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Tailoring kappa/iota-hybrid carrageenan from *Mastocarpus stellatus* with desired gel quality through pre-extraction alkali treatment

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ABSTRACT

Mastocarpus stellatus seaweeds were subjected to different alkali pre-treatments to investigate the effect of alkali type, concentration and treatment duration on the chemical structure and the gelling properties of extracted kappa/iota-hybrid carrageenan (KI). Increasing the concentration in KOH and the pre-treatment time gives KI with lower amounts of nu-carrageenan units, which have a direct impact on the improved gelling properties. However, excessive KOH concentration and pre-treatment duration give KI with smaller molecular mass and depressed gel properties. NaOH is more efficient in converting nu-carrageenan to iota-carrageenan, but no correlation between the chemical structure and gel properties was found. An interaction between the alkali concentration and the pre-treatment duration complicates the rationalization of NaOH efficiency. A set of KI with 60–75 mol% kappa-carrageenan and tailored gelling properties for a wide range of applications is delivered.

1. Introduction

Carrageenans are industrially important hydrocolloids obtained from numerous red seaweeds (Rhodophyta). They are built on a disaccharide backbone of alternating 3-linked β-D-galactopyranose (**G**) and 4-linked α-D-galactopyranose (**D**). Several types of carrageenans are recognized according to the position of sulphate/s (**S**) in the disaccharide repeating unit, cyclization of the **D** units forming an anhydro ring (**DA**), and presence of pyruvate (**P**) on **G** units. Colloquially, carrageenan types are named based on the Greek alphabet, but a new nomenclature based on chemical structure, with corresponding alphanumeric codes, has been proposed (Knutsen, Myslabodski, Larsen & Usov 1994). Different types have distinct gel and solution characteristics that may be suitable for specific texturing applications in food (essentially dairy products), cosmetics and pharmaceutics (Bixler & Porse, 2011; Campo, Kawano, da Silva, & Carvalho, 2009). Commercially available types include the gelling kappa- and iota-carrageenan (**G4S-DA** and **G4S-DA2S**, respectively) and the non-gelling lambda-carrageenan (**G2S-D2S6S**). A relatively new commercial carrageenan product is the kappa/iota-hybrid carrageenan (KI, also known as kappa-2 carrageenan), which has more and more gained recognition in food industry with its diversified functionality as texturing agent (Bixler & Porse, 2011; van de Velde, 2008; Villanueva, Mendoza, Rodrigueza, Romero, & Montaño, 2004). In general terms, kappa-carrageenan gels are hard, strong and brittle, whereas iota-carrageenan forms soft and weak gels that are shear reversible. So, the rheological implication of the hybridization is the weakening of the hard, brittle gel of kappa carrageenan by the soft, elastic gel character of iota carrageenan (Falshaw, Bixler, & Johndro, 2003). As shown in Fig. 1, the chemical structure of KI can be modelled as a copolymer possessing a statistical distribution of sequences (blocks) of kappa- and iota-carrageenan, and also sequences of carrageenan biological precursors such as mu- and nu-carrageenans (**G4S-D6S** and **G4S-D2S6S**, respectively). The length and distribution of sequences depend on the family and the life stage of seaweeds (van de Velde et al., 2005). This comes as no surprise as the quality of commercial kappa- and iota-carrageenan is known to be affected by seaweed culture conditions (Hayashi et al., 2007; Hurtado, Critchley, Trespoey, & Bleicher-Lhonneur,

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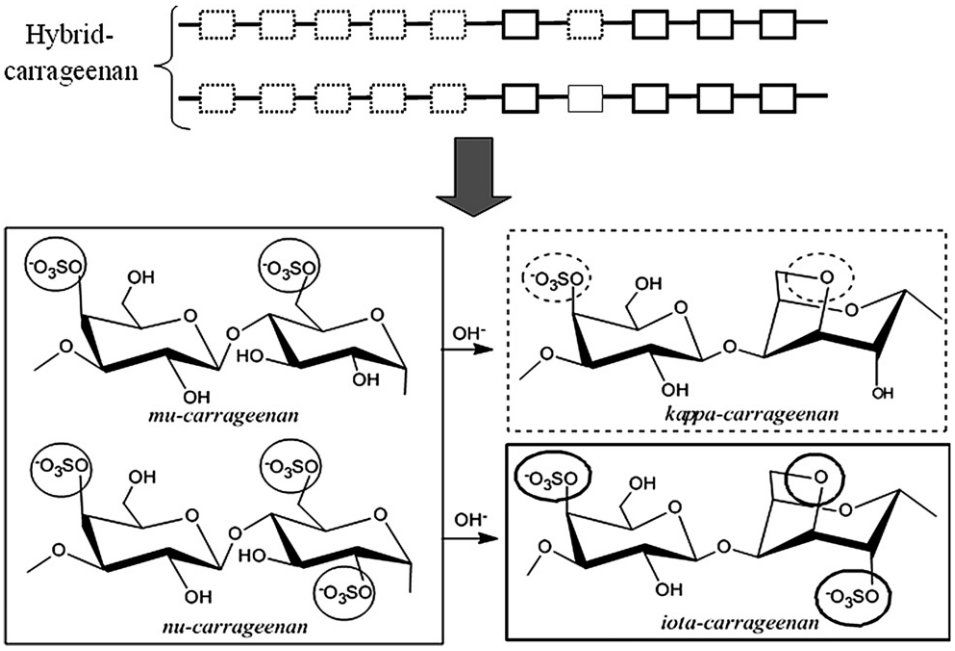

Fig. 1. Chemical structure of kappa/iota-hybrid carrageenans (top) showing blocks of kappa-carrageenan (**G4S-DA**, dashed boxes) and iota-carrageenan (**G4S-DA2S**, thick boxes), which are separated by disaccharide units or blocks of mu-carrageenan (**G4S-D6S**, thin boxes) and nu-carrageenan (**G4S-D2S6S**, thin boxes) prior to alkali treatment.

