Agar from *Gracilaria gracilis* (Gracilariales, Rhodophyta) of the Patagonic coast of Argentina – Content, structure and physical properties

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ABSTRACT

Milled summer thalli of *Gracilaria gracilis* from Argentina were sequentially extracted with water at room temperature (RTW1-3), 70 °C (W701-3) and 90 °C (W901-2). Both W701 and W901 consisted of high molecular weight polysaccharides (ca. 540,000 Da), but polydispersity was higher for the major product W701 (yield, 72% of the recovered). Structural analyzes by methylation and ^{13}C NMR spectroscopy revealed that W701 was mainly agarose. Alkaline treatment, together with structural analyzes, indicated a negligible proportion of precursor L-galactose 6-sulfate residues in this product, while they were clearly detected in the ^{13}C NMR spectra of RTW2-3. The presence of floridean starch in W901 had an antagonistic effect on its gel strength, which resulted nearly three times lower than that of fraction W701.

Ultrastructural observation by transmission electron microscopy showed that, after extraction with hot water, a partial loss of cell wall stratification and disorganization of the cuticle had occurred. Final cellular debris exhibited swelling in the microfibrillar component.

After this first thorough study of the chemical composition and physical properties of the products of *G. gracilis* from Bahía Bustamante we conclude that a good quality agarose is obtained in high yield after extraction with water at 70 °C without the requirement of alkaline pretreatment, which usually produces degradation of the polysaccharide.

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1. Introduction

The major non-fibrillar polysaccharides extracted from marine red macroalgae are sulfated galactans essentially consisting of linear chains of alternating 3-linked β-D-galactopyranosyl and 4-linked α-L-galactopyranosyl units. The 3-linked unit always belongs to the D-series, whereas the 4-linked unit may have the D or the L configuration, often occurring as a 3,6-anhydrogalactopyranosyl moiety. Thus, these galactans are classified either as carrageenans if the 4-linked residue is in the D configuration or as agars if the 4-linked residue is in the L configuration. According to Knutsen et al. (1994), the structure of alternating 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose is termed agaran, but when all the 4-linked residues are in the 3,6-anhydro form, the repeating backbone is called agarose (Fig. 1). Additionally, agarans may have variable amounts of different O-linked groups, particularly methyl ether, sulfate ester, pyruvate acetal or β-D-xylopyranosyl residues.

The proportion and distribution of the aforementioned substituents in the skeletal chain of the galactan modify the physico-chemical properties of the polysaccharide (Yaphe and Duckworth, 1972; Lahaye and Rochas, 1991). Of particular interest are the viscosity and the gelling capacity, which confer these hydrocolloids multiple industrial applications as thickeners, stabilizers and gelling agents (Usov, 1992).

Though the best quality agar is extracted from the genus *Gelidium*, its high cost and the gradual exhaustion of natural prairies led in search of new natural sources for this polysaccharide. *Gracilaria* (Gracilariales, Rhodophyta), a cosmopolitan genus, became an excellent substitute for *Gelidium* agar in food industry, especially after discovering that low gel strength could be notably improved by alkaline treatment with sodium hydroxide which converts L-galactose 6-sulfate into 3,6-anhydro-L-galactose (Armisen, 1995; Noseda et al., 2000; Marinho-Soriano, 2001; Melo et al., 2002; Freile-Pelegrin and Murano, 2005; Marinho-Soriano and Bourret, 2005).

Composition and properties of *Gracilaria* agars depend on, among others, the species, physiological factors, life cycle stage,

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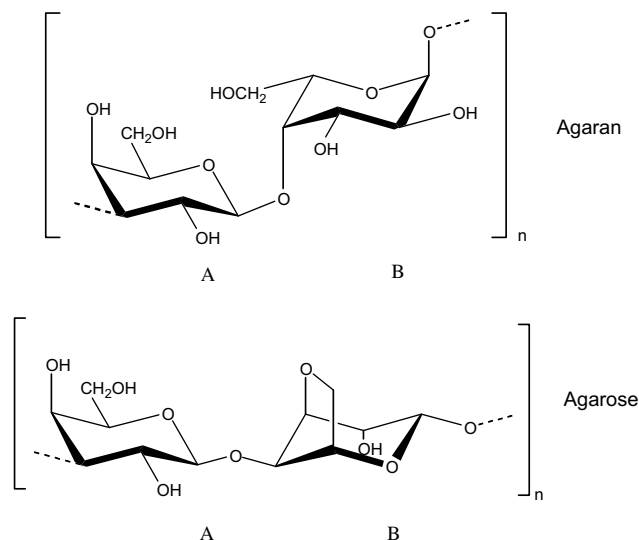*E-mail address:* cristina@qo.fcen.uba.ar (M.C. Matulewicz).

Fig. 1. Repeating unit of an agaran and agarose. 3-Linked and 4-linked units in the diads are indicated by A and B, respectively.

environmental conditions, including seasonal and geographical aspects, extraction procedures and postharvest storage (Marinho-Soriano and Bourret, 2003; Marinho-Soriano et al., 2006; Romero et al., 2008).

The characterization of agars from different origins is then crucial if they are destined for food additives fitting international norms such as those of the FAO or of the World Health Organization (Armisen, 1995).

Gracilaria gracilis (Stackhouse) Steentoft, Irvine and Farnham prairies from Bahía Bustamante (Chubut Province, Argentina) are being exploited for industrial agar, but its polysaccharides have not been thoroughly studied.

This paper describes the characterization of the polysaccharides isolated from summer specimens by sequential extraction with water at room temperature, 70 °C and 90 °C; and the physical properties and gelling capacity of the main products obtained at 70 °C and 90 °C. In addition, changes in the cell walls after extraction steps were monitored by transmission electron microscopy (TEM).

2. Methods

2.1. Materials

G. gracilis was collected from the subtidal in Bahía Bustamante (45°08′S, 66°32′W) (Chubut Province, Argentina) in summer (February 2005). At harvest time, water temperature was 16.7 °C, pH 8.27 and salinity 32.9‰. Algae were dried in the open and carefully hand sorted. A voucher specimen was deposited at BBB herbarium.

2.2. Extractions

Thalli (20 g), previously milled, were extracted with mechanical stirring in: (a) water (2 l) at room temperature (×3) for 24 h (extracts RTW1-3); (b) water (1 l) at 70 °C (×3) for 4 h (fractions W701-3) and (c) water (1 l) at 90 °C (×2) for 4 h (fractions W901-2). After each extraction step, the residue was removed by centrifugation and the supernatant was concentrated and freeze-dried. Extracts obtained at room temperature were thoroughly dialyzed using Spectra/Por tubing (Spectrum Laboratories Inc., USA) with a molecular weight cutoff of 3500 Da before freeze-drying.

2.3. General methods

Carbohydrate content was analyzed by the phenol–H_2SO_4 method (Dubois et al., 1956) without previous hydrolysis of the polysaccharide, using galactose as standard. Sulfate was measured with the turbidimetric method of Dodgson and Price (1962) after hydrolysis of the samples with 1 M HCl for 4–5 h at 105–110 °C. The content of protein was estimated by the method of Lowry et al. (1951). Pyruvic acid was determined by the method of Koepsell and Sharpe (1952).

Reductive hydrolysis of the extracts and the permethylated sample, and further acetylation of the sugar mixtures were performed as described by Stevenson and Furneaux (1991).

GLC of alditol acetates was carried out on a Hewlett–Packard 5890A gas chromatograph (Hewlett Packard, USA) equipped with a flame-ionization detector and fitted with a fused-silica column (0.25 mm id × 30 m) WCOT-coated with 0.20 μm film of SP-2330 (Supelco, USA). Chromatography was carried out at: (a) 220 °C isothermally for alditol acetates and (b) from 180 °C (2-min hold) to 230 °C at 1 °C min^{-1}, followed by a 30-min hold, for partially methylated alditol acetates. Nitrogen was used as carrier at a flow rate of 1 ml min^{-1}. The split ratio was 80:1. The injector and detector temperature was 240 °C.

Conversion of GLC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory (Sweet et al., 1975). For 1,2,4,5-tetra-O-acetyl-3,6-anhydrogalactitol and 1,4,5-tri-O-acetyl-3,6-anhydro-2-O-methylgalactitol values of 0.72 and 0.64, respectively, were used (Stevenson and Furneaux, 1991).

GLC-MS of the partially methylated alditol acetates was carried out on a GCMS-QP 5050A gas chromatograph/mass spectrometer (Shimadzu Corp., Japan) working at 70 eV. Chromatography was performed on the SP-2330 capillary column using the program temperature (b). The He total flow rate was 4.4 ml min^{-1}, the head pressure 12 psi; the injector temperature 250 °C and the split ratio 10:1. Mass spectra were recorded over a mass range of 30–600 Da.

Percentages of D- and L-galactose and the absolute configuration of 6-O-methyl-D-galactose were determined by hydrolysis of the samples with 2 M TFA at 121 °C for 2 h and further derivatization of the monosaccharides with (S)-1-amino-2-propanol to the diastereomeric 1-deoxy-1-(2-hydroxypropylamino)alditols. The acetylated derivatives were analyzed by GLC on an Ultra 2 column (Agilent Technologies, USA) under the previously described conditions (Cases et al., 1995). The configurations of 2,6-di-O-methylgalactose and 2-O-methylgalactose were determined using (S)-1-phenylethylamine (Cases et al., 1995; Errea et al., 2001).

Proton decoupled ^{13}C NMR spectra (100.61 MHz) were recorded on a Bruker Avance DRX400 spectrometer (Bruker, Germany) on samples in D_2O solution. Polysaccharide samples (20 mg ml^{-1}) were analyzed using a 5 mm inverse probe at 70 °C. Spectra were recorded using a 90° pulse (7.1 μs), a relaxation delay of 0.10 s, an acquisition time of 0.59 s, a spectral width of 31.0 kHz, 32 K data points, for 60,000–80,000 scans. ^{13}C NMR DEPT spectra were obtained at θ_z = 135° where CH and CH$_3$ signals appear in a positive phase with CH$_2$ in a negative phase. Chemical shifts (ppm) were measured relative to internal acetone (CH$_3$ at 31.1 ppm).

2.4. High performance size exclusion chromatography (HPSEC) at 70 °C

HPSEC analyzes were performed with a liquid chromatography system equipped with a Shimadzu refractive index detector (Model RID-10A), a Shimadzu LC-10AD pump, and a Shimadzu column oven (CTO-10A) at 70 °C (Shimadzu Corp., Japan). Polysaccharides were analyzed using four Waters Ultrahydrogel columns (2000, 500, 250 and 120; 300 × 7.8 mm) connected in series (Waters,

USA). Polysaccharide samples (1 mg ml^{-1}) were dissolved in ultra-pure water under magnetic stirring for 2 h and filtered through a 0.45 μm nitrocellulose membrane (GSWP, Millipore) (Millipore Corp., USA). Polysaccharide solutions (20 μl) were injected into the column using an injection manual valve and eluted with degassed ultra-pure water, containing NaN$_3$ (200 ppm) as preservative, at a flow rate of 0.6 ml min^{-1}.

2.5. HPSEC–multi-angle laser light scattering analysis (MALLS)

The determination of the homogeneity was performed on a Waters high-performance size exclusion chromatograph (Waters, USA) consisting of a Waters pump (Model 515) and multi-detection with a differential refractometer (Model 2410 RID) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering Detector (Wyatt Technologies Corp., USA). The four Waters Ultra-hydrogel columns (2000, 500, 250 and 120) were connected in series and 0.1 M NaNO$_2$ solution, containing NaN$_3$ (200 ppm) as preservative, was used as eluent at a flow rate of 0.6 ml min^{-1}. The samples (1 mg ml^{-1}) were dissolved in the same solvent under magnetic stirring for 2 h and filtered through a 0.45 μm and 0.22 μm nitrocellulose membrane (GSWP, Millipore). HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The light scattering signal was detected simultaneously at 11 scattering θ angles, ranging from 35° to 132°. These experiments were carried out at 25 °C.

2.6. Gel properties

Cylindrical specimens of the gels (1.8 cm diameter × 10 cm height) were prepared from agar solutions (1.5% w/v in water, 10 ml) and allowed to gel overnight at room temperature. The gel strength (g cm^{-2}) was measured with a Stable Microsystems TA-XT2i Texture Analyzer (Texture Technologies Corp., USA) using a cylindrical probe (P/36R 36 mm diameter). The elasticity index, resilience and adhesiveness were also determined.

The gelling and melting temperatures of W701 were determined according to Murano et al. (1992). The gelling temperature was measured by cooling a 1.5% (w/v) hot agar solution (10 ml) placed in a glass test tube (2.0 cm × 9 cm, 10 ml). The temperature drop was of about 0.5 °C min^{-1} and a glass rod was periodically introduced in the agar solution; the temperature at which a permanent hole was observed was considered as the gelling temperature.

The melting temperature was determined by heating the 1.5% (w/v) agar gel with a temperature increase of about 0.5 °C min^{-1} recording the temperature at which a glass bead (15.78 mm diameter, 4.93 g) placed on top of the gel sank.

2.7. Methylation analysis

The sample (30.5 mg) was dissolved in DMSO (2 ml) and methylation was carried out using NaOH–iodomethane (Ciucanu and Kerek, 1984). The methylated derivative was recovered by dialysis, with tubing of molecular weight cutoff of 3500 Da, and freeze-drying (yield, 12.2 mg).

2.8. Alkaline treatment

The one-pot alkaline treatment technique described by Navarro and Stortz (2003) was used. The polysaccharide (30 mg) was dissolved in water (15 ml) and NaBH$_4$ (5 mg) was added. After 1 h, 5 M NaOH (5 ml) was added and the resulting solution was heated at 80 °C in a water bath. Aliquots (0.8 ml) were taken at 30, 60, 120, 180, 240 and 300 min and the solutions were neutralized with 4 M CF$_3$CO$_2$H TFA (0.2 ml). The solvent was evaporated-off and the residue was derivatized to the acetylated alditols and analyzed by GLC as described previously (Stevenson and Furneaux, 1991).

2.9. TEM

For TEM observations, samples were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) containing 0.25 M sucrose. Fixation was followed by a series of rinses in 0.1 M Na-cacodylate buffer with a gradually decreased concentration of sucrose, post-fixation for 2 h in 2% OsO$_4$ in 0.1 M Na-cacodylate buffer, dehydration in acetone, and infiltration in Spurr's resin. Sections were stained with aqueous uranyl acetate followed by lead citrate and observed in a Jeol 100CX-II transmission electron microscope (Jeol Ltd., Japan) at the Centro de Investigaciones Básicas y Aplicadas (CRIBABB), Bahía Blanca, Argentina.

3. Results

3.1. Analysis of the native polysaccharide

The seaweed was sequentially extracted with water at room temperature, 70 °C and 90 °C. Table 1 depicts the yield and composition of the different extracts obtained at each temperature. Highest yield (ca. 27%) was found after the first extraction at 70 °C (W701). Sulfate analysis indicated considerable degree of sulfation in RTW1-3 and W702-3. RTW1 contained an important amount (20.4%) of ashes and low carbohydrate content (25.6%). The monosaccharide composition of all the extracts showed mainly the presence of galactose and 3,6-anhydrogalactose. Minor quantities (3–11%) of 6-O-methylgalactose were identified. In addition, small

Table 1
Yields and analyzes of the products obtained by extraction with water at room temperature, 70 °C and 90 °C

Product	Yield (%)	Sulfate (% SO$_4$)	Carbohydrate (%)	Gal:AnGal:6-Me Gal:Sulfate (molar ratio)	Protein (%)	Monosaccharide composition (mol%)						
						D-Gal	L-Gal	L-AnGal	6-Me Gal	3-Me Gal	Xyl	Glc
RTW1[a]	1.9	15.9	25.6	1.00:0.20:0.06:2.12	20.6	38	16	11	3	1	6	25
RTW2	1.3	13.7	40.0	1.00:0.28:0.05:1.10	18.3	39	18	20	3	1	6	13
RTW3	0.5	16.8	34.4	1.00:0.26:0.05:1.59	19.9	36	20	17	3	–	9	15
W701	26.7	6.8	68.1	1.00:0.95:0.16:0.41	5.7	40	4	42	7	tr[b]	tr	7
W702	3.4	11.8	68.6	1.00:0.81:0.24:0.85	13.5	31	6	30	9	3	1	20
W703	0.6	15.2	60.6	1.00:0.67:0.19:1.07	19.5	34	9	29	8	3	2	15
W901	1.8	4.9	75.1	1.00:1.06:0.32:0.35	26.5	28	6	36	11	3	tr	16
W902	1.1	4.6	69.6	1.00:1.09:0.34:0.33	29.8	28	4	35	11	3	3	16

[a] Ashes 20.4%.
[b] Percentages lower than 1% are considered as traces (tr).

amounts of xylose and 3-O-methyl-/4-O-methyl-galactose were also detected. No pyruvate was found.

The configurational analysis of monosaccharides revealed the mol% of D- and L-galactose shown in Table 1. Additionally, the totality of 6-O-methylgalactose appeared as the D-enantiomer, indicating that this sugar could only derive from the 3-linked A moiety of the repeating unit (Fig. 1). The absolute configuration of 3,6-anhydrogalactose was assigned as belonging to the L-series from the ^{13}C NMR spectra (see below).

Significant amounts of glucose were also detected in all the extracts except in W701 and according to the ^{13}C NMR spectra it derived from floridean starch (see below).

3.2. Physical properties

SEC of the gelling extracts W701 and W901 gave remarkably high weight-average molecular weights (M_w), with values of 541,800 and 546,800 for the first and second extract, respectively. The polydispersity index (PI = M_w/M_n, M_n, being the number-average molecular weight) reflected the broadness of the molecular weight distribution curve for W701 (2.8) but a considerably narrower distribution for W901 (1.7).

W701 had higher gel strength (437 g cm^{-2}) than W901 (108 g cm^{-2}). Accordingly, resilience was higher for W701 (0.503) than for W901 (0.358) even when the elasticity indexes were similar (0.91–0.95) and in both cases no adhesiveness was observed. The gelling and melting temperatures of the major extract were 31 °C and 85 °C, respectively.

3.3. ^{13}C NMR spectroscopy

RTW2-3, W701-2 and W901 were analyzed by ^{13}C NMR spectroscopy. Table 2 indicates the assignment of the main diads and Fig. 2 shows the spectra of RTW2, W701 and W901. Besides allowing the identification of pure agarose as the main product in W701-2 and W901, ^{13}C NMR analysis confirmed: (a) minor degree of methyl substitution on the 6-position of the 3-linked unit (Usov et al., 1983); (b) absence of sulfate substitution on the 6-position (no signal at 67.8 ppm; Usov et al., 1983), in agreement with the negligible increment of 3,6-anhydrogalactose observed after the alkaline treatment of W701 (Noseda et al., 2000); and (c) that the significant amount of glucose in extracts W702-3 and W901 (ca. 16–20%, Table 1) derived from floridean starch (Knutsen and Grasdalen, 1987; van de Velde et al., 2004).

In the spectrum of RTW2 and in minor proportion in RTW3 (Fig. 2, Table 2), in addition to the signals of agarose, resonances corresponding to the G → L6S diad were clearly observed (Usov et al., 1983), indicating the presence of precursor units typical of agarans of low gelling capacity (Fig. 1).

Table 2
^{13}C NMR assignment (ppm) of the diads present in the spectra of RTW2-3, W701-2 and W901

Unit[a]	C-1	C-2	C-3	C-4	C-5	C-6	O-Me	Present in
G[b]	102.9	70.7	82.7	69.2	75.8	61.9		RTW2-3, W701-2
LA[b]	98.7	70.4	80.6	77.8	76.1	69.9		and W901
G6M[c]	102.9	70.7	82.7	69.2	74.1	72.2	59.5	W701-2
LA[c]	98.7	70.4	80.6	77.8	76.2	69.9		
G[d]	103.9	70.2	81.3	69.2	76.3	61.8		RTW2-3
L6S[d]	101.6	69.3	71.2	79.1	70.2	67.9		
F.starch	100.7	72.7	74.3	78.7	72.5	62.2		W702 and W901

[a] Diad nomenclature after (Knutsen et al., 1994).
[b] Agarose diad (major).
[c] Substitution with O-methyl groups on the 6-position of 3-linked unit (minor).
[d] Diad with precursor unit.

3.4. Linkage analysis of W701

Methylation analysis of the major extract W701 (Table 3) also confirmed the presence of the agarose backbone determined by ^{13}C NMR spectroscopy. The presence of minor amounts of 2,4- and 2,6-di-O-methylgalactose, 2-O-methylgalactose and 3,6-anhydro-2-O-methylgalactose was in agreement with the small percentage of sulfate detected in the native extract; the small content of galactose (2%) could be attributed to undermethylation.

A portion of the permethylated product was hydrolyzed and subjected to reductive amination with (S)-1-phenylethylamine which showed that 2-O-methylagalactose was in the D configuration while galactose was in the L configuration and that the 2,6-di-O-methyl-D-:-L-galactose ratio was 1.0:0.5 (Table 3).

3.5. Ultrastructural observations

The cell wall of *G. gracilis* is composed of an inner and an outer stratum. The epidermal cells are covered externally by a wall covering or "cuticle" (Fig. 3a). After extractions, it can be observed that the cells in the residue lack the "cuticle", there is a significant reduction of the outer stratum with finely parallel fibrillar material and a notable swelling of the inner one, where fine fibrillar material has an irregular disposition (Fig. 3b).

4. Discussion

Feasibility for the exploitation of an agar producing species should consider aspects such as natural population dynamics, industrial processing, yield and quality of the gelling polysaccharide. To the best of our knowledge, this analysis has not been fulfilled for *G. gracilis* from Argentina.

G. gracilis, collected in summer in the Patagonic coast of Argentina biosynthesizes almost pure agarose (W701-3 and W901-2) in high yield (~34%), and only minor amounts (~2%) of low gelling capacity agarans containing G → L6S (RTW2-3). Rebello et al. (1997a,b) reported a yield of 32.6% for an Argentinean sample of *G. gracilis* (sample 7), but unfortunately neither the site nor the season of collection were indicated. They employed an aqueous extraction with boiling water followed by a freezing and thawing purification step. Assuming that the products equivalent to our extracts RTW1-3 would have been eventually drained off after freeze-thawing purification, their yield was similar to that of the present study. Moreover, except for the slightly higher sulfate content in W701, the 3,6-anhydrogalactose and methoxyl contents reported by these authors were also similar to ours but no information about the monosaccharide composition was given.

With a similar extraction procedure, Marinho-Soriano (2001) and Marinho-Soriano and Bourret (2003) analyzed seasonal variations in agar yield of *G. gracilis* from natural stocks from Thau lagoon (43°24′N, 03°32′E) in the Mediterranean Sea. The highest yield corresponded to spring specimens (30%) and the lowest (19%) to autumn ones. Higher summer yields were also observed by Mollet et al. (1998).

Room temperature extracts contain galactose 6-sulfate precursor units that are not firmly cross-linked in the amorphous domain of the cell walls. Sulfation of L-galactose takes place early during the biosynthesis, while ring closure (following the action of sulfohydrolases) and methylation occur at a later stage (Hemmingson and Furneaux, 2000).

In *Gracilaria* agar gelling temperature is directly related to the content of 6-O-methyl-D-galactose and 3,6-anhydro-2-O-methyl-L-galactose (Guiseley, 1970; Murano et al., 1992; Falshaw et al., 1998). Even though important amounts of 6-O-methyl-D-galactose have been previously reported for agars of several *Gracilaria*

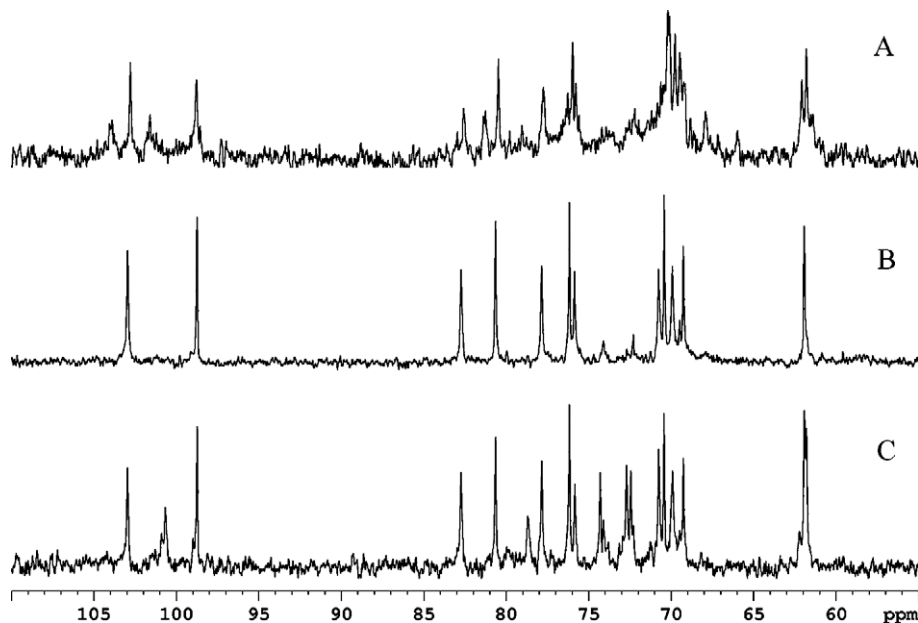**Fig. 2.** ^{13}C NMR spectra of (A) RTW2, (B) W701 and (C) W901.

Table 3
Methylation analysis (mol%) of W701

Monosaccharide	Deduced unit and substitution pattern	mol%[a]	Repeating unit[b]
2-Me AnGal	LA	40	B
2,4,6-Me$_3$ Gal	G + G6M	37	A
2,6-Me$_2$ D-Gal	G4S + G6M4S	4	A
2,6-Me$_2$ L-Gal	L3S	2	B
AnGal	LA2S	5	B
2,4-Me$_2$ Gal	G6S	2	A
2-Me D-Gal	G4S, 6S	8	A
3-Me Gal	–	tr[c]	B
L-Gal	–	2	B

[a] Normalized to exclude glucose (7%).
[b] See Fig. 1.
[c] Percentages low than 1% are considered as traces (tr).

species (Tako et al., 1999; Villanueva and Montaño, 1999; Melo et al., 2002), only small quantities were present in our extracts.

The degree of methyl substitution (considering the D-unit of the dimer as the sum of D-galactose plus 6-O-methyl-D-galactose, Table 1) of W701 was 0.13, which means that ~13% of each repeating dimer sequence G-LA is methylated on C-6.

The gelling temperature of W701 was lower than that reported (~55 °C) by Rebello et al. (1997a) for the Argentinean sample. Marinho-Soriano and Bourret (2003) observed for agars extracted from *G. gracilis* from Thau lagoon that the gelling temperature varied significantly between the seasons, ranging from 48 °C (autumn) to 37 °C (spring), being both values higher than that determined for W701.

The melting temperature is related to molecular weight. The higher the molecular weight, the higher the probability of forming stable interactions within gelling sequences in the polymer, with the consequent increment in melting temperature. Similar melting temperatures to that of W701 were reported for the sample (85 °C) studied by Rebello et al. (1997a) and for the native agar (80 °C) from *Gracilaria dura* (C. Agardh) J. Agardh (Murano et al., 1992).
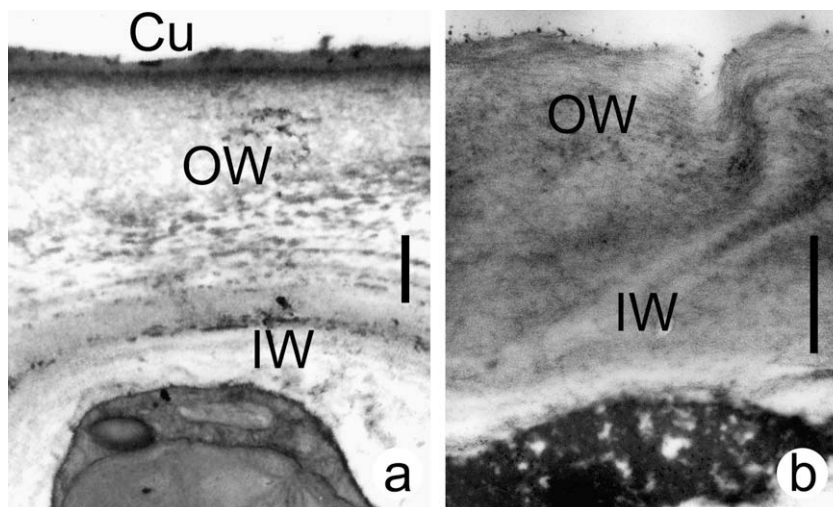

Fig. 3. Fine structure of *G. gracilis* cell wall. (A) Control epidermal cell wall consists of a cuticle and outer and inner wall strata. (B) After extractions residues lack the cuticle, the outer stratum reduces and the inner one swells. Cu, cuticle; IW, inner wall; and OW, outer wall. (a, b) Scale bar, 1 μm.

For the latter, the weight-average molecular weight (393,000) is comparable to ours. The weight-average molecular weights and polydispersity indexes herein found for W701 and W901 fall within the range previously reported for *Gracilaria* agars: 176,000–420,000 g mol^{-1} and 1.9–3.3, respectively (Lahaye, 2001).

On the other hand, sulfate substitution on the 6-position of L-galactose is negatively correlated with gel strength. Structural analysis of our products revealed a negligible percentage of L-galactose 6-sulfate in the gelling products W701-3 and W901-2. Accordingly, an insignificant increment in 3,6-anhydrogalactose content was observed after alkaline treatment of W701. This treatment was also ineffective for the Argentinean sample, of similar gel strength (~500 g cm^{-2}), studied by Rebello et al. (1997a). Meanwhile, low values were obtained by Marinho-Soriano and Bourret (2003) for *G. gracilis* from Thau lagoon (828 g cm^{-2}). The fact that an agarose rich fraction, with no need of further alkaline treatment, was isolated in the present study is very important, since this treatment usually leads to a decrease in molecular weight (Murano, 1995).

Floridean starch is usually extracted with hot water; and its signals were clearly observed in the ^{13}C NMR spectra of W702 and W901. The presence of floridean starch together with agarans has been already reported in other species of the family Gracilariaceae, i.e., *Gracilaria chilensis* Bird, McLachlan and Oliveira (Hemmingson et al., 1996) and in *G. corticata* J. Agardh (Mazumder et al., 2002). Even though W701 and W901 had similar molecular weights, the gel strength of the latter was notably lower indicating a negative effect of floridean starch on gel quality. Rice starch has been informed to be antagonistic to gel strength in agarose (Lai et al., 1999). In a similar fashion, when comparing major products W701 and W901, protein content was also inversely related to gel strength.

W701 contained minor amounts (5.7%) of protein but values of 13.5–29.8% were detected in the rest of the extracts. Marinho-Soriano and Bourret (2003) reported nitrogen contents in the range of 1.90–5.64% for *G. gracilis* from Thau lagoon, with lower values in spring/summer; Marinho-Soriano (1999) also found an inverse relationship between agar yield and nitrogen content which indicated that protein synthesis was limited in favor of polysaccharide synthesis (Dawes et al., 1974). A similar relationship was found by Bird et al. (1981) for agars extracted from *Gracilaria tikvahiae* McLachlan.

Linkage analysis and ^{13}C NMR spectroscopy of W701 indicated that the galactan backbone consisted mainly of G → LA, but that the G6M → LA diad was also present (Table 2). In addition, monosulfated and disulfated residues were also detected (Table 3).

Sulfation on the 6-position and on the 4-position of D-galactose was found in agars from *Gracilaria multipartita* (Clemente) Harvey and *Gracilaria bursa-pastoris* (S. Gmelin) P. Silva, respectively (Murano, 1995), while the agar of *Gracilaria edulis* (S. Gmelin) Silva exhibited sulfation on the 4-position of 6-O-methyl-D-galactose (Villanueva and Montaño, 1999). The presence of L-galactose 3-sulfate and 3,6-anhydro-L-galactose 2-sulfate residues has been previously reported for different agarans (Duarte et al., 2002; Kolender and Matulewicz, 2002).

The fine structure of the cell wall of *G. gracilis* observed in this study agrees with walls described in *Gracilaria verrucosa* (Huds.) Papenfuss, *G. tikvahiae*, *Gracilaria cornea* J. Agardh and *G. chilensis* (Verdus et al., 1985; Dawes et al., 2000; Leonardi et al., 2006). As was indicated by Hanic and Craigie (1969) and Craigie (1990) in red algae, protein is the major component of the wall covering or "cuticle". In *G. gracilis* the absence of "cuticle" after extractions is correlated with the presence of protein in all the products (Table 1). Moreover, the shrinking of the external layer of the wall is in agreement with the extraction of agarose at 70 °C. In fact, the first step of extraction at this temperature produces the highest yield of

this polysaccharide (Table 1). Consequently, a finely parallel fibrillar material can be distinguished in the external layer. On the other hand, hydration of fibrillar material after hot water treatment would lead to the observed increase in size of the inner layer, in which a fine fibrillar (presumably cellulose) component is observed.

This first thorough study of the chemical composition and physical properties of the products of *G. gracilis* from Bahía Bustamante demonstrates that a good quality agarose in high yield (71.6% of the recovered) is obtained after extraction with water at 70 °C without the requirement of alkaline pretreatment.

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