structural determination of the soluble polysaccharide from tetrasporic plants of the green variant of *G. skottsbergii*. Also, the relevance of the antioxidant activity in vitro of the polysaccharide toward oxygen-centered radicals (peroxyl and hydroxyl), chelating activity, and the $ABTS^{\cdot+}$ assay based on electron transfer mechanism was studied. These results were compared with those obtained for the commercial λ-carrageen, which is widely used as an ingredient in the food industry.

2. Results and discussion

Extraction of tetrasporic plants of the green variant of *G. skottsbergii* afforded 50 dry weight percent of a polysaccharide, hereinafter named as GVT polysaccharide, that presents 64% of reducing sugars, 25.9% of $-SO_3$ groups and 2.5% of protein. It showed a molecular weight of $1 \times 10^6 \pm 1.2 \times 10^5$ determined spectrophotometrically by the reducing end method. Its IR spectrum presented bands at 1254 cm^{-1}, attributed to the S=O vibration of the sulfate groups, and a broad band in the region of 850–800 cm^{-1}. The second-derivative spectrum showed bands at 942 and 896 cm^{-1} indicative of the presence of α- and β-linked galactopyranosyl residues, respectively; and bands at 830 cm^{-1} assigned to absorption of equatorial secondary 2-sulfate group and at 819 cm^{-1}, due to 6-sulfate group on galactopyranosyl residues.[14] Reductive hydrolysis of the GVT polysaccharide followed by acetylation and GC analysis only indicated the presence of hexa-*O*-acetyl-galactitol. 3,6-Anhydrogalactitol acetate was not

* Corresponding author. Tel.: +56 2 7181159; fax: +56 2 681 2108.
E-mail address: betty.matsuhiro@usach.cl (B. Matsuhiro).

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detected, which is in agreement with the absence in the IR spectrum of the polysaccharide of a band at 930 cm^{-1} due to 3,6-anhydrogalactosyl residues.[14] Configuration of galactose was determined by GC analysis of diastereoisomeric amines prepared by reductive amination and acetylation of the total hydrolysis product of GVT polysaccharide. Comparison of the single signal with standards compounds derived from D- and L-galactose indicated that the polysaccharide is only composed of D-galactose. Taken together, these results indicate the presence of a sulfated galactan of carrageenan family with a galactose/sulfate molar ratio of 1:0.65.

The GVT polysaccharide was submitted to partial acid hydrolysis due to its high molecular weight for the analysis by NMR spectroscopy. The ^1H NMR spectrum in D_2O of the partially hydrolyzed polysaccharide (figure not shown) presented in the α-anomeric region, four signals that probably are due to sulfated and unsulfated α-D-galactopyranosyl residues. Assignments of these signals were possible using ^{13}C NMR spectrum and 2D homonuclear (COSY and NOESY) and heteronuclear (HSQC and HMBC) experiments, and literature data (Table 1).[15,16] Noteworthy is the presence of signals ascribed to 3-*O*-methyl-α-D-galactopyranose. Signals at 4.66 and 4.61 ppm in the ^1H NMR spectrum may be attributed to the anomeric protons of β-D-galactopyranosyl residues, although the latter is a very small signal and could not be properly assigned. Better results were obtained in the NMR spectra of the desulfated polysaccharide. 2D NMR spectra allowed the complete assignments of major ^1H NMR signals assigned to alternating →3)-β-D-galactopyranosyl- and →4)-α-D-galactopyranosyl units. The ^{13}C NMR spectrum (figure not shown) presented 12 major signals corresponding to desulfated λ carrageenan which are in very good agreement with those reported by Van de Velde et al.[16] Smaller signals were also visible in the ^{13}C NMR spectrum, which were assigned with the aid of 2D NMR spectra and literature data to 3-*O*-methyl-α-D-galactopyranosyl residue.[17–19] Figure 1 illustrates the $^1H/^{13}C$ connectivities in the HSQC spectrum and Table 1 shows the assignments of the signals.