2008), season when collected (Hung, Hori, Nang, Kha, & Hoa, 2008; Wakibia, Bolton, Keats, & Raitt, 2006), seaweed health or disease state (Mendoza, Montaño, Ganzon-Fortes, & Villanueva, 2002), state of maturity of the seaweed material (Mendoza, Ganzon-Fortes, Villanueva, Romero, & Montaño, 2006; Rivera-Carro, Craigie, & Shacklock, 1990), and post-harvest storage (Hilliou et al., 2011; Romero, Villanueva, & Montano, 2008). The extraction method (temperature, pH, duration) used to isolate KI from the seaweed also critically affects the chemical structure and thus the gel properties (Hilliou, Larotonda, Abreu et al., 2006; Hilliou, Larotonda, Sereno, & Gonçalves, 2006).

The chemical structure of KI can further be modified with the employment of alkali treatment prior to extraction (Pereira & van de Velde, 2011). Alkali treatment is a step in the industrial processing of seaweed galactans (carrageenan and agar) which converts the 'precursor', L/D-galactose-6-sulphate, moieties in the galactan backbone to 3,6-anhydrogalactose (Rees, 1972), with corresponding increase in gel strength of the extract. The precursor units serve as kinks in the galactan which restrains the formation of double-helix network for the gelation process (Rees, 1969). Depending on the extent of conversion of the precursor units to 3,6-anhydrogalactose, a spectrum of gel properties can be obtained when different levels of alkali treatment conditions were employed. Expectedly, a native extract (no alkali pre-treatment) is at the low end of the gel quality spectrum, while alkali pre-treatment with high alkali concentration and long duration would yield carrageenan whose gel quality is placed at the high end (provided no degradation occurs at certain extreme conditions).

Here, we determine the effect of alkali concentration and pre-treatment duration on the yield and physico-chemical properties of KI extracted from the red seaweed, *Mastocarpus stellatus* (Stackhouse) Guiry, collected in north-western Portugal. The main objective is to optimize the extraction of a KI with virtually no precursor on the polysaccharide backbone, thus extending earlier results (Hilliou, Larotonda, Abreu et al., 2006) to different types of alkali and to the chemical characterization of the raw seaweed. Optimized KI extracts, with different counter ions, will facilitate future investigation into the relationship between chemical structure and gelling properties, much needed for this type of carrageenan (Souza, Hilliou, Bastos, & Gonçalves, 2011; van de Velde, 2008). In this respect, *M. stellatus* can be considered a model seaweed for the following reasons: First, only the gametophytic life stage of the seaweed can be harvested, since the tetrasporophytic life stage with inherent highly sulphated carrageenans (lambda-, mu-, nu-carrageenan, etc.) is crustose and hard to remove from inter tidal rocks; Second, *M. stellatus* shows a wide geographical distribution with high cover and biomass in northern Portugal, with limited variability in the chemical structure of extracted KI attributable to collection site conditions (Pereira & van de Velde, 2011); Third, *M. stellatus* is a promising candidate for Integrated Multitrophic Aquaculture (Abreu, Pereira, Mata, Nobre, & Sousa Pinto, 2011; Domingues, 2010) and thus a future sustainable resource for the production of KI in northern Portugal, with presently an important industrial relevance (Bixler & Porse, 2011).

2. Experimental

2.1. Seaweed sampling and postharvest storage

M. stellatus gametophytes were collected from inter tidal rocks at Mindelo, Vila do Conde, Portugal (41°18′37.5″ N, 8°40′33.0″ W) during low tide in April 2010. At the lab, samples were washed with freshwater, cleaned of extraneous matter (sand, rocky material clinging to the holdfast, other seaweeds, and invertebrates – mollusks and crustaceans). The seaweeds were then dried and stored as detailed elsewhere (Hilliou et al., 2011) to ensure optimum post-harvest preservation until extraction of kappa/iota-hybrid carrageenan. Remaining fresh seaweeds were used to conduct the separate study of bioremediation efficiency in an integrated multi-trophic aquaculture (Domingues, 2010).

2.2. Alkali pre-treatment and carrageenan extraction

Dried seaweeds were ground to a powder and 1.5 g was soaked in 50 ml NaOH or KOH alkali solution of different concentrations (0,

0.5, 1, 2, 3, 5 and 10% w/v) at 80 °C and during 1, 3 and 5 h. Distilled water (50 ml) was added to cool down the suspensions to 40 °C, and the pH was adjusted to 8–9 with 5 M HCl to stop the alkali action during the extraction process. The carrageenan extraction consisted heating the suspension to 90 °C for 1 h. Then the suspension was cooled to 50–55 °C and treated with amylase (1 mg/g algal material; amyloglucosidase from *Aspergillus niger*, Fluka Biochemika) for 1 h with stirring to digest any Floridean starch present. After this, the temperature was elevated to 80 °C to facilitate the centrifugation of the viscous suspension at 8000 rpm for 10 min. The supernatant was then precipitated in 2.5 volumes of 96% ethanol. Precipitate was collected using cotton clothes and washed twice with ethanol, then oven-dried at 60 °C. The dried carrageenan was weighed to obtain the yield and then pulverized using a coffee grinder. Extractions were performed in triplicates for each alkali concentration and pre-treatment duration.

2.3. Spectroscopic analyses

The Diffuse Reflectance Infrared Fourier Transform (DRIFT) method was used for the qualitative and semi-quantitative characterization of the native seaweed and the extracted KI. Dried seaweeds were directly scratched onto the abrasive pad of the DRIFT accessory of the FTIR Spectrometer (Spectrum 100, PerkinElmer Ltd., United Kingdom), whereas KI powder samples were deposited on the DRIFT accessory. Each recorded spectrum is the average of 16 scans acquired at 4 cm^{-1} resolution. The absorbance of peaks attributed to total sulphate (1240 cm^{-1}) and 3,6-anhydrogalactose (**DA**, 930 cm^{-1}) were normalized with respect to that of peak attributed to C–H (2920 cm^{-1}, referring to the total sugar content) and the resultant ratios, respectively A_{1240}/A_{2920}, and A_{930}/A_{2920} served as semi-quantitative indices of the chemical composition of extracts (respectively sulphate content and **DA** contents). In addition, the peak absorbance assigned to 2-sulphate of **DA** (**DA2S**, 805 cm^{-1}) was normalized with respect to that assigned to 4-sulphate of **G** (**G4S**, 845 cm^{-1}) giving an absorbance ratio A_{805}/A_{845} indicative of the relative content in iota-carrageenan.

Nuclear Magnetic Resonance spectra were recorded non-spinning at 80 °C and 500 MHz using a Varian VNMRS spectrometer (Agilent Inc., USA). The probe used was a 5 mm HCX triple probe equipped with triax gradients of which only the z-gradient was used. The samples were prepared at 0.6–1% w/v and 3-(trimethylsilyl)propionic-2,2,3,3-D4 acid sodium salt (TSP) was added as an internal chemical shift reference ($\delta_H = -0.017$ ppm; $\delta_C =$ 0.18 (van de Velde, Pereira, & Rollema, 2004)). Proton and HMQC experiments were acquired using standard Varian pulse sequences. HMQC spectra were acquired within 17 h with 4096 complex points in the direct dimension (^1H, $\delta = 12--1$ ppm), 256 time increments (^{13}C, $\delta = 120--10$ ppm) and 16 transients per increment. Gaussian weighting was applied to both dimensions with time constants gff1 = 0.015 and gff2 = 0.073, 1024 complex points were used in each dimension for the 2D Fourier transform. The inter-scan delay was 1.5 s and 64 steady-state dummy scans were used. In addition, ^1H NMR spectroscopy was performed using a Varian Unity plus 300 Spectrometer (Agilent Inc., USA) operating at 300.13 MHz for the quantitative determination of carrageenan fractions. Spectra of 0.5% (w/v) polysaccharide solutions in D_2O were obtained at 60 °C. Chemical shifts (ppm) were referenced to the D_2O lock signal (4.41 ppm) and further adjusted to match the chemical shift for kappa-carrageenan (5.09 ppm), as prescribed by van de Velde et al. (2004). The molar fractions (% mol) of the carrageenan repeating units (kappa and nu) are calculated as the integrated intensity of the corresponding ^1H NMR peak (5.09 and 5.5 ppm, respectively) over the sum of the integrated intensities of all assigned carrageenan anomeric protons.

2.4. Gels preparation and viscoelastic characterization

KI extracts (1% w/v) were dissolved in 1 M NaCl (for alkali pre-treatment carried out with NaOH) or 0.1 M KCl (for alkali pre-treatment carried out with KOH) during 1 h under strong stirring at 80 °C. These concentration and ionic strength were chosen in order to obtain gels with sufficient rigidity at 20 °C and with no water release, while keeping a single cation (K^+ or Na^+) to promote the gel formation. Hot KI solutions were directly poured in the pre-heated (at 80 °C) Couette accessory (CC27) of a Paar Physica MCR300 rheometer (Anton Paar, Austria), and the surface geometry was covered with dodecane to prevent water loss. Measurements were performed in triplicates to determine the averages and standard deviations of the gel setting temperature T_g, the gel elasticity G_0 and the gel melting temperature T_m, using an experimental protocol described elsewhere (Souza et al., 2011), but cooling down to 20 °C. The intrinsic viscosity [η] of selected extracts was measured in triplicates with a capillary viscometer (501 – 10 Type I, SCHOTT, Germany) using KI solutions prepared as above but with concentrations ranging from 0.06% w/v to 0.01% w/v in 0.1 M NaCl at 25 °C and using the Huggins' equation to fit the data.

2.5. Statistical analysis

A two-way analysis of variance (ANOVA) was used to determine the main and interactive effects of alkali concentration and pre-treatment duration on carrageenan yield, gel and chemical properties. Tukey's studentized range (HSD) test was carried out as *post hoc* comparison procedure. Microcal Origin software (Microcal Software, Inc., Northampton, MA) version 8.0 was used to carry out the statistical analysis.

3. Results and discussion

3.1. Chemical structure of M. stellatus and extracted KI

The DRIFT spectrum of *M. stellatus* displayed in Fig. 2 shows all the characteristic bands of carrageenan but the one assigned to **DA2S**, which is specific to iota-carrageenan. This is in contrast with all the other spectra gathered in Fig. 2 which indicate that all the extracts have kappa- and iota-carrageenan disaccharide units in their backbone, even when no alkali pre-treatment is employed. In addition, a shoulder located at 975 cm^{-1} for the seaweed turns into an absorption band for the KI extracts. The shoulder is related to highly sulphated carrageenans, whereas a strong band is reported for alkali modified iota-carrageenans and agars (Chopin, Kerin, & Mazerolle, 1999). Thus, the spectra in Fig. 2 confirm that *M. stellatus* is essentially a carrageenophyte from which KI are extracted.

A qualitative assessment of the effect of alkali pre-treatment on the chemical structure of extracted KI can be inferred from the HMQC spectra presented in Fig. 3. The spectrum of KI extracted without alkali pre-treatment (Fig. 3A) presents signals which match the reported shifts for nu- and mu-carrabiose moieties (**D2S6S** and **D6S**, respectively) obtained from enzymatic degradation of hybrid carrageenans (Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010; Jouanneau, Guibet et al., 2010). Interestingly, data in Fig. 3A do not show signals of anomeric protons of **D2S6S** and **D6S** which are the hallmark of nu- and mu-carrageenans, respectively. The high viscosity of the sample probably explains the low resolution of the HMQC spectrum and the consecutive absence of HMQC signal in the 100 ppm region where the anomeric proton of

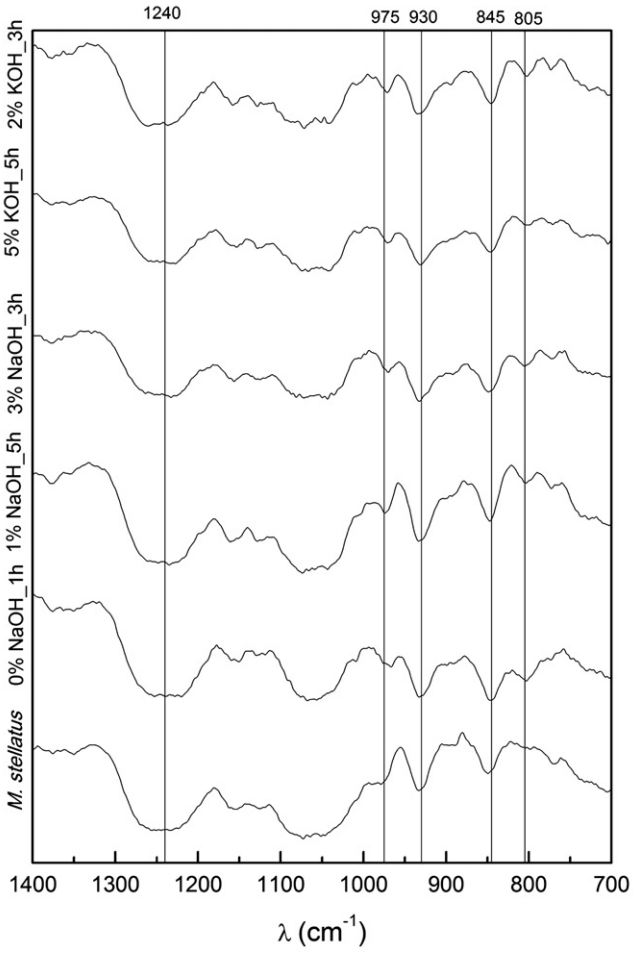

Fig. 2. DRIFT spectra of *M. stellatus* native seaweed and carrageenans extracted using different concentrations of NaOH or KOH and different alkali pre-treatment durations. Bands assigned to total sulphate (1240 cm^{-1}), alkali modified galactose (975 cm^{-1}), 3,6-anhydrogalactose – **DA** (930 cm^{-1}), **G4S** (845 cm^{-1}) and **DA2S** (805 cm^{-1}) are marked accordingly with vertical lines.

kappa-carrageenan (**DA1**) is resolved. However, separate ^{1}H NMR spectra show the peak associated with nu-carrageenan (see also Fig. 4 for a quantitative analysis of this peak), together with a shoulder between the peaks assigned to iota- and kappa-carrageenan, which confirms a low amount of mu-carrageenan. Thus the native KI of *M. stellatus* is made of kappa-, iota-, mu- and nu-carrageenan with no agarocolloids, since no 6-O-methyl substitution at around 3.42, 61.6 ppm (^{1}H, ^{13}C) is resolved (Villanueva, Sousa, Gonçalves, Nilsson, & Hilliou, 2010). After an alkali pre-treatment of 5 h and 5% w/v KOH, an HMQC spectrum devoid of any signal originating from carrageenan precursors is obtained, and the KI in Fig. 3B shows peaks corresponding to signals reported in the literature for kappa- and iota-carrageenan (van de Velde et al., 2004). The absence of any signal at 1.44 ppm and 5.35 in ^{1}H NMR spectra (results not shown) also suggests that the KI obtained with the strongest KOH treatment is free of any pyruvate and floridean starch, respectively. A similar result is obtained with NaOH. This is in contrast with a recent report (Pereira & van de Velde, 2011) where a milder alkali treatment with NaOH was used with no amylase digestion, and validates the pertinence of the optimization of the alkali pre-treatment carried out here. The qualitative chemical characterization presented in Fig. 3 suggests that the conversion of **D6S** into **DA** and **D2S6S** into **DA2S** using KOH or NaOH in hot pre-treatment is more efficient than a cold pre-treatment using Na_2CO_3 (see for instance Fig. 1 in Hilliou, Larotonda, Abreu et al., 2006, where NMR signal assigned to nu-carrageenan is resolved).

3.2. Effect of alkali pre-treatment

Yield of carrageenan is affected significantly by the alkali pre-treatment (see Tables 1 and 2). The yield for native carrageenan (treated with 0% alkali) is significantly smaller than the yield achieved with 0.5–5 % w/v NaOH, but larger from those treated with more than 3% w/v KOH (Table 3). Overall, longer alkali pre-treatment favours the recovery of more KI (Table 3) and yields obtained with NaOH are larger than those achieved with KOH (40–50% against 30–43%, data not shown). The yield does not differ significantly when using different concentration of NaOH (within the range studied here) or using 0.5–3 % w/v KOH. However, the yield for seaweeds treated with 5% KOH drops significantly (Table 3). Indeed, a degradation of KI under such strong alkali

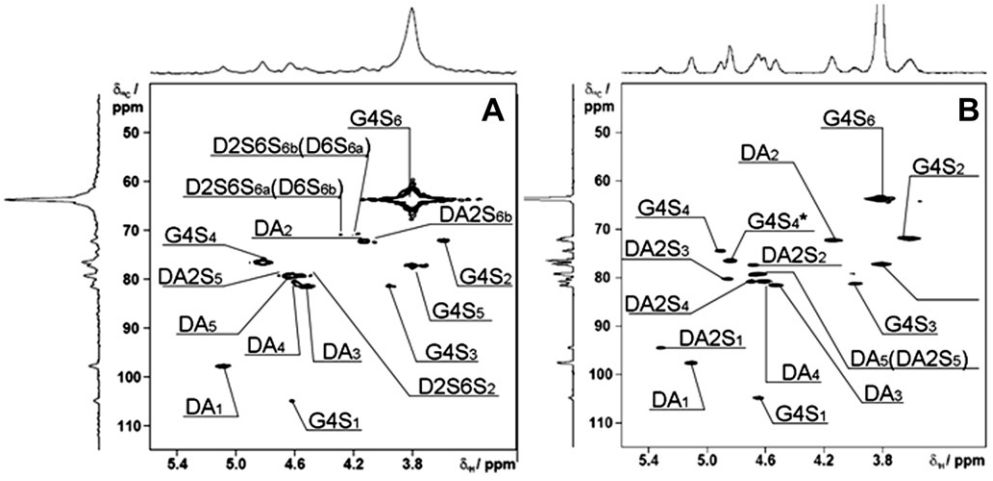

Fig. 3. HMQC spectra with ^{1}H (top) and ^{13}C (left) projections of carrageenans extracted from *M. stellatus*, without any alkaline pre-treatment (A) and employing 5 h alkaline pre-treatment with 5% w/v KOH (B). The asterisk (*) indicates that the signal is assigned to kappa- and mu-carrageenan whereas the other signal labelled $G4S_4$ is assigned to iota-carrageenan. Numerical subscripts refer to the carbon number in each residue. In (B), the missing labelled signal is G_4S_5.

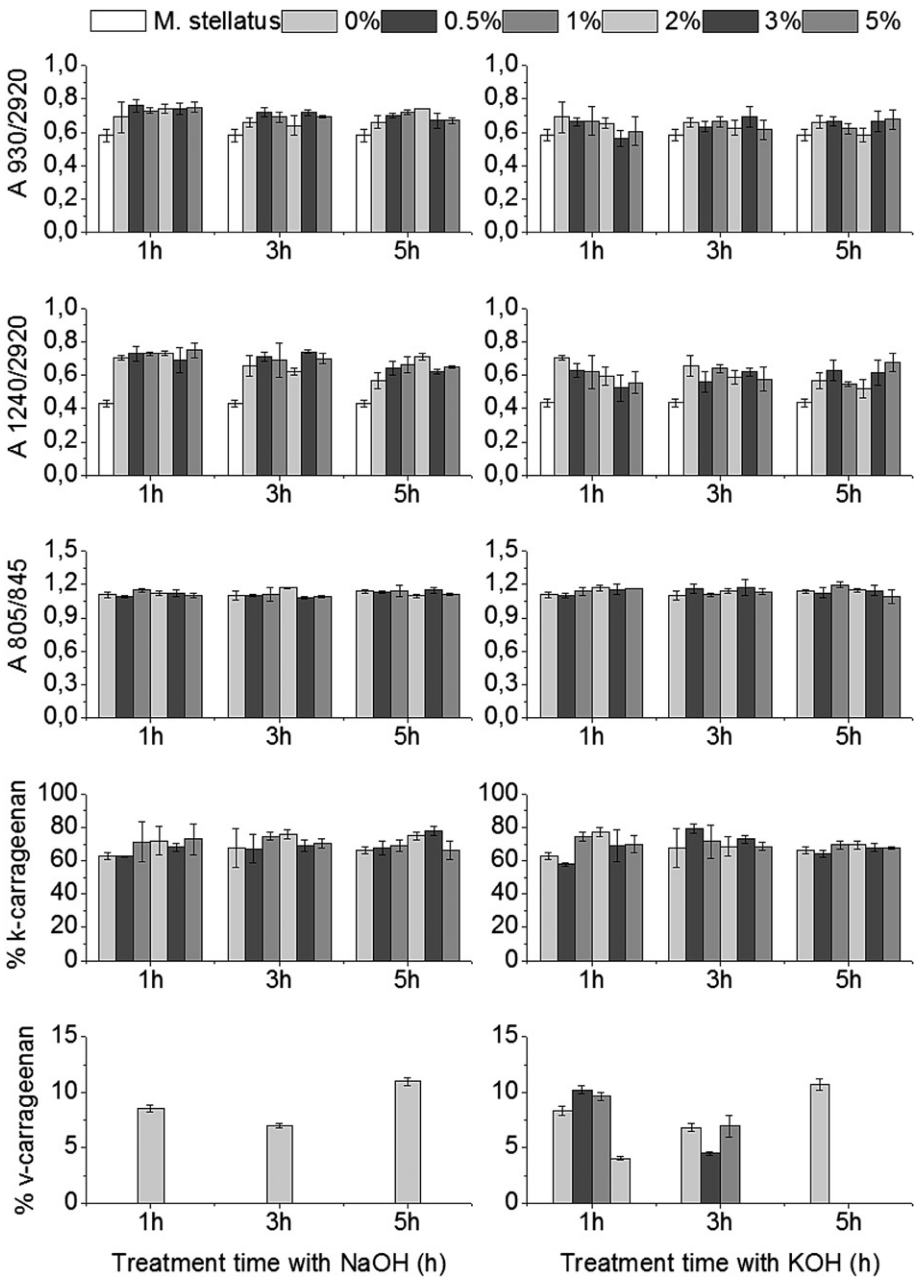

Fig. 4. Chemical properties (mean ± s.d., $n = 3$) of carrageenans extracted from *M. stellatus* pre-treated with different alkali concentrations (left column, NaOH; right column KOH) for different durations. The molar fraction (% mol) of the carrageenan repeating units is defined as the integrated intensity of the corresponding ^{1}H NMR peak over the sum of the integrated intensities of all assigned carrageenan anomeric protons. Differences among concentrations and pre-treatment durations are presented in Table 3 for NaOH and Table 4 for KOH.

pre-treatment is suspected (see also below) as gelatinous precipitates in alcohol were produced which are hard to collect, whereas long fibrous precipitates were easily recovered at smaller alkali concentration. These results are not in agreement with those observed in *Eucheuma isiforme*, showing increasing yields with increasing KOH concentrations (Freile-Pelegrín, Robledo, & Azamar, 2006). Differences in protocols used to separate KI solutions from seaweeds and to recover the precipitates may explain such discrepancy.

Ratios of DRIFT bands and of ^{1}H NMR integrated peak intensities are presented in Fig. 4, and the statistical analysis of corresponding data is reported in Tables 1 and 2. The concentration in NaOH has no significant effect on the relative contents in iota-carrageenan, sulphate and **DA**. Longer pre-treatment duration leads to a significant increase in A_{805}/A_{845} and to a significant decrease in A_{1240}/A_{2920}, which indicates that the conversion of the alkali-labile sulphate at 6-position in **D2S6S** into **DA2S** is a rather slow process, within the time range studied here. The relative content in **DA** is significantly decreasing with the increasing pre-treatment time. This result is hard to reconcile with the statistical analysis of both A_{805}/A_{845} and A_{1240}/A_{2920}. However, the significant interaction between NaOH concentration and pre-treatment duration (see Table 1) suggests that opposite effects of one parameter on the performance of the other could explain the overall drop of A_{930}/A_{2920}. The semi-quantitative chemical analysis carried out with DRIFT data is not sensitive enough to extract any information on the

Table 1
Two-way ANOVA on the effect of NaOH alkali concentration (A) and pre-treatment duration (B) on properties of carrageenan from *M. stellatus*. The degrees of freedom (df) for $N = 54$ and $n = 3$ is: factor $A = 5$; factor $B = 2$; interaction $A \times B = 10$ and Total = 53.

Carrageenan property	NaOH concentration (A)		Pretreatment duration (B)		$A \times B$	
	F	p	F	p	F	p
Carrageenan yield	7.636	<0.001*	17.979	<0.001*	1.517	<0.001*
A_{805}/A_{845}	1.834	0.131	4.605	0.0166*	3.658	0.00193*
A_{930}/A_{2920}	1.938	0.112	7.465	0.00194*	2.202	0.0407*
A_{1240}/A_{2920}	2.397	0.0563	11.466	<0.001*	4.599	<0.001*
% kappa-carrageenan	1.994	0.128	0.646	0.536	0.650	0.754
% nu-carrageenan	3481.63	<0.001*	59.187	<0.001*	59.187	<0.001*
G_0	28.330	<0.001*	1.723	0.208	11.129	<0.001*
T_g	9.950	<0.001*	7.650	<0.001*	2.000	0.0960
T_m	15.310	<0.001*	8.850	<0.001*	2.960	<0.001*

*Significant ($p < 0.05$).

effect of KOH pre-treatment on the chemical structure of isolated KI. Table 2 shows that no significant variation in the DRIFT ratios could be related to either KOH concentration or alkali pre-treatment duration. Assuming that KOH essentially converts mu-carrageenan into kappa-carrageenan (see below), we suspect that the low amount of **D6S** moieties inferred from the ^{1}H NMR carried out with native extracts will not be detected in DRIFT, and thus its conversion into **DA** will not affect the band ratios studied in Table 2. However, earlier reports on the effective alkali conversion of nu-carrageenan into iota-carrageenan using KOH (Freile-Pelegrín et al., 2006) do not support the KOH specificity hypothesized here. We note however that these authors conducted the alkali pre-treatment on *E. isiforme* (Solieriaceae, Rhodophyta) at room temperature and the hot extraction of the iota-carrageenan was performed under alkali conditions.

The quantitative analysis obtained from the ^{1}H NMR spectroscopy essentially shows that NaOH is more efficient than KOH in converting nu-carrageenan to iota-carrageenan as lower alkali concentration and pre-treatment time are needed to reduce the signal assigned to the alpha-anomeric proton of **D2S,6S** (5.5 ppm) below the detection limit of NMR (see Fig. 4 and corresponding values of F-ratios for nu-carrageenan in Tables 1 and 2). The alkali pre-treatment with KOH or NaOH does not significantly affect the molar fraction of kappa-carrageenan (Tables 1 and 2), which suggests that the conversion of mu-carrageenan (see signals labelled **D6S6a** and **D6S6b** in Fig. 3) remains quantitatively below the sensitivity of NMR, either because there is not enough **DS6** moieties to convert or because both NaOH and KOH are not efficient in the conversion of this carrageenan precursor. Table 2 also suggests that the pre-treatment duration (F-ratio 59.2) is a less significant parameter compared to NaOH concentration (F-ratio 3481.6), whereas for KOH both parameters show roughly similar importance. Overall, the ^{1}H NMR analysis is in harmony with the DRIFT analysis, and equally points towards the importance of optimizing the alkali pre-treatment as interactions between concentration and pre-treatment duration are significant. The optimization of the alkali pre-treatment leads to the extraction of KI containing 60–75 mol% of kappa-carrageenan and 40–25 mol% of iota-carrageenan. This is in agreement with Bixler (1996) but is slightly above the contents documented by Pereira and van de Velde (2011) and by Hilliou, Larotonda, Sereno et al. (2006), who did not optimize the NaOH alkali treatment (for the former report) or used Na_2CO_3 (for the latter report).

Fig. 5 shows the elastic modulus G_0 of gels obtained with all KI extracts. As expected, the alkali pre-treatment significantly improves G_0 for both types of alkali (Tables 1–3). However, above a critical concentration of alkali, the gel elasticity is depressed. This behaviour is more striking with NaOH, as larger concentrations in KOH were needed to probe the corresponding maximum in G_0. As such, optimal alkali concentration and pre-treatment duration are needed to extract KI with maximized gel elasticity. A maximum in the viscosity of iota-carrageenan solutions extracted from *E. isiforme* alkali treated with KOH was also reported by Freile-Pelegrín et al. (2006), and the drop in the viscosity with increasing alkali concentration was attributed to the degradation of the polysaccharide. To check for a possible KI depolymerisation at longer time and higher concentration, the intrinsic viscosity $[\eta]$ of selected samples was determined and results are displayed in the insets of Fig. 5. The maximum in G_0 coincides with a maximum in $[\eta]$. For higher alkali concentrations, a clear decrease in $[\eta]$ is seen which indicates that KI with smaller molecular mass is recovered. We thus conclude that strong alkali pre-treatment with NaOH or KOH causes the depolymerisation of extracted KI. Strong alkali pre-treatment yields smaller quantity of KI (only in the case of KOH, see Tables 1–3) and KI with $[\eta]$ smaller than 4 dL/g forms significantly weaker gels.

Both T_g and T_m significantly depend on the concentration in NaOH and on the duration of the alkali pre-treatment (Tables 1 and 2). However, this trend cannot be simply transposed to KOH, as no significant variation in T_m is seen and the duration of alkali pre-treatment with KOH does not affect T_g (Table 2). As the gel setting temperature is of relevance in specific applications (such as in pharmaceutics), Fig. 6 displays the spectrum of T_g achieved with the different extracted KI, together with the range of gel elasticity in NaCl and KCl. This figure further demonstrates that tailoring the alkali pre-treatment parameters allows the delivery of a set of gelling KI with tuned properties, viz., with gel elasticity ranging from 10 to 7000 Pa and gelling temperatures ranging from 30 to 60 °C, thus satisfying a broad spectrum of applications. Evidently, the type and concentration of counterion incorporated in the gelling solvent, and which comes along with the alkali treatment of KI have a preponderant role in the variation of gel elasticity. We thus compared the gel elasticity of 2 KI samples obtained with nearly similar concentrations in KOH or NaOH, and which gave the best gel elasticity in their respective salt KCl and NaCl. The result of this comparison is displayed in the inset of Fig. 6. The mechanical spectra of both gels obtained in a mixed salt solution (0.05 M KCl plus 0.05 M NaCl) with a concentration of 1% w/v are identical,

Table 2
Two-way ANOVA on the effect of KOH alkali concentration (A) and pre-treatment duration (B) on properties of carrageenan from *M. stellatus*. In this case the degrees of freedom (df) for $N = 54$ and $n = 3$ were: factor $A = 5$; factor $B = 2$; interaction $A \times B = 10$ and Total = 53.

Carrageenan property	KOH concentration (A)		Pretreatment duration (B)		$A \times B$	
	F	p	F	p	F	p
Carrageenan yield	9.038	<0.001*	7.967	<0.001*	3.848	<0.001*
A_{805}/A_{845}	2.165	0.0798	0.411	0.666	0.363	0.955
A_{930}/A_{2920}	2.343	0.0611	0.285	0.754	1.098	0.390
A_{1240}/A_{2920}	2.323	0.0630	0.0737	0.929	1.470	0.191
% kappa-carrageenan	1.473	0.247	2.097	0.152	2.268	0.0627
% nu-carrageenan	61.731	<0.001*	65.831	<0.001*	22.845	<0.001*
G_0	49.184	<0.001*	60.923	<0.001*	16.900	<0.001*
T_g	3.53	<0.001*	0.760	0.483	0.530	0.850
T_m	0.720	0.610	2.030	0.170	0.640	0.770

*Significant ($p < 0.05$).

Table 3
Tukey's studentized range (HSD) test groupings of different alkali concentrations with NaOH and KOH, and pre-treatment durations on properties of carrageenan extracted from *M. stellatus*. Treatments with the same letter (case specific) are not statistically different ($p > 0.05$). 'A' and 'a' represent the highest values, 'D' and 'b' being the lowest. See Figs. 4 and 6 for data.

Carrageenan property	NaOH									KOH									
	Concentration (% w/v)						Time (h)			Concentration (% w/v)							Time (h)		
	0	0.5	1	2	3	5	1	3	5	0	0.5	1	2	3	5	10	1	3	5
Carrageenan yield	B	A	A	A	A	A	b	b	a	A	A	A	A	A	B	C	b	ab	a
A_{805}/A_{845}	–	–	–	–	–	–	b	b	a	–	–	–	–	–		ND	–	–	–
A_{930}/A_{2920}	–	–	–	–	–	–	a	b	b	–	–	–	–	–	–	ND	–	–	–
A_{1240}/A_{2920}	–	–	–	–	–	–	a	a	b	–	–	–	–	–	–	ND	–	–	–
% nu-carrageenan	A	B	B	B	B	B	b	c	a	A	B	B	C	C	C	ND	a	b	c
G_0	C	C	AB	A	AB	B	b	ab	a	B	B	B	A	A	A	C	c	b	a
T_g	C	BC	A	A	AB	AB	b	ab	a	B	A	AB	AB	AB	A	C	–	–	–
T_m	C	B	AB	A	AB	B	b	a	a	–	–	–	–	–			–	–	–

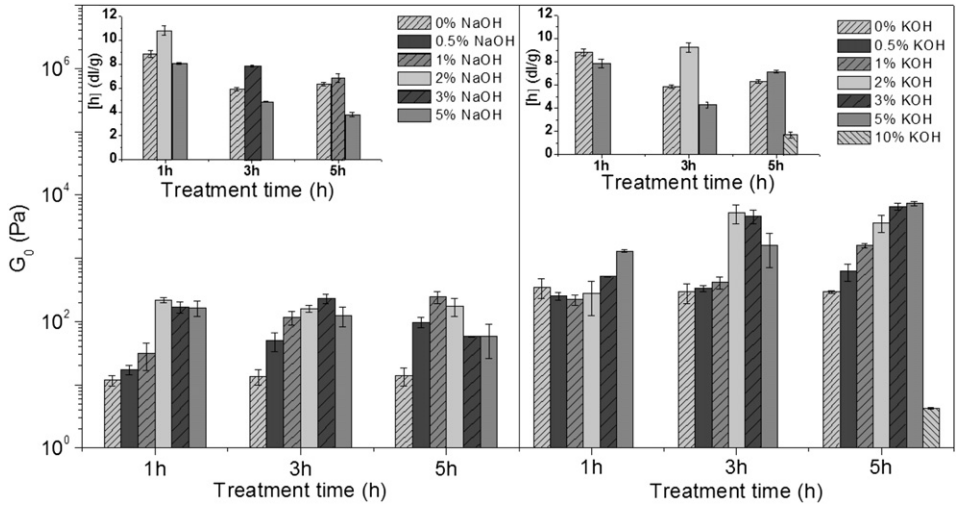

Fig. 5. Gel elastic moduli of kappa/iota-hybrid carrageenans from *M. stellatus* obtained with alkali pre-treatments using NaOH (left panel) and KOH (right panel). Insets present the intrinsic viscosity of selected extracts.

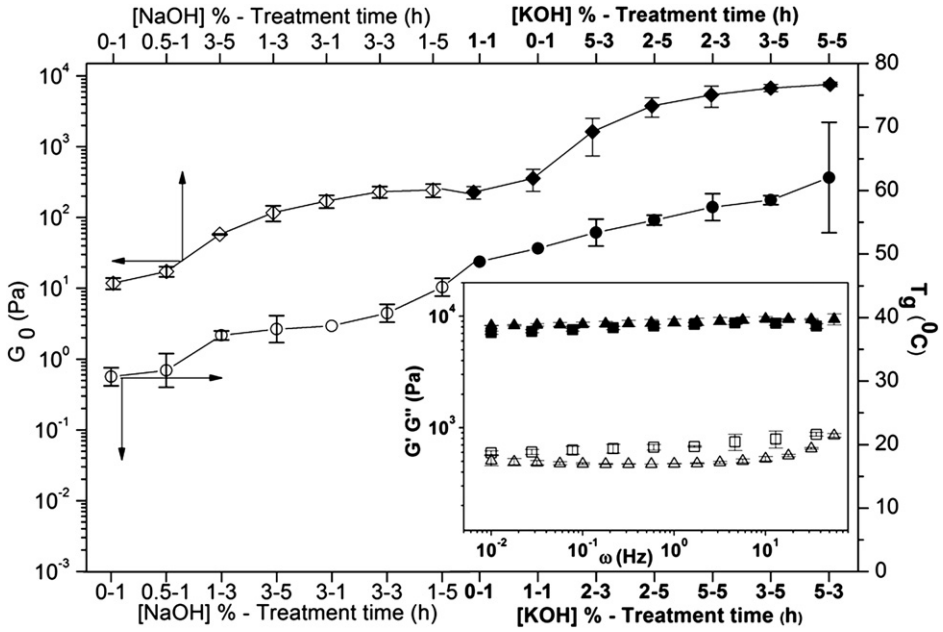

Fig. 6. Spectrum of gel elasticity displayed by kappa/iota-hybrid carrageenans extracted from *M. stellatus* using different alkali concentration, type (open symbols, NaOH; solid symbols, KOH) and pre-treatment duration. Inset: mechanical spectra (G', solid symbols; G'', open symbols) of 2 gels obtained in a mixed salt (0.05 M NaCl + 0.05 M KCl) at 20 °C and with 1% w/v kappa/iota-hybrid carrageenan extracted with 5% w/v KOH for 5 h (triangles) and 3% w/v NaOH for 3 h (squares).

Table 4
Pearson's correlation coefficient matrix of gel and chemical properties of carrageenan extracted from *M. stellatus*, using NaOH for alkali pre-treatment. $N = 54$.

	T_g	T_m	A_{805}/A_{845}	A_{930}/A_{2920}	A_{1240}/A_{2920}	% nu-carrageenan	Carrageenan yield
G_0	0.730*	0.800*	0.126	−0.384	−0.193	−0.150	0.163
T_g	1	0.883*	−0.0624	−0.382	−0.105	−0.219	0.230
T_m		1	0.0901	−0.377	−0.152	−0.312	0.251
A_{805}/A_{845}			1	−0.428	−0.779*	0.0564	0.319
A_{930}/A_{2920}				1	0.728*	−0.211	−0.167
A_{1240}/A_{2920}					1	−0.488	−0.209
% nu-carrageenan						1	−0.553*

*Significant ($p < 0.05$).

Table 5
Pearson's correlation coefficient matrix of gel and chemical properties of carrageenan extracted from *M. stellatus*, using KOH for alkali pre-treatment. $N = 54$.

	T_g	% nu-carrageenan	Carrageenan yield
G_0	0.554*	−0.590*	0.153
T_g	1	−0.518*	0.367
% nu-carrageenan		1	−0.158

*Significant ($p < 0.05$).

within the experimental errors of both rheometer and concentration determination of three replicates. This result shows that NaOH and KOH alkali treatments are equally good in yielding KI gels with similar elasticity and chemical composition (70 mol% kappa-carrageenan, 30 mol% iota-carrageenan, mu- and nu-carrageenan below ^{1}H NMR detection limit, see Fig. 4).

3.3. Relationships between chemical and gel properties of KI

Having produced a palette of KI with distinct chemical structures and gelling properties, we are now in a position to look for potential relationships. Tables 4 and 5 present the Pearson's correlation coefficients computed from the data presented in Figs. 4 and 5 which showed significant variation with the NaOH or the KOH alkali pre-treatment parameters. Beside the expected strong correlation between gel elasticity and gel thermal properties, no relationship between gel thermoelastic properties and KI chemical structure is found for KI extracted using NaOH. This result comes as no surprise since a complex interplay between molecular mass, chemical composition and gel properties was reported (Hilliou, Larotonda, Sereno et al., 2006). The lack of simple correlations actually motivated a dedicated study on the separate effects of these properties on the gel elasticity and thermal properties of KI extracted from *M. stellatus* (Souza et al., 2011), but such study where KI with similar molecular mass distribution or iota-carrageenan content is beyond the scope of this paper. In contrast to the lack of significant Pearson's coefficients in Table 4, the nu-carrageenan content in KI extracted with KOH negatively correlates with both gel elasticity and gel setting temperature (see Table 5). This is expected as such behaviour justifies the alkali pre-treatment used in the industry. We conjecture that the slower kinetics of nu-carrageenan conversion by KOH is responsible for the observed correlation, