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Chemical and rheological characterization of the carrageenans from *Hypnea musciformis* (Wulfen) Lamoroux

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ABSTRACT

This work reports a chemical and rheological study of the carrageenans from *Hypnea musciformis*, a red seaweed commercially known for its production of κ-carrageenan. The polysaccharides were extracted with water both at room temperature and at 90 °C: the yield of the latter was about six times larger than the former. Fractionation with KCl yielded a large proportion (50–67%) of a precipitate with 0.125 M of this salt for both extracts, with characteristics of a nearly pure κ-carrageenan, as determined by methylation analysis and NMR spectroscopy. Smaller amounts of fractions precipitating at higher concentrations showed a basic κ-carrageenan structure, but included some ι-carrageenan diads. The KCl-soluble polysaccharides showed a larger complexity, containing D- and L-galactans or D/L-hybrids. Some differences in the rheological properties of these carrageenans have been found. Although all KCl-precipitating polysaccharides form true gels at 10 °C in presence of KCl, those extracted with hot water form stronger gels than those extracted at room temperature. Both purified κ-carrageenans show lower gelling and melting temperature than the whole polysaccharides from which they were originated.

1. Introduction

Carrageenans are galactose-rich polysaccharides found in certain red seaweeds. Their structure is based on a linear chain of a repeating pair of a 3-linked β-D-galactopyranosyl residue and a 4-linked α-D-galactopyranosyl residue, the last occurring many times as a 3,6-anhydro-α-D-galactopyranosyl moiety. They differ from agarans, which are found in other red seaweeds, in the configuration of the 4-linked residue, which belongs to the series L-in agarans. A third group, in which the α-linked galactose is present in both configurational series are rare, and are known as D-/L-hybrids (Stortz & Cerezo, 2000). Either of these galactans can be substituted, mainly, by sulfate ester groups in different positions, and less by pyruvic acid ketals, methyl ethers, or single stubs of monosaccharides (usually β-D-xylopyranose). These last features are more common in agarans. Thus, carrageenans are only seldom pyruvylated, methoxylated or branched. Their classification is done according to their sulfation pattern and/or the presence or absence of the 3,6-anhydro residue or its chemical precursor, galactose 6-sulfate (Stortz & Cerezo, 2000). From the commercial/industrial point of view, there are three major types of carrageenans: κ-carrageenan, with a 3-linked, 4-sulfated residue and a 4-linked 3,6-anhydro moiety; ι-carrageenan, with a structure analogous to the former, but with an additional sulfate ester group on C-2 of the 3,6-anhydrogalactose residue; and λ-carrageenan, with a 2-sulfated 3-linked galactose unit, and a 2,6-disulfated 4-linked galactose unit (Stortz & Cerezo, 2000).

These carrageenans are widely used in the industry for their rheological properties as stabilizers and thickening or gelling agents. In the food industry, for example, they are used for the improvement of the texture of cottage cheese or as binders and stabilizers for the manufacture of sausages and low-fat hamburgers (Campos, Kawano, Silva, & Carvalho, 2009). They are also used in the pharmaceutical, cosmetics, printing and textile industries. It was reported their use as renewable, ecological and nontoxic mobility control agents to increase sweep efficiency for oil recovery in dwindling petroleum reserves (Iglauer, Wu, Shuler, Tang, & Goddard, 2011). It is widely known that κ- and ι-carrageenan can form gels upon cooling or in the presence of K^+ or Ca^{2+} counterions (Lahaye, 2001). On the other hand, λ-carrageenan hardly gels in the presence of K^+ (Stortz & Cerezo, 1993). Thus, it is used as a thickening agent.

For the gelling carrageenans, it is accepted that gelling is produced when carrageenans in solution transform from random coil shapes to helical, ordered structures, as temperature decreases.

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These helices can interact, via intermolecular association, between themselves forming small independent domains and crosslinking of the chains (Thrimawithana, Young, Dunstan, & Alany, 2010). In presence of cations, the formation of the domains is enhanced due to the interaction between the cation and the sulfate ester groups.

Hypnea musciformis is a widely-distributed red seaweed known to produce large amounts of κ-carrageenan (Alves et al., 2012). It has been commercially exploited in Brazil (McHugh, 2003), and is considered a potentially profitable species due to its high yields of κ-carrageenan, fast growth rate and tolerance to different environmental conditions (Vázquez-Delfin, Robledo, & Freile-Pelegrin, 2013). Although several works have previously studied the sulfated galactans from this seaweed, they usually focused on single aspects, such as their structure (Bi, Arman, Hassan, & Iqbal, 2006; Greer, Shomer, Goldstein, & Yaphe, 1984; Hamilton & Carrol, 1962; Knutsen et al., 1995), rheological properties (Andrade, Azero, Luciano, & Gonçalves, 2000; Bi, Hassan, Arman, Iqbal, & Mahmood, 2007; Mahmood et al., 2007) or biological activities as elicitors of plant defense mechanisms (Arman, 2011; Arman & Ul Qader, 2012; Bi, Arman, Ali, & Iqbal, 2009; Bi & Iqbal, 1999, 2000, 2002, 2005; Bi, Iqbal, Ali, Aman, & Hassan, 2008; Bi, Iqbal, Arman, Ali, & Hassan, 2011), antioxidant (Alves et al., 2012), antiinflamatory (De Brito et al., 2013; Rodrigues, de Araújo, de Paula, Vanderlei, et al., 2011) or cytotoxic (Alves et al., 2012; Rodrigues, de Araújo, de Paula, Lima, et al., 2011). In most of these works, the non-purified extracted polysaccharide (considered to be κ-carrageenan) was used. Only a few of them took some purification steps, and none attempted to analyze the products accompanying the κ-carrageenan. The aim of this work is to present the isolation of carrageenans extracted from *H. musciformis* by room temperature- and boiling water, their fractionation with potassium chloride, and their full chemical and rheological characterization, in order to assess its potential as a commercial source of carrageenans.

2. Materials and methods

2.1. Material

Samples of *H. musciformis* (Wulfen) Lamoroux Foslie were collected near Natal (Rio Grande do Norte), Brazil (5°37′52″S, 35°13′03″W). The seaweeds were sorted, air dried, cleaned manually and milled to a fine powder before extraction. All chemical reagents were of analytical grade.

2.2. General methods

Total carbohydrates were determined by the phenol-H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using galactose as standard. The percentages of sulfate were measured by turbidimetry (Dodgson & Price, 1962) after hydrolysis with 1 M HCl, and the soluble proteins were determined by the procedure of Lowry, Rosebrough, Farr, and Randall (1951).

The proportions of monosaccharides constituting the polysaccharides were determined using a reductive hydrolysis procedure (Stevenson & Furneaux, 1991) slightly modified as shown elsewhere (Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999; Navarro & Stortz, 2003), in order to avoid the destruction of 3,6-anhydrogalactose. After acetylation (CF_3COOH:Ac_2O 1:1, 10 min, 50 °C), the alditol acetates were analyzed by GLC on an HP 5890A apparatus fitted with a capillary column SP 2330 (30 m × 0.25 mm i.d., thickness 0.20 μm), equipped with a flame ionization detector operating at 240 °C. The injector temperature was 240 °C and the oven temperature started at 200 °C, rose 2 °C/min to 230 °C, and stayed at this temperature for 20 min. Nitrogen was used as the carrier gas at a head pressure of 15 psi. Aliquots were injected with a split ratio of 80:1. Cyclizable 6-sulfate was determined by 3,6-anhydrogalactose measurement (Navarro & Stortz, 2003) after alkaline treatment (NaOH 1 M for 5 h at 80 °C). Other aliquot of the hydrolyzates was converted to the acetylated aminodeoxyalditols using (*S*)-1-amino-2-propanol and (*S*)-α-methylbenzylamine (Cases, Cerezo, & Stortz, 1995) and analyzed by GLC as stated therein. The configuration of the 3,6-anhydrogalactose was determined after mild hydrolysis and derivatization with (S)-α-methylbenzylamine, as described (Navarro & Stortz, 2003). When necessary, GLC–MS analyses were carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using similar conditions to those described above, but using He as gas carrier and a split ratio of 60:1. The molar percentage of the different monosaccharides was calculated by considering that the FID responses are proportional to the molecular weight of the derivatives.

2.3. Extraction and fractionation

The extraction procedure is similar to that reported by Estevez, Ciancia, & Cerezo (2000, 2004). The milled seaweed (20 g/L) was extracted first with water at room temperature, with mechanical stirring for 24 h. After centrifugation (13,100 × *g*), the supernatant was concentrated and poured into 3.5 vols of isopropanol. The precipitated polysaccharides were washed with isopropanol, air-dried, redissolved in water, and the remaining isopropanol was removed by rotary evaporation. Final freeze-drying led to carrageenan R. The residue was reextracted with water at 90 °C for 8 h yielding, by the above-mentioned procedure, carrageenan H.

For fractionation, the carrageenans R and H were dissolved in water (2.5 g/L) and centrifuged (13,100 × *g*). To the supernatant solid ground KCl was added in small portions, with vigorous stirring, up to a KCl concentration of 0.125 M. The stirring was continued for 6 h to ensure equilibration, and the solutions were centrifuged. The precipitates were dialyzed (mol. weight cut-off 6000–8000), concentrated and lyophilized. To the supernatants, more KCl was added in small portions, so that the concentration was increased by 0.1–0.25 M each time. After each addition the stirring was continued, and the solutions were centrifuged again (13,100 × *g*). When a noticeable precipitate appeared, it was isolated as described above. The residual solution (upper limit 2 M KCl) was dialyzed, concentrated and centrifuged as stated above. In this way, from the R carrageenan we isolated a fraction not solubilized in water (R-0), two precipitating by KCl in the intervals 0–0.125 M and 0.8–1 M (R-0.125 and R-0.8-1, respectively), and a fraction soluble in KCl (R-2S). Similarly, the H carrageenan yielded fractions H-0.125, H-1-1.25 and H-2S.

2.4. Gel permeation chromatography

GPC analysis was performed with a high performance liquid chromatography system equipped with a Shimadzu refractive index detector (Model RID-10A), and a Shimadzu LC-20AT pump. Polysaccharides were analyzed using two columns connected in series: a Supelco Progel-TKS G4000 and a Waters Ultrahydrogel 250 (300 × 7.8 mm). A degassed solution of 0.05 M $NaNO_3$ prepared with ultra-pure water and containing 0.02% NaN_3 was used as solvent and eluant. 100 μl of polysaccharide solutions (5 mg/ml) were filtered through a 0.22 μm PVDF membrane (GV, Millipore) and injected into the column using a manual valve. The eluant flow rate was of 0.6 ml/min and the temperature was held at 25 °C. The column was calibrated using dextran standards of different molecular weights (Sigma–Aldrich, USA).

Table 1
Composition and yields of the carrageenans extracted with room-temperature- and hot water from *Hypnea musciformis* and of the subfractions isolated from them.

	Fraction				Fraction			
	R	R-0.125	R-0.8-1	R-2S	H	H-0.125	H-1-1.25	H-2S
Yield (%)[a]	5.5 ± 0.7	45.8 ± 4.2	7.3 ± 1.5	18.9 ± 2.8	39.4 ± 2.3	69.0 ± 1.5	2.9 ± 0.3	15.9 ± 1.3
Protein (%)	8.8	2.7	2.4	17.9	6.4	2.8	2.5	13.9
Molar ratio Gal:3,6-AnGal:S	1:0.82:1.03	1:0.89:1.21	1:0.72:1.67	1:0.16:1.31	1:0.80:1.14	1:0.96:1.31	1:0.67:1.39	1:0.09:0.61
Molar ratio Gal:Gal 6-sulfate	1:0.04	1:0	1:0.05	1:0	1:0.08	1:0	1:0.10	1:0.12[b]
MW[c] (kDa)	287	88	186	W[d]	197	215	248	W[d]
Monosaccharides (mol/100 mol)								
D-Gal	51	52	57	39	53	50	58	45
L-Gal				16				20
3.6 An-D-Gal	41	46	41	6	42	49	39	4
3.6 An-L-Gal				3				2
D-Xyl	3	1	1	12	1	tr.	1	11
D-Glc	3	1	1	14	3	1	1	15
D-Man				2	1			1
L-Ara	1			1			tr.	tr.
L-Rha	1			1				
L-Fuc				2				1
2-O-Me-D-Gal				2				2
3-O-Me-D-Gal				2				1

[a] Yields for R and H given in g/100 g dry seaweed. Yields for the subfractions given in g/100 g original fraction (R or H). Average and standard deviations are shown ($n = 2$).
[b] Similar amount of both enantiomers.
[c] Molecular weight, as determined for the maximum value of the GPC peak.
[d] Very wide peak, with more than one maximum.

2.5. Methylation analysis

The triethylammonium salts of the fractions (5 mg) were methylated as described by Ciucanu and Kerek (1984), using NaOH and CH_3I in dimethyl sulfoxide. The methylated products (isolated by dialysis and lyophilization) were hydrolyzed and derivatized as described above (Section 2.2), analyzed by GLC as described elsewhere (Cases, Stortz, & Cerezo, 1994), and characterized by GLC–MS.

2.6. Nuclear magnetic resonance

The NMR spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz provided with a 5-mm probe, at room temperature, using ca. 20 mg polysaccharide in 0.7 mL of D_2O. Acetone was added as internal standard (referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm in ^{13}C, 2.22 ppm in ^{1}H). ^{1}H NMR spectra were carried out with an acquisition time of 4.36 s, a pulse angle of 30°, and a pulse delay of 1 s. Typically, 16 scans were used. The ^{1}H–^{13}C HSQC (Heteronuclear single quantum correlation) spectra were carried out with the Bruker program 'hsqcetgpsi2', with a FID size of 512 (F1) × 1024 (F2), using 24 scans and adjusting the $^1J_{C-H}$ to 144.9 Hz.

2.7. Oscillatory rheological assays

Samples of 0.04 g of each suitable polysaccharide fraction were dissolved in 45 mM KCl aqueous solution in order to obtain 2.00% (w/v) systems, with aid of vortexing. Temperatures of at least 50–60 °C were needed to give true solutions. Enough volume of a sample solution heated to 85 °C was transferred to the 25-mm-diameter serrated parallel plate of an MCR300 Paar Physica rheometer (Germany) and a gap of 500 μm was used. The temperature control was performed in the rheometer through a peltier unit (Viscotherm VT2 Physica, Germany) and evaporation was avoided through a chamber. The dynamic viscoelastic functions, shear storage (G') and loss (G'') moduli were measured at stationary state as a function of temperature, time and frequency for each polysaccharide fraction, according to the following steps: (a) an 85.0 °C isothermal period of 60.0 s; (b) cooling ramp from 85.0 to 10.0 °C at a rate of 10.0 °C/min (1 Hz, 0.02% of strain); (c) isothermal period of 900 s (1 Hz, 0.02%-constant strain); d) frequency sweep from 1 to 100 rad/s at linear viscoelastic condition (0.02%-constant strain); and e) heating ramp from 10.0 to 85.0 °C at 10.0 °C/min (1 Hz, 0.02% of strain). The setting and melting temperatures were respectively determined in the steps b and e, respectively. During the cooling ramp (step b), the setting temperature corresponded to the point where G'' values crossed down G' ones, after which the elastic modulus was above G''. Similarly, the melting temperature was determined from the heating ramp (step e) when G'' crossed over G'. Measurements were performed in triplicate.

2.8. Statistical analysis

The results of the rheological analysis were reported as the average and standard deviation for three sample replicates. Results were analyzed through ANOVA (α: 0.05) followed by multiple comparisons evaluated through least square difference significant difference test, using the Statgraphic package (Statgraphic Plus for Windows, version 5.0, 2001, Manugistic Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Extraction and fractionation of the carrageenans

The red seaweed *H. musciformis* was extracted with water at room temperature, yielding polysaccharide R with a 5.5% yield. The residue was reextracted with water at 90 °C, giving rise to the more abundant polysaccharide H (39.4% yield). The analyses of these products are given in Table 1. Both R and H show typical analytical characteristics of κ-like carrageenans, with galactose:3,6-anhydrogalactose:sulfate molar ratios very close to 1:1:1, and small amounts of cyclizable 6-sulfate which, together with a Gal/AnGal ratio barely larger than 1 indicate the presence of only few precursor (μ-like) units. The analytical differences between R and H are small; the latter appears to be slightly more sulfated; the molecular weight at the HPLC "peak" of the room temperature extract is slightly higher (Table 1 and Fig. 1A), but the shape of the peak is less Gaussian (Fig. 1A), suggesting the presence of more low-molecular weight material. In their study of *H. musciformis* from European origin, Knutsen et al. (1995) have found a larger yield of carrageenans in the cold-water extract (25%) and a

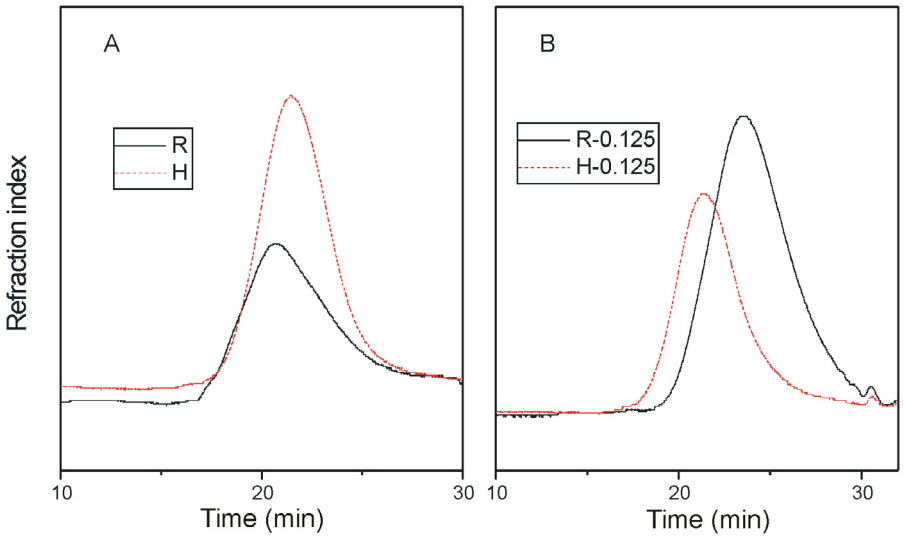

Fig. 1. GPC profile of carrageenans R and H (A), and fractions R-0.125 and H-0.125 (B). A sequence of two columns (a Supelco Progel-TKS G4000 and a Waters Ultrahydrogel 250) was used. The solvent and eluant was a degassed solution of 0.05 M $NaNO_3$/0.02% NaN_3 prepared with ultra-pure water.

smaller yield in the hot-water extract (7%). The differences should be ascribed to an acid pretreatment made by the Norwegian-Italian group, which increases the extracting power of the cold water (Knutsen et al., 1995).

Fractionation of carrageenan R by means of KCl precipitation yielded a major κ-fraction, as deduced from its precipitation range (0–0.125 M) and its analytical characteristics (Table 1), a minor amount of a second fraction, precipitating at concentrations of KCl between 0.8 and 1 M, characterized by higher sulfate and lower 3,6-anhydrogalactose contents (Table 1), and a substantial proportion (18.9%) of a soluble fraction, with analytical characteristics which suggest the presence of carrageenans (D-Gal, 3,6-An-D-Gal) together with other polysaccharides, as agaroids (L-Gal, 3,6-An-L-Gal, mono-*O*-methylated sugars), D/L-hybrids, glucans, xylans or xylose-branched galactans and moderate amounts of protein.

Fractionation of carrageenan H presented a similar qualitative picture. However, the yield of the κ-like fraction was larger (69.0% against 45.8% in R), the second precipitating fraction needed more KCl to precipitate and showed a larger amount of 6-sulfated precursor units (Table 1). The fraction soluble in KCl presented the same heterogeneity observed for the R-2S fraction, although some precursor units (not appearing in R-2S) are suggesting the presence of μ/ν-carrageenans or equivalent agarose precursors.

Both κ-fractions (precipitated at 0.125 M KCl) showed Gaussian HPLC peaks, but the peak of that extracted by hot water (H-0.125) looked narrower and with a higher molecular weight (Fig. 1B).

3.2. Chemical characterization of the carrageenans

The more homogeneous fractions R-0.125, R-0.8-1, H-0.125, and H-1-1.25 were permethylated and submitted to ^1H and HSQC NMR analysis. The results of the methylation analysis are shown in Table 2. R-0.125 shows 2,6-di-*O*-methylgalactose (51%) and 3,6-anhydro-2-*O*-methylgalactose (39%) as main constituents after hydrolysis. These two sugars are indeed those expected to appear in equal amounts from the hydrolysis of a permethylated idealized κ-carrageenan. The small deviations that appear originate from the presence of non-methylated 3,6-anhydrogalactose in a proportion of 6%, and from a slightly larger proportion of galactoses than 3,6-anhydrogalactoses. The former result can be explained either in terms of a small proportion of ι-carrageenan structures interspersed in the molecules or by slight undermethylation; the latter result could arise from partial destruction of the 3,6-anhydrogalactose during the reductive hydrolysis procedure, as no evidence of non-cyclized α-galactose units is observed, but for the scarce presence of 2,3,6-tri-*O*-methylgalactose (1%). The other κ-like carrageenan, isolated in larger amounts from the hot water extraction (H-0.125) does not show any ι-character, as almost all 3,6-AnGal appears 2-*O*-methylated; however other small anomalies appear, as the presence of 6% of non-sulfated β-galactose units, and 2% of non-sulfated, non-cyclized α-galactose units. 2D NMR spectroscopy (HSQC) of these two fractions shows that the main peaks are characteristic of κ-carrageenan (Table 3, Tojo & Prado,

Table 2
Composition of partially methylated monosaccharides produced by permethylation of R-0.125, R-0.8-1, H-0.125, and H-1-1.25.

Monosaccharide[a]	R-0.125	R-0.8-1	H-0.125	H-1-1.25
2,3,6-Gal	1	1	2	1
2,4,6-Gal		3	6	1
2,3-Gal		1		
2,4-Gal	1	2	1	1
2,6-Gal	51	45	45	48
2-Gal	1	2	3	1
4-Gal		1		
6-Gal		1		1
2-AnGal	39	30	42	38
AnGal	6	11	1	7

[a] Mol/100 mols of monosaccharides having methyl groups at the positions indicated.

Table 3
^1H and ^{13}C NMR assignment of the signals of fractions R-0.125 and H-0.125.

		^1H NMR	^{13}C NMR
Gal	1	4.70	102.5
	2	3.65	69.4
	3	4.06	78.2
	4	4.91	73.7
	5	3.87	74.8
	6[a]	3.86	61.2
3,6-AnGal	1	5.15	94.7
	2	4.19	69.7
	3	4.58	79.2
	4	4.65	78.4
	5	4.70	76.7
	6	4.10, 4.19	69.7

[a] Protons 6 and 6′ could not be differentiated.

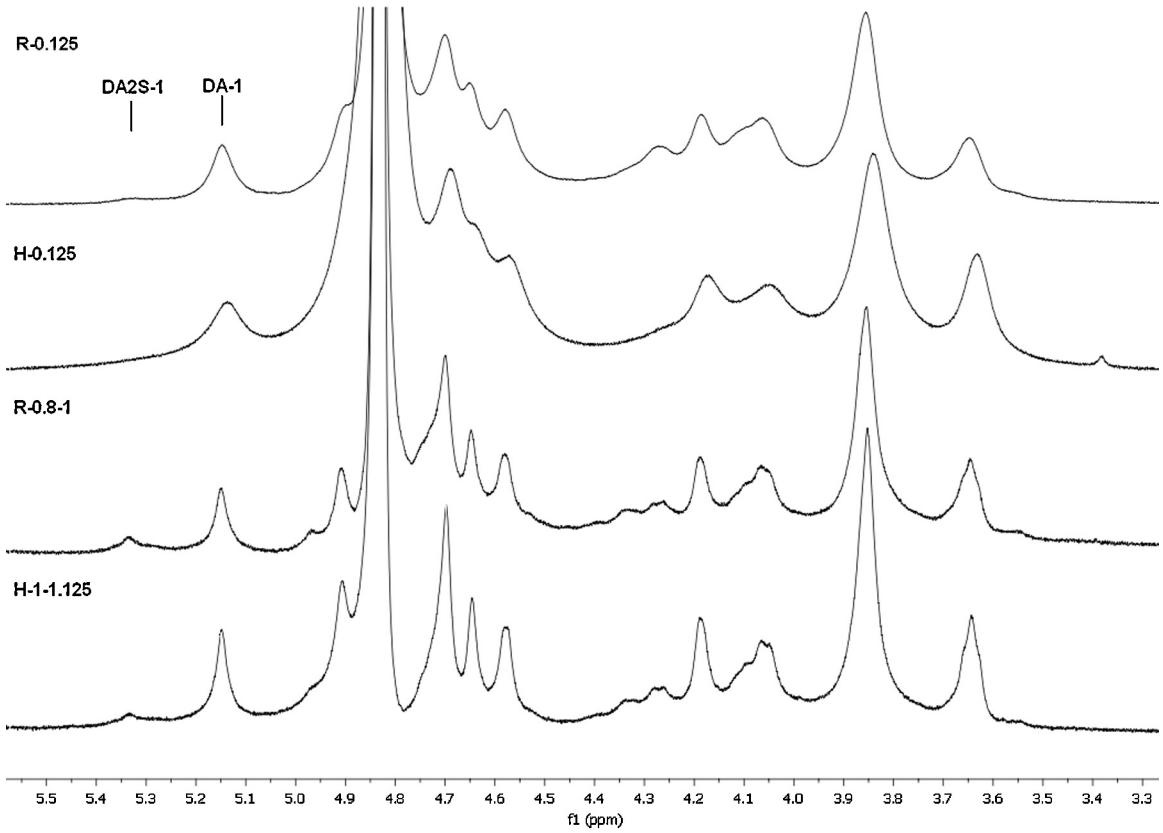

Fig. 2. 500 MHz ^{1}H NMR spectra of the main fractions obtained from carrageenans R and H. The location where the anomeric protons of the main α-units appear is indicated.

2003; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). This was also confirmed by ^{1}H NMR spectroscopy, which also helped to quantify minor peaks (Tojo & Prado, 2003). The ^{1}H NMR (Fig. 2) of R-0.125 shows a small peak at 5.34 ppm that corresponds to the anomeric proton of the 3,6-anhydrogalactose-2-sulfate (DA2S-1, Stortz, Bacon, Cherniak, & Cerezo, 1994). It represents about 7% of the total 3,6-AnGal. No equivalent peak was observed for H-0.125, suggesting that the later is quite pure κ-carrageenan, as also indicated by the methylation analysis. The fractions precipitating at higher KCl concentrations (R-0.8-1 and H-1-1.25) also show a methylation pattern characteristic of a κ-carrageenan, with 3-linked, 4-sulfated units in 45–48% proportion. The 3,6-anhydrogalactose (41–45% summing both derivatives) appears mainly methylated at *O*-2, but a substantial amount (7–11%) appears non-methylated, suggesting that if undermethylation is discarded, more ι-structures appear in the molecules, probably causing the delayed KCl precipitation of these samples. The presence of ι-structure was confirmed by ^{1}H NMR (Fig. 2). Up to about 30% of ι-structures appear in R-0.8-1, and up to 20% in H-1-1.25. Appearing only slightly above the baseline, a signal at 5.57 ppm, characteristic of ν-carrageenans (Stortz et al., 1994) is observed for both fractions. No indication of the presence of μ-carrageenans was observed. Methylated sugars indicating other abnormalities appear only in small proportions (Table 2).

The fractions soluble in 2 M KCl show only small amounts of 3,6-anhydrogalactose and a heterogeneous mixture of sugars which exhibit L-galactose and xylose as main components. These complex mixtures have already been shown to appear in non-commercial tetrasporic seaweeds (Stortz, Cases, & Cerezo, 1997; Stortz & Cerezo, 1993), cystocarpic seaweeds (Ciancia, Matulewicz, & Cerezo, 1993, 1997; Craigie & Rivero-Carro, 1992), and also in commercial, κ-carrageenan-producing seaweeds (Estevez, Ciancia, & Cerezo, 2000, 2004). Separation of the components (Estevez, Ciancia, & Cerezo, 2004; Stortz et al., 1997) showed complex mixtures of carrageenans, agarans and hybrid D/L galactans with unusual features. Knutsen et al. (1995) reported that the KCl-soluble fractions from *H. musciformis* show IR spectra which suggested the presence of a mixture of galactans; they found low sulfation and the presence of xylose, but no further studies were carried out. The fine analysis of fractions R-2S and H-2S will be the subject of further work.

3.3. Rheological characterization of the carrageenans

The rheological characterization of the carrageenan fractions was carried out using 2.00% (w/v) solutions in 45 mM KCl at pH 6.05–6.12, as it has been shown that ionic strength and pH the influence the mean hydrodynamic diameter (*Z*-average) of polysaccharide solutions (Carneiro-da-Cunha, Cerqueira, Souza, Teixeira, & Vicente, 2011).

It has been reported that all carrageenans are soluble in hot water and that only the Na^+ salts of κ- and ι-carrageenans are soluble in cold water (Imeson, 2000). True solutions for each carrageenan fraction were obtained at high temperatures. Hence, they were submitted to dynamic assays performed as a function of temperature, at constant frequency (1 Hz) and strain (0.02%). Cooling carried out for each carrageenan fraction produced the profiles shown in Fig. 3A, C and E. It can be observed that the elastic modulus (G') increased upon cooling for the six carrageenan samples shown in Fig. 3A and C. This behavior corresponds to an increase in hydrogen bonding and also to the formation of electrostatic junction zones, in this case mediated by sulfate groups and potassium ions of the adjacent carrageenan helical structures formed upon cooling (Imeson, 2000). Both R and H fractions showed a similar gelling temperature upon cooling, 40.8 and 42.0 °C, respectively, in KCl solution (Table 4). On cooling, κ- and ι-carrageenan

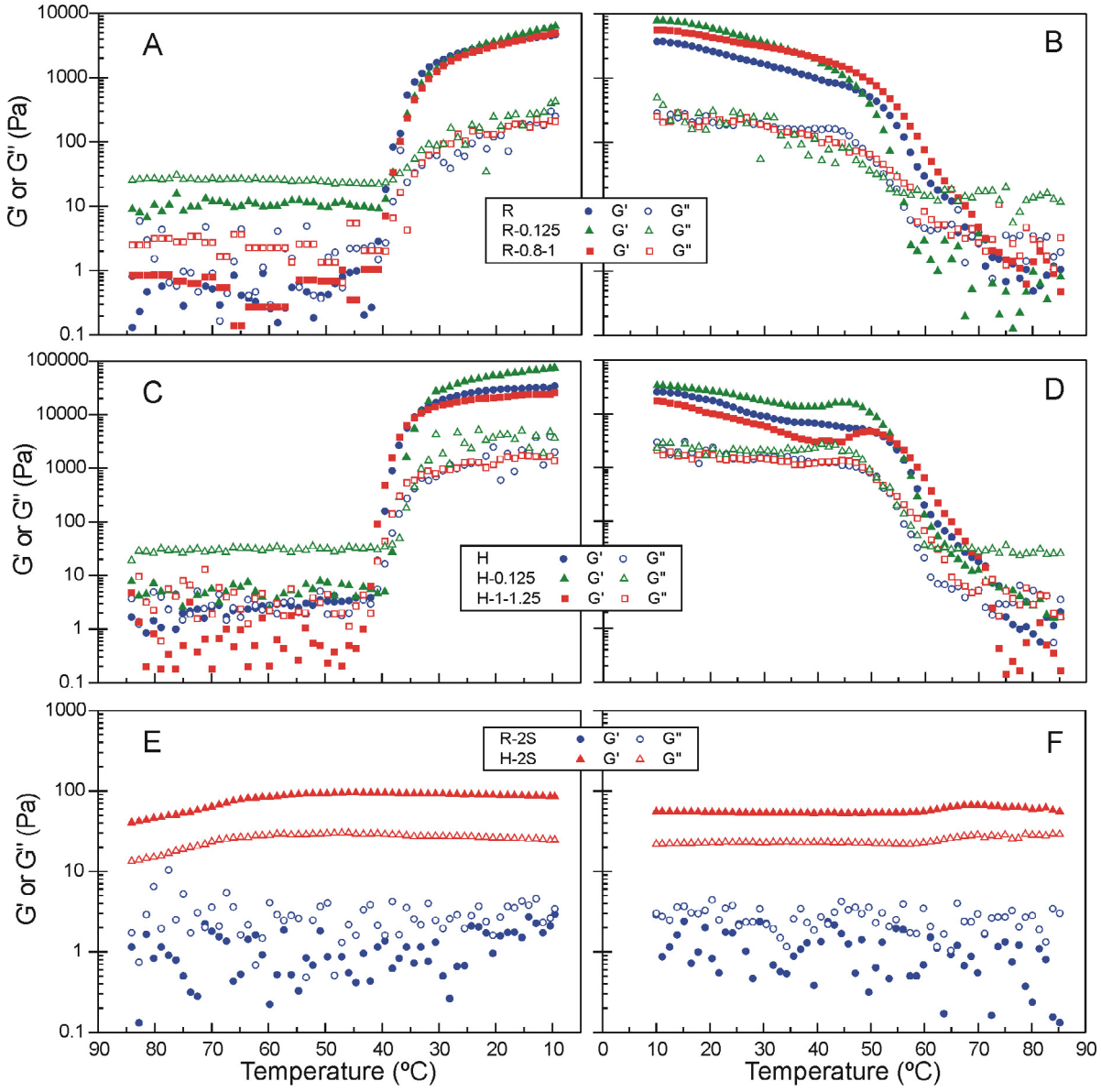

Fig. 3. Cooling ramp performed at 1 Hz and 10 °C/min constant for the fractions indicated (A), (C) and (E); and heating ramp performed under the same conditions (B), (D) and (F).

Table 4
Gelling and melting temperatures, elastic (G') and loss (G'') moduli[a] obtained for carrageenans R and H, and for their subfractions in 2.00% solutions.

Fraction	$T_{gelling}$ (°C)	G' (Pa)	G'' (Pa)	$T_{melting}$ (°C)
R	40.8 ± 0.2^{F}	3860 ± 25^{F}	272 ± 13^{D}	64.9 ± 0.4^{C}
R-0.125	38.2 ± 0.3^{H}	7860 ± 37^{D}	294 ± 7^{D}	56.0 ± 0.1^{D}
R-0.8-1	39.5 ± 0.2^{G}	5470 ± 30^{E}	201 ± 18^{E}	72.5 ± 0.3^{B}
R-2S	–	3^{H}	3^{G}	–
H	42.0 ± 0.3^{E}	$28{,}100 \pm 82^{B}$	2530 ± 11^{B}	73.7 ± 0.2^{A}
H-0.125	38.2 ± 0.3^{H}	$37{,}700 \pm 46^{A}$	2850 ± 15^{A}	63.6 ± 0.2^{C}
H-1-1.25	42.6 ± 0.1^{E}	$18{,}600 \pm 51^{C}$	1620 ± 9^{C}	72.5 ± 0.2^{B}
H-2S	–	85 ± 3^{G}	26 ± 2^{F}	–

[a] Mean and standard deviations are shown ($n = 3$). The same capital letter within a column indicates non-significant differences ($p < 0.05$). Gelling and melting temperatures are considered as one column.

undergo a random coil to helix conformational transition which generates gelation. Cations such as potassium bind specifically to the double-helix, increasing stability and promoting chain aggregation (Andrade et al., 2000). After the sharp gelation process, cooling profiles were characterized by G' values that increased monotonically throughout the cooling regime (Fig. 3A, C and D). Despite the cooling rate assayed (10 °C/min), the slope of the trace subsequent to gelation suggests that network conversion is achieved over a narrow time/temperature range and, although substantial internal rearrangements may occur later, these are not revealed by large changes in the measured G' modulus (Fig. 3A and C) (Richardson & Goycoolea, 1994). When reaching ≈10 °C, the G' was one order of magnitude higher for H (≈ 35,000 Pa) than for R fraction (≈4600 Pa), with the loss modulus (G'') an order of magnitude lower than G', as expected for solid viscoelastic materials like true gels.

These networks showed no dependence on time (G', G'') along the isothermal (10.0 °C) 900 s-period for both R and H carrageenan samples (Fig. 4A and C). Afterwards, the mechanical spectra (linear viscoelastic condition, 0.02% constant strain) recorded between 1 and 100 rad/s of angular frequency (ω), at 10.0 °C, confirmed that the viscoelastic structure formed was a true gel (Doublier, Launay, & Cuvelier, 1992), with that obtained from the H carrageenan fraction being more solid or elastic ($G' \approx 28{,}000$ Pa) than that produced

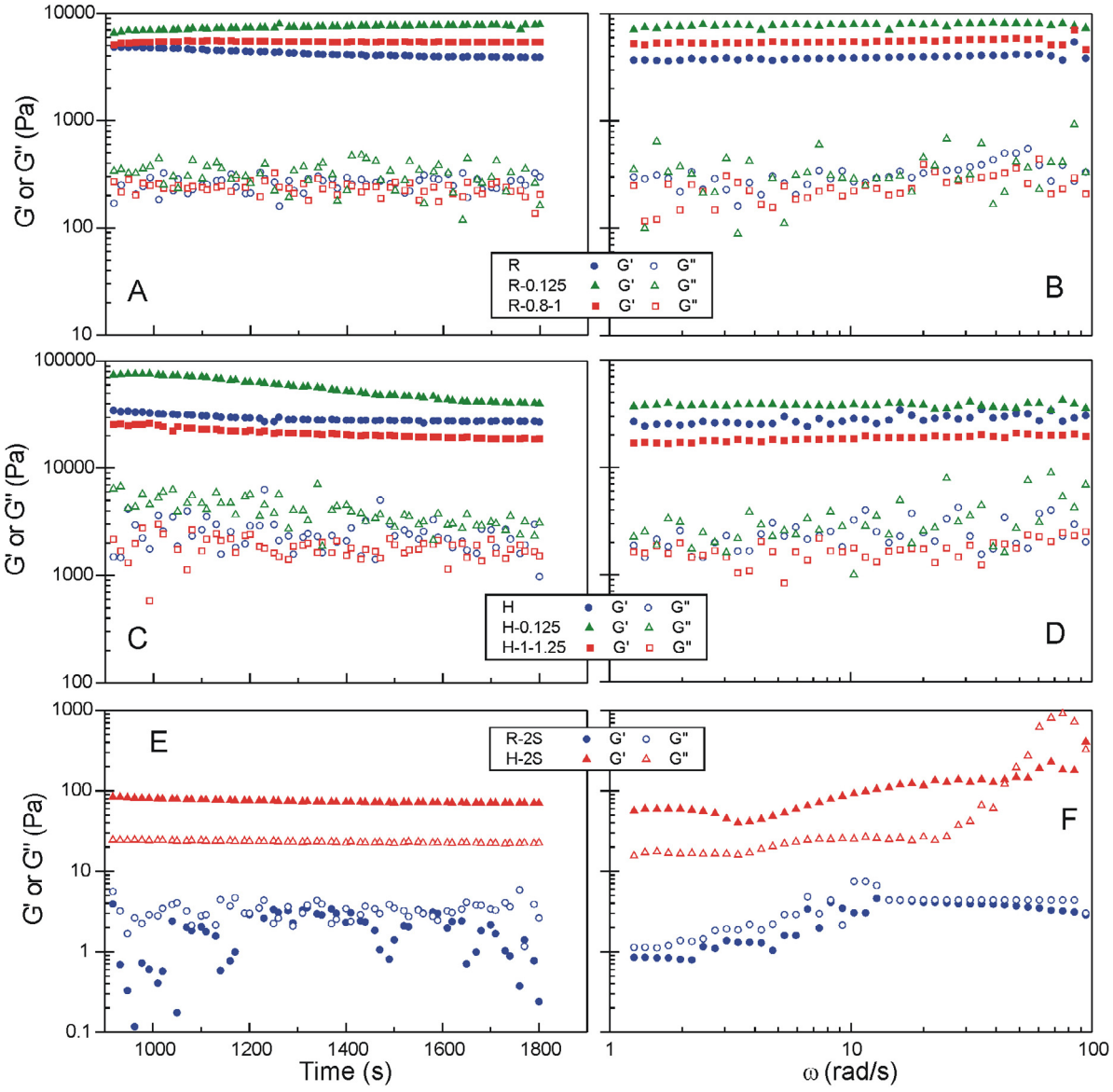

Fig. 4. Isothermal period (1000 s) of gels at 10.0 °C (A), (C) and (E); frequency sweep carried out at 10.0 °C under lineal viscoelasticity (B), (D) and (F).

by the R fraction ($G' \approx 3860$ Pa) (Fig. 4B and D and Table 4). R and H fractions are κ-like carrageenans with similar analytical characteristics. R showed higher molecular weight than H (Table 1), but the latter produced stronger gels (Table 4). Taking into account that R and H are actually mixtures of polysaccharides with different structures, the higher value of G' for H may be due to the higher proportion of gelling carrageenans present in this fraction (Table 1).

When gels were then submitted to a heating ramp (10.0 °C/min), it was observed that the G' modulus decreased slowly with the increase in temperature up to ≈ 51 °C, being almost paralleled by G'' (Fig. 3B and D). After this, G' decreased faster than G'', until the moduli crossed over at 64.9 °C for R and 73.7 °C for H carrageenan gels (Table 4). Each of these points corresponded to the melting temperature (Fig. 3B and D); the H gel showed some higher thermal stability than R one. During the melting process, breaking of some hydrogen bonds occurred and double helices change their conformation giving rise to dissociation of aggregates, which results in loss of the gel network (Núñez-Santiago & Tecante, 2007). As a conclusion, both carrageenan fractions constituted thermo-reversible gels, showing hysteresis between cooling (Fig. 3A and C) and heating (Fig. 3B and D).

Similar cooling profiles were obtained for R-0.125 and H-0.125 carrageenan fractions dissolved in 45 mM-KCl (Fig. 3A and C). Lower gelling temperatures (38.2 °C) than those observed for R and H were obtained for R-0.125 and H-0.125 carrageenans (Table 4). Lower setting temperatures may be related to a delay in the aggregation or a more temperature dependent process of conformational change for these fractions. As mentioned before, R-0.125 and H-0.125 are fractions essentially constituted by κ-carrageenan, whereas R and H also included some proportions of ι- and ν-carrageenans, together with other polysaccharides and small amounts of protein (Table 1). It has previously been shown that an increase in the proportion of κ-carrageenan in κ/ι-hybrids lowers the gelling temperature (Souza, Hilliou, Bastos, & Gonçalves, 2011). As observed for H and R, G' was one order of magnitude higher for H-0.125 (≈74,100 Pa) than for R-0.125 system (≈6300 Pa) when reaching 10 °C, with the loss modulus (G'') an order of magnitude lower than G' (Fig. 3A and C). The G' values observed for these purified carrageenan fractions were higher than those determined for R and H, as expected considering their larger proportion of κ-diads. The structure formed on cooling of R-0.125 solution was not dependent on time in the 900 s subsequent isothermal (10.0 °C)

period considered (Fig. 4A), but some dependence was observed for H-0.125, where G' decreased with an increase in resting time (Fig. 4C). This behavior may be ascribed to syneresis and consequent slippage (Richardson & Goycoolea, 1994). After, they showed to be frequency-independent and, hence, true gels were developed on cooling. The gel produced by the H-0.125 carrageenan fraction was more elastic ($G' \approx 38{,}500$ Pa) than that developed by R-0.125 ($G' \approx 7950$ Pa) (Fig. 4B and D), and both of them were more elastic than those obtained from R and H solutions. Fractions more enriched in κ-carrageenan produced firmer gels. H-0.125 was constituted by nearly pure κ-carrageenan and showed higher molecular weight than R-0.125, characteristics that coincided with a higher value of G' modulus for H-0.125. R-0.125 fraction showed some ι-carrageenan character. When subsequently submitted to heating, these gels showed similar profiles to those previously observed for R and H systems (Fig. 3B and D). Syneresis and consequent slippage observed during the 900-s resting time of H-0.125 gel is later revealed during heating through the peak observed at ≈45 °C (Fig. 3D). The reduction in G' modulus with time (Fig. 4C) could be attributed to continued helix-helix aggregation on holding of pure κ-carrageenan, resulting in increased syneresis and consequent slippage, and the anomalous increases of G' in Fig. 3D to reduction in slippage as the network become weaker during heating. The observed melting temperatures were 56.0 °C for R-0.125 and 63.6 °C for H-0.125 carrageenan gels (Table 4). Again, the H-0.125 gel showed higher thermal stability (with higher molecular weight) than R-0.125 gel but both derivatives were slightly less stable than R and H fractions.

R-0.8-1 and H-1-1.25 fractions were also studied rheologically in the presence of KCl (Fig. 3A and C). Gelling temperatures of 39.5 and 42.6 °C were observed upon cooling for R-0.8-1 and H-1-1.25, respectively (Table 4). Approaching ≈10 °C, G' was one order of magnitude higher for H-1-1.25 (≈25,600 Pa) than for R-0.8-1 fraction (≈4970 Pa), and the loss modulus (G'') was once again one order of magnitude lower than G' (Fig. 3A and C). These values are more similar to those occurring for R and H, respectively. Considering that these networks developed upon cooling, the R-0.8-1 system showed no dependence on time along the 900 s period studied at 10.0 °C of constant temperature (Fig. 4A). On the other hand, the H-1-1.25 carrageenan sample was slightly dependent, with G' decreasing with the rise in holding time, as occurred for H-0.125 (Fig. 4C). This fact was associated to some degree of syneresis (Richardson & Goycoolea, 1994). The mechanical spectra recorded between 1 and 100 rad/s of ω, at 10.0 °C, once confirmed true gels. That developed from the H-1-1.25 fraction was again more elastic ($G' \approx 19{,}600$ Pa) than the gel produced by the R-0.8-1 fraction ($G' \approx 5500$ Pa) (Fig. 4B and D). The H-1-1.25 gel showed the lowest elasticity (G') relative to the other equivalent fractions. In comparison with R-0.125 and H-0.125, H-1-1.25 and R-0.8-1 are enriched in ι-carrageenan. H-1-1.25 contained less ι-diads than R-0.8-1 and has a higher molecular weight. These facts may contribute to increase the elastic character of the H-1-1.25 gels with respect to those developed by the R-0.8-1 fraction. R-0.8-1 and H-1-1.25 gels melted upon heating (10 °C/min) at the same higher temperature (72.5 °C) (Fig. 3B and D and Table 4). As occurred for H-0.125, a peak was also observed (≈52 °C) during heating of H-1-1.25 gel (Fig. 3D), which may indicate syneresis and consequent slippage (Richardson & Goycoolea, 1994). However, the time dependence of H-1-1.25 gel is lower than that observed for H-0.125 (Fig. 4C) as ι-carrageenan is affecting this gelation process. Later, the anomalous increases of G' in Fig. 3D may be attributed to reduction in slippage as the network become weaker during heating.

R-2S and H-2S fractions showed very different behaviors upon cooling. H-2S fraction constituted a gel just at 85.0 °C, with G' above G'' along the complete temperature sweep (Fig. 3E) and resting period (Fig. 4E), whereas R-2S did not set on cooling even after aging for a 900 s-period (Fig. 4E). The mechanical spectrum indicated an unstructured system (Fig. 4F). Afterwards, the mechanical spectrum recorded from H-2S between 1 and 100 rad/s of angular frequency (ω), at 10.0 °C, showed a profile that can be ascribed to a weak gel system, not to a true gel, with both moduli of the same order of magnitude and dependent on frequency (Fig. 4F). G' was above G'' up to 43.8 rad/s and its value increased with frequency from ≈62 to ≈132 Pa. Beyond 43.8 rad/s, G'' was above G' up to 100 rad/s. This may be caused by the characteristic relaxation times of the H-2S weak gel network, which could be shorter than the times involved at the highest experimental frequencies. Heating of H-2S system also presented G' above G'' and both had the same order of magnitude (Fig. 3F), and a melting temperature was not observed. R-2S and H-2S differed from the other fractions in their higher heterogeneity, containing mixtures of carrageenans, agarans, D/L-galactans, other polysaccharides, and even co-extracted proteins giving rise to a wide distribution of molecular weights (Table 1).

Andrade et al. (2000) have made a comprehensive rheological study on carrageenans from *H. musciformis*. However, the data is not comparable to those of the current study, as the purification method was different, and they used a 0.5% carrageenan solution in 0.1 M KCl, yielding lower G' (≈4000 Pa) and G″ values, and higher melting and gelling temperatures (55 °C) than those determined in the present work.

The distinct carrageenan structures differ in 3,6-anhydrogalactose and ester sulfate content as well as in their molecular weights. Variations in these components influence hydration, gel strength and texture, melting and setting temperatures, syneresis and synergism (Imeson, 2000). Considering the chemical composition of the isolated carrageenan fractions it can be concluded that both the elasticity and thermal stability of gel networks developed from KCl aqueous solution increases with the κ-carrageenan content. Besides, all the KCl-precipitated fractions produced stable gels at 10 °C. It has been reported that κ-carrageenan forms a firm gel with potassium ions, whereas ι-carrageenan is only slightly affected (Al-Alawi, Al-Marhubi, Al-Belushi, & Soussi, 2011). In the present work, an increase in the ι-carrageenan proportion shows a trend toward an increase of the gelling and melting temperatures, as already shown by Souza et al. (2011). This can be explained in terms of their gelation mechanisms: both ι- and κ-carrageenan form threefold, right-handed, parallel double helices stabilized by interchain hydrogen bonds, but their juxtaposition in κ-carrageenan is significantly different because they are offset from the half-staggered arrangement by a 28° rotation and a 1.0-Å translation (Millane, Chandrasekaran, Arnott, & Dea, 1988). This fact, together with its lower sulfation, bring on less hydrogen bonds stabilizing the κ-carrageenan double helix (Chandrasekaran, 1998) which is, besides, more dependent on the K^+ concentration and temperature than the ι-carrageenan double helix. Therefore, it is expected that the gelification of κ-carrageenan will be affected by the presence of ι-carrageenan. Given the low K^+ concentration herein used (45 mM) and the lower hydrogen-bonding in κ-gels, it makes sense that the gelling and melting temperatures are lower when the κ-carrageenan/ι-carrageenan ratio increases. Hence, as R-0.125 and H-0.125 fractions are essentially constituted by κ-carrageenan, they showed the lowest gelling and melting temperatures (Table 4). The slightly higher melting temperature for the H-0.125 gel than for R-0.125 may be related to its higher molecular weight (Table 1). As the ι-character increased in the remaining isolated fractions, the gelling and melting temperatures also rose (Table 4). These facts remark the importance of temperature as a relevant factor in deciding which carrageenan should be used in a pharmaceutical or food formulation (Andrade et al., 2000; Imeson, 2000).

4. Conclusion

H. musciformis is a valuable source of gelling carrageenans, especially κ-carrageenan. Extraction with cold and hot water and fractionation by potassium chloride precipitation allowed to find high yields of an essentially pure κ-carrageenan (H-0.125), together with lower amounts of a κ-carrageenan with a small ι-character (R-0.125), and even lower proportions of two κ/ι-hybrids containing small amounts of precursor *ν*-carrageenan units (R-0.8-1 and H-1-1.25). Deviant heterogeneous fractions have also been isolated, soluble in potassium chloride (R-2S and H-2S). The last two fractions either did not gel or produced very weak type gels. The rest of the isolated fractions formed true gels at 10 °C, and melted by heating above 55 °C. The fractions extracted with hot water formed stronger gels (almost one order of magnitude larger G') than those extracted at room temperature. Within each group, gel elasticity increased as fractions were purified in κ-carrageenan, at the same time that the gelling and melting temperature decreased. Thus, the purified fractions yielded much better gelling behavior than the crude carrageenans. The carrageenans isolated from *H. musciformis* are valuable biopolymers to be used in pharmaceutical, chemical and food industries, and may also be promising as ecological mobility control agents in dwindling petroleum reserves.

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