Confirmation of the presence, in low proportion of a residue corresponding to 3-*O*-methyl-galactose in the polysaccharide was obtained by ethylation analysis of the native polysaccharide. The polysaccharide was submitted to three rounds of ethylation, followed by total hydrolysis and the resulting ethylated derivatives were analyzed by GC–MS as alditol acetates (Table 2). 3-*O*-Methyl-2,6-di-*O*-ethyl-galactitol acetate (4.4%) was identified; furthermore, ethylation analysis gave information about glycosidic linkages and sulfate positions on the native polysaccharide. Galactose was found to be involved in (1→3) and (1→4) linkages as expected for carrageenan-type polysaccharides. However, only 9.6% of ethylated alditol showed sulfation at position *O*-6; for →4)-D-galactopyranosyl residues in λ carrageenan total sulfation was expected at this position. The presence of 3,6-di-*O*-ethyl-galactitol acetate indicates sulfation at position *O*-2 in →4)-linked galactopyranosyl residues. It can be deduced that 40.4% of 2,4,6-tri-*O*-ethyl-galactitol acetate comes from 3-linked D-galactopyranosyl residues. About 3.8% of the latter residue is sulfated at position *O*-2. Therefore, unlike the normal tetrasporic plants of *G. skottsbergii*, the green variant produces a sulfated D-galactan with a backbone similar to λ carrageenan but with much lower sulfation at position *O*-6 of the α-residue and at position *O*-2 of the β-residue. A simple relation of the structure of the polysaccharide with the pigmentation could not be proposed; it can be mentioned that the green variant of cystocarpic *G. skottsbergii* mainly produced κ carrageenan,[20] the expected polysaccharide for carrageenophytes in the cystocarpic phase.

The peroxyl radical ($ROO^{\cdot}$) scavenging capacity toward GVT polysaccharide was measured by the ORAC method using fluorescein and C-phycocyanin as fluorescent probes.[21,22] Figure 2 shows the decrease of the fluorescein (Fl) emission mediated by peroxyl radicals in the absence (I_F^o) and presence (I_F) of increased GVT polysaccharide concentrations, expressed as mg/mL. These data show that in the absence of the polysaccharide the fluorescence probe decreases with negligible inhibition time, and the polysaccharide addition decreases the probe consumption rate. Kinetic profiles with C-phycocyanin as fluorescent probe were similar. In both cases, the area under the curve of the kinetic plots was linearly related to the amount of polysaccharide addition (Fig. 2 insert). The ORAC values, taking ascorbic acid as reference, were $3.1 \pm 0.4 \times 10^{-3}$ and $0.72 \pm 0.05 \times 10^{-3}$ for fluorescein and C-phycocyanin, respectively, and they are lower than that measured for the commercial λ-carrageenan ($5.6 \pm 0.3 \times 10^{-3}$ and $1.1 \pm 0.1 \times 10^{-3}$). In both cases, the ORAC value is five times higher for fluorescein than C-phycocyanin. The lower reactivity of the protein toward peroxyl radicals can be explained considering that the first step of the reaction of $ROO^{\cdot}$ with the probe is the abstraction of a hydrogen atom.[23] The complex structure of the protein[24] retards the access of peroxyl radicals toward the reactive center in the protein. On the other hand, ORAC values are higher for λ carrageenan. A sulfate content of 33–38% has been reported for this polysaccharide,[25,26] which is quite higher than that found for GVT polysaccharide (25.9%). Moreover, considering that the hydrogen abstraction occurs from the anomeric hydrogen atom, the presence of the sulfate group on C-2 should increase the hydrogen abstraction rate. Indeed, λ carrageenan contains sulfate groups on C-2 of α- and β-D-galactopyranosyl residues, whereas GVT polysaccharide is mainly sulfated at the C-2 position of α-D-galactopyranosyl residue. These facts suggest that the number as well as the position of sulfate groups play a role in the antioxidant activity of these polysaccharides toward the peroxyl radicals.

The scavenging activity toward hydroxyl radicals produced by the Fenton reaction has been assessed by the competitive reaction of these radicals with Brilliant Green (BG). Kinetics of the reaction of GVT polysaccharide with hydroxyl radicals showed complex behavior. Figure 3 shows that the BG absorbance decay becomes

Table 1
Assignments of chemical shifts (ppm) in the ^1H and ^{13}C NMR spectra of partially hydrolyzed and desulfated GVT polysaccharides

GVT polysaccharide[a]	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	*O*-CH_3
→-4-α-D-Galp-2*S*→	5.45/94.66	4.52/75.90	4.11				
→-4-α-D-Galp-3*M*→	5.36/100.13	3.63					
→-4-α-D-Galp-6*S*→	5.30/95.81	3.95				4.32/68.09	
→-4-α-D-Galp→	5.19/95.98	3.98					
→-3-β-D-Galp→	4.66/104.73	3.74				3.77/61.42	
Desulfated GVT							
→-3-β-D-Galp-1→	4.64/104.80	3.75/70.58	4.26/78.97	4.14/65.66	3.66/75.36	3.80/61.21	
→-4-α-D-Galp-1→	5.15/96.23	3.94/69.28	4.25/70.91	3.75/78.51	4.02/70.41	3.83/61.45	
→-4-α-D-Galp-3*M*→	5.35/100.10	3.75/71.85	3.62/77.86	3.62/72.12	3.96/73.76	3.61-3.52/63.45	3.73/55.85

[a] M: $-CH_3$; S: $-SO_3^-$.

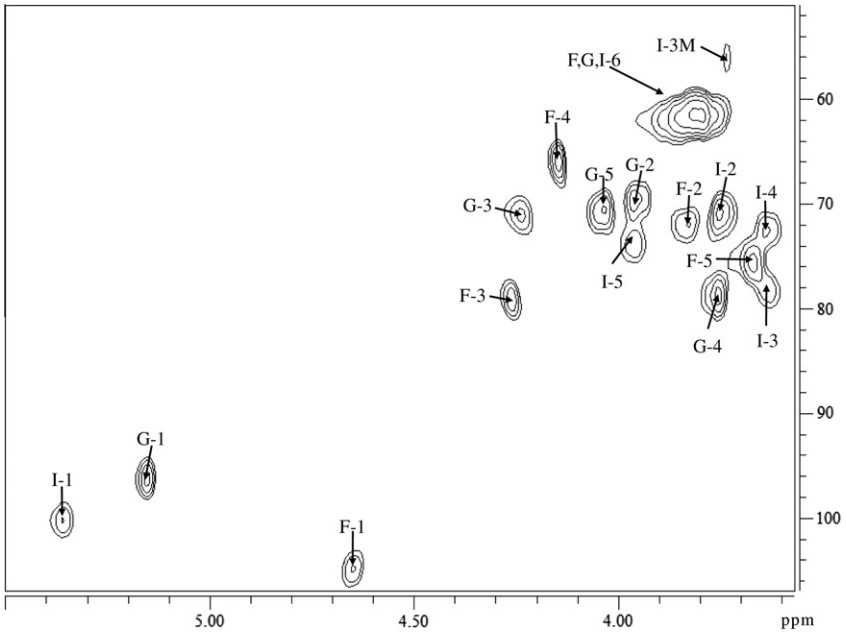

Figure 1. ^{13}C–^{1}H HSQC NMR spectrum in D_2O of desulfated polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii.* F-1 means C-1 and H-1 of unit F.

Table 2
Linkage analysis of the constituent sugars of GVT polysaccharide

Alditol acetate	Deduced unit and substitution pattern	Polysaccharide[a] Ethylation
2,4,6-Tri-*O*-ethyl-Gal	→3-Gal	40.4
2,3,6-Tri-*O*-ethyl-Gal	→4-Gal	18.3
4,6-Di-*O*-ethyl-Gal	→3-Gal 2 S	3.8
3,6-Di-*O*-ethyl-Gal	→4-Gal 2 S	23.5
2,3-Di-*O*-ethyl-Gal	→4-Gal 6 S	9.6
3-*O*-Ethyl-Gal	→4-Gal 2,6 S	–
3-*O*-Methyl-2,6-Di-*O*-ethyl-Gal	→4-Gal 3 M[b]	4.4

S: sulfate.
[a] Normalised mol % of monosaccharide having methyl or ethyl groups at the positions indicated.
[b] According to GC–MS analysis.

faster in the presence of the polysaccharide. This behavior is not expected from the inhibition of the probe reaction by the polysaccharide, and indicates the generation of free radicals in the reaction of GVT polysaccharide with hydroxyl radicals, followed by a slower reaction of these formed reactive species with the probe. According to these reaction pathways, the absorption spectrum monitored during the reaction showed a fast consumption of the dye and the presence of a strong absorption at wavelengths lower than 420 nm, indicating a new product formation (Fig. 3 insert). A decrease of the probe consumption with increasing substrate concentration also has been reported for some polysaccharides.[26–28] Commercial λ-carrageenan also exhibited a descending curve, suggesting that free radical generation and not inhibition occurs.[20] On the other hand, the absorbance decay of the BG in the presence of polysaccharide is well fitted to an exponential decay, indicating that the reaction follows pseudo-first order kinetics. Interestingly, the apparent rate constant (k_{app}) increases linearly with increasing the polysaccharide concentration, giving rate constant values of 0.28 ± 0.05 and 0.73 ± 0.03 $(mg/mL)^{-1}$ min^{-1} for commercial λ-carrageenan and GVT polysaccharide, respectively. The slower reactivity of λ-carrageenan does not agree with the higher sulfated content at C-2 of β-D-galactopyranosyl residues. Recently reported works on the scavenging activity by hydroxyl radicals, measured at fixed end point, for polysaccharides from marine algae show that the antioxidant capacity increases with the sulfate content.[28–30] The faster reactivity of hydroxyl radicals with GVT polysaccharide with respect to that of commercial λ-carrageenan suggests that other parameters also contribute to the reactivity of the complex reaction of polysaccharides with hydroxyl radicals. Kinetic studies have confirmed that the reaction of $HO^{\cdot}$ radicals with polysaccharides is close to the diffusion control, considering this high reactivity, it can be expected to have low selectivity.[31] Thus, the reaction of $HO^{\cdot}$ radicals can occur with different groups on the polysaccharide. All of these facts indicate that hydroxyl radical scavenging determined at a fixed end point is not strictly related to results obtained from kinetic data. Furthermore, it has been proposed that $HO^{\cdot}$ radicals scavenger capacity can occur by two mechanisms, the suppression against hydroxyl radical generation and the chemical reaction of radicals with the substrate. In the former, one of the most important processes is the inhibition of $HO^{\cdot}$ radical generation by chelation of the metal ion with the macromolecule.

Figure 4 shows the absorbance of the ferrozine–Fe^{2+} complex[32] in the absence and presence of GVT polysaccharide. These data show that the polysaccharide addition inhibits the formation of the ferrozine–Fe^{2+} chelating complex. In the absence of polysaccharide the complex is formed in a few minutes and then, its absorbance remains constant. In the presence of polysaccharide, the complex formation is slower and reaches an almost constant absorbance value at longer times. The rate of the ferrozine–Fe^{2+} complex formation decreases with increasing polysaccharide concentration, but at longer times, the absorbance is similar to that in the absence of the polysaccharide. The same behavior was found for commercial λ-carrageenan. These findings suggest that the ferrozine–Fe^{2+} complex is formed but at slower rate. A plausible explanation is the electrostatic interactions between the Fe^{2+} and the negatively charged sulfate groups in the polysaccharide. This can contribute, at least in part, to the antioxidant capacity of GVT polysaccharide and commercial λ-carrageenan in the assay with hydroxyl radicals.

Additionally, the antioxidant capacity of sulfated polysaccharides was measured using the $ABTS^{\cdot+}$ assay based on electron transfer mechanism, with the cation radical as oxidant.[33] Changes of the

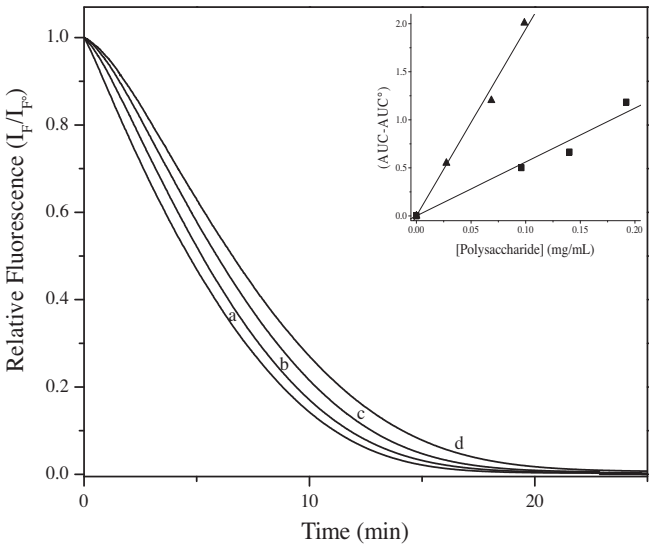

Figure 2. Fluorescence decay of fluorescein probe in the presence of AAPH at different concentrations of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii* at 37 °C, and pH 7.0. (a) In the absence and in the presence of (b) 0.03 mg/mL, (c) 0.07 mg/mL, and (d) 0.10 mg/mL of polysaccharide. Insert shows the dependence of the net area under the curve versus concentration for native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii* with fluorescein probe (▲), and C-phycocyanin probe. (■).

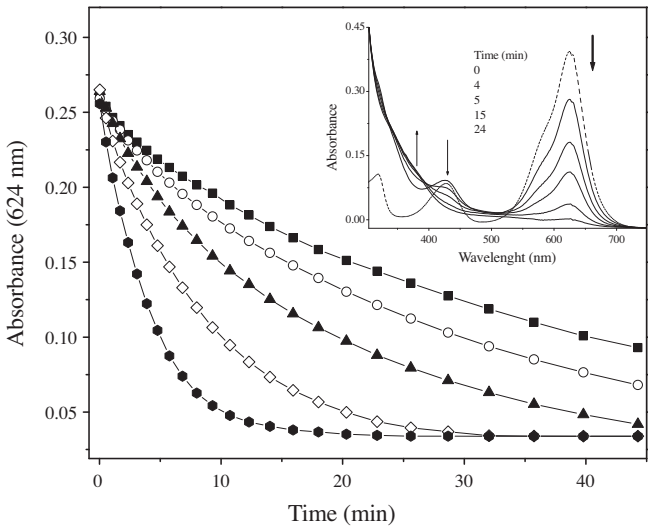

Figure 3. Decrease of Brilliant Green absorbance at 624 nm in the presence of different concentrations of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii*. In the absence of polysaccharide (■) and in the presence of 0.06 mg/mL (○), 0.07 mg/mL (▲), 0.12 mg/mL·(◇), and 0.24 mg/mL (●). Insert shows the absorption spectra of Brilliant Green in the absence of H_2O_2 (- -), and in the presence of H_2O_2 and 0.24 mg/mL of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii* recorded at different reaction times (from 0 to 24 min).

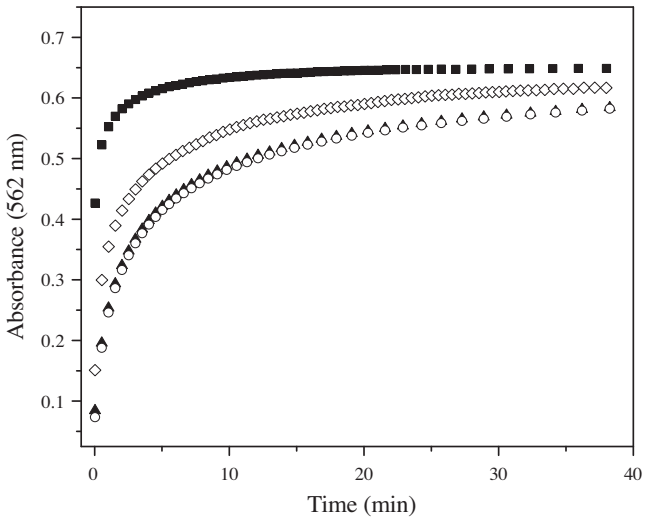

Figure 4. Ferrozine complex formation in absence of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii* (■) and in the presence of 0.09 mg/mL (◇), and 0.55 mg/mL (▲); in the presence of 0.55 mg/mL of commercial λ-carrageenan (○).

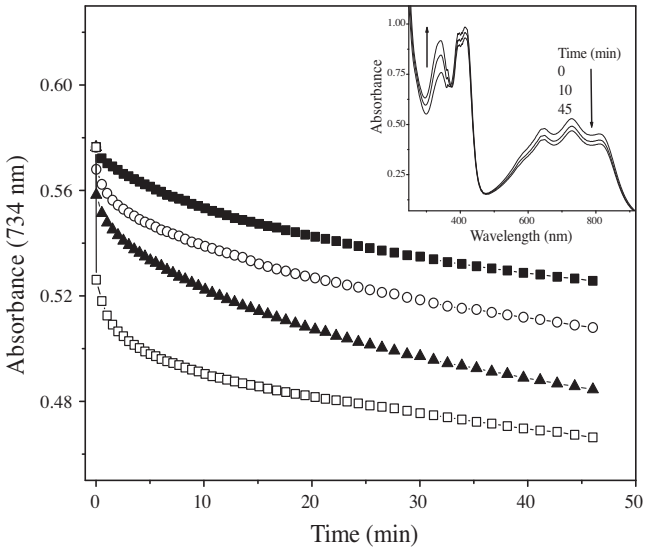

Figure 5. Decrease of ABTS radical cation absorbance at 734 nm in the presence of different concentrations of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii*. In the presence of 0.47 mg/mL (■), 0.97 mg/mL (○), 1.41 mg/mL (▲), and 1.90 mg/mL (□). Insert shows the absorption spectra of ABTS radical cation in the presence of 1.90 mg/mL of native polysaccharide from tetrasporic plants of the green variant of *Gigartina skottsbergii*, recorded at different reaction times (from 0 to 45 min).

$ABTS^{\cdot+}$ absorbance radical cation with GVT polysaccharide showed fast initial kinetics followed by a slow reaction (Fig. 5). The extension of the fast process was very small; it was completed within five minutes. Previously, the same behavior was found for commercial λ-carrageenan.[20] The $ABTS^{\cdot+}$ absorbance decay could be well fitted to biexponential kinetics with lifetimes in the range of 0.4–1.8 min and 20–31 min, depending on the polysaccharide concentration. This biphasic kinetics suggests a very fast reaction of polysaccharide with the cation radical to form a product that is followed by a slow reaction to form other products. Recently, this behavior also has been reported for some phenolic compounds,[34,35] vitamin B derivatives,[36] and thiol derivatives[37] and it has been related to the formation of side products. Further information on the side products was obtained from the absorption spectra of the $ABTS^{\cdot+}$ monitored at different reaction times (Fig. 5, insert). The absorption bands with maximum at 734 nm and 410 nm decrease and the band at 340 nm increases. Probably, the absorption at 340 nm corresponds to the second reaction since its enhancement is maintained at long reaction times. These findings indicate that to obtain comparative values of antioxidant capacity of a series of compounds it is necessary to know the kinetic pattern involved in the reaction with $ABTS^{\cdot+}$. Reported data for some marine algae indicate a higher reactivity for sulfated species.[28,38,39] A comparison of results obtained for the

$ABTS^{\cdot+}$ bleaching by GVT polysaccharide and commercial λ-carrageenan shows that, even though the activity is quite moderate, is it slightly higher than that of the GVT polysaccharide.

3. Conclusions

The polysaccharide synthesized by individuals of the green variant of tetrasporic *G. skottsbergii* was different from λ carrageenan, this polysaccharide shows a novel pattern of sulfation and methylation units, which is unusual in sulfated galactans from carrageenophytes. It can be concluded that the ORAC assay is an adequate method to measure the antioxidant capacity of aqueous soluble polysaccharides. However, this assay measures the antioxidant capacity toward peroxyl radicals but not the total antioxidant capacity. It was found in the ORAC assay that antioxidant capacity was dependent on the target molecule employed and on the content of sulfate groups and their position. λ Carrageenan had higher ORAC value and sulfate group content next to glycosidic bond, than the native polysaccharide. Kinetic studies indicated that the antioxidant capacity with $ABTS^{+\cdot}$ and $OH^{\cdot}$ radicals was contrary to the content of sulfate groups, because in both cases the native polysaccharide was the most active. The behavior of the native polysaccharide and commercial λ-carrageenan was influenced by the radical used to measure the antioxidant capacity.

4. Experimental

4.1. Material and general procedures

Tetrasporic plants of the green variant of *G. skottsbergii* (Gigartinales, Rhodophyta) were collected in spring in Fuerte Bulnes (53° 37′ S, 70° 55′ W), Southern Chile. Specimens were deposited in Herbario de Criptógamos, Universidad de Magallanes, Punta Arenas, Chile. ^{1}H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra of the polysaccharides were recorded in D_2O, after isotopic exchange (3 × 0.75 mL) at 70 °C on a Bruker Avance DRX 400 spectrometer using the sodium salt of 3-(trimethylsilyl)-1-propane-d_4-sulfonic acid) as the internal reference. All two-dimensional experiments were acquired using a pulse field gradient incorporated into NMR pulse sequence. The two-dimensional homonuclear ^{1}H/^{1}H COSY experiment was acquired with 128 × 2048 data points having a spectral width of 1200 Hz and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in the two-dimensions. The two-dimensional heteronuclear single quantum coherence correlation (HSQC) spectra were acquired with 128 × 1024 data point and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in ^{1}H and close to 2.4 Hz/point in ^{13}C. The number of scans in each experiment was dependent on the sample concentration. FT-IR spectra in KBr pellets were registered in the 4000–400 cm^{-1} region using a Bruker IFS 66v instrument according to Leal et al.[40] Absorbance was measured in a Genesys 5 Thermospectronic spectrophotometer.

Gas–liquid chromatography (GC) was carried out on a Shimadzu GC-14B chromatograph equipped with a flame ionization detector using a SP 2330 column (0.25 mm × 30 m) and performed with an initial 5 min hold at 150 °C and then at 5 °C min^{-1} to 210 °C for 10 min. The helium flow was 20 mL min^{-1}. GC–MS analysis was performed with an Agilent Technologies 7890A GC fitted with an Agilent 19091S-433 column (30 m × 250 um × 0.25 um) interfaced to a Agilent Technologies 5975C INERT XL M8D mass spectrometer. Conversion of GC areas to molar basis was calculated for the partially ethylated alditol acetates according to the effective carbon response theory of Sweet et al.[41] Unless otherwise stated all the reagents and chemicals were from Merck (Darmstadt, Germany).

4.2. Extraction

The dried algal material (25 g) was extracted with distilled H_2O (1.25 L) at 90 °C during 3 h with stirring and centrifuged (4000×g). The solid was extracted with 1 L of distilled water, and the extraction process was repeated once. The aqueous layers were dialyzed against distilled water using Spectra Por (MWCO 3500) membrane, water was exchanged four times. The retentate was concentrated in vacuo, poured over 1 L of ethyl alcohol, and the precipitate was separated by centrifugation at 8500×g, dissolved in distilled water and freeze-dried, affording 12.5 g of white cotton-like solid.

4.3. Chemical analysis

Reducing sugars were determined by the phenol–sulfuric acid method using D-galactose as standard.[42] Molecular weight was determined by the reducing end method as previously described.[43] Sulfate content was determined by a modification of Dodgson and Price method;[44] a conditioning solution containing glycerine instead of gelatine was used (6 g NaCl, 20 mL H_2O, 0.5 mL HCl, and 25 mL glycerine). The configuration of galactose was determined through the formation of diastereomeric derivatives with (*S*)-1-amino-2-propanol and GC analysis of the corresponding 1-deoxy-1-1-(2-hydroxy-propylamino) alditol acetates on a Ultra 2 column according to Cases et al.[45] The corresponding derivatives of D- and of L-galactose were used as standards. Nitrogen was determined by microanalysis in Facultad de Química, Pontificia Universidad Católica de Chile, and the protein content was calculated by multiplying the percentage of nitrogen by the 6.25 factor.[46]

4.4. Reductive hydrolysis

The native polysaccharide (5 mg) was heated with 0.1 M trifluoroacetic acid (1 mL) for 3 h at 80 °C. After cooling the trifluoroacetic acid (TFA) was evaporated and the residue was washed with distilled water. One milliliter of aqueous $NaBH_4$ (10 mg/mL) was added and the solution was stirred for 1 h at room temperature; then, the reaction was stopped by addition of two drops of acetic acid, and the resulting syrup was washed with methanol. The solid was suspended in 2 M TFA (1 mL) and the reaction was kept for 2 h at 120 °C. The excess acid was removed by co-distillation with water, and the reduction and washing with methanol was repeated. The alditols were acetylated in Ac_2O–pyridine and analyzed by GLC.

4.5. Partial hydrolysis

The native polysaccharide (100 mg) was stirred in concentrated HCl (1 mL) and concentrated sulfuric acid (0.1 mL) at 25 °C. After 15 min, the solution was poured into acetone, and the precipitate was washed thrice with portions of acetone (2 mL) and dissolved in distilled H_2O. The resulting solution was dialyzed against 0.1 M sodium acetate followed by distilled H_2O and freeze-dried.

4.6. Desulfation

The native polysaccharide was desulfated according to Falshaw and Furneaux.[47] Briefly, the polysaccharide (50 mg) was transformed to the pyridinium salt form by dialysis against 0.1 M pyridinium hydrochloride, then it was freeze-dried. The solid was dissolved in 89:10:1 v/v Me_2SO–MeOH–pyridine (0.4 mL/mg), and heated at 100 °C for 4 h. The mixture was dialyzed against distilled water for 48 h and freeze-dried.

4.7. Ethylation analysis

Ethylation analysis was conducted according to Ciucanu and Kerek.[48] Briefly, the native polysaccharide (50 mg) in dimethylsulfoxide (5 mL) was stirred with finely powdered NaOH (400 mg) for 2 h at rt, then CH_3CH_2 I (5 mL) was added and the mixture was stirred for 1 h. The addition was repeated twice. The reaction was stopped by addition of 2 mL of water, dialyzed against distilled water and freeze-dried. The solid obtained was submitted twice to the same ethylation procedure. The ethylated polysaccharide was hydrolyzed with 2.0 M TFA for 2 h at 120 °C, and the partially ethylated monosaccharides were reduced with $NaBH_4$, and acetylated with Ac_2O–pyridine. The partially ethylated alditol acetates were analyzed by GC and GC–MS.

4.8. Determination of antioxidant capacity in vitro of sulfated polysaccharides

4.8.1. Oxygen radical absorbance capacity (ORAC) assay

The consumption of fluorescein or C-phycocyanin associated with its incubation with AAPH [2,2′-azo-bis(2-amidinopropane) dihydrochloride] was estimated from fluorescence measurements.[49] A reaction mixture containing 10 mM AAPH with and without the native polysaccharide or commercial λ-carrageenan with different concentrations (0.001–1.0 mg/mL) 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 in the sample fluorescence intensity (excitation 491 nm, emission 512 nm). C-phycocyanin (300 μM) consumption was evaluated based on the decrease in the sample fluorescence intensity (excitation 610 nm, emission 645 nm). Fluorescence measurements were conducted using a Fluorolog-Spex 1681/0.22 m spectrofluorimeter (Spex, Metuchen, NJ, USA). Bandwidths of 1.25 nm were used for excitation and emission slits. Values of the intensity of fluorescence F in relation to initial value $F°$ ($I_F/I_{F°}$) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that ($I_F/I_{F°}$) reached a value close to zero. ORAC values were obtained from the slopes of concentration versus AUC curves of the polysaccharide and ascorbic acid.

4.8.2. Hydroxyl radical scavenging activity assay (HRS)

Hydroxyl radicals were generated by the Fenton reaction at 20 °C.[20] The absorbance at 624 nm of aqueous solutions of 0.435 mM Brilliant Green (BG) (Carlo Erba, Milano, Italy), 0.25 mM solution of $FeSO_4$, and varying concentrations of the native polysaccharide and commercial λ-carrageenan (0–0.24 mg/mL) were measured as time function immediately after the addition of H_2O_2. Absorption spectra were measured using an HP8453 diode array spectrophotometer (Hewlett Packard, Waldbronn, Germany).

4.8.3. Ferrous ion chelating ability

The method reported by Decker and Welch was used to investigate the ferrous ion chelating ability.[50] The native polysaccharide and commercial λ-carrageenan (0.54 mg/mL) were mixed with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine solutions. The absorbance was measured at 562 nm where the complex of Fe_2–ferrozine showed strong absorbance.

4.8.4. Antioxidant capacity (ABTS)

The ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) aqueous solution with 2.45 mM potassium persulfate at room temperature for 16 h.[51] The $ABTS^{\cdot+}$ solution was diluted with PBS, pH 7.0 to an absorbance of 0.70 at 734 nm. To 20–500 μL solution of native polysaccharide and commercial λ-carrageenan (0.0075–0.377 mg/mL), 500 μL of $ABTS^{\cdot+}$ was added. Absorbance was registered in order to measure the kinetics of the reaction.

4.9. Statistical analysis

The data obtained were means ± S.D. of three determinations, and followed by the Student's t-test. Differences were considered to be statistically significant if $P < 0.05$. All assays were performed in triplicate and repeated at least three times on different days.

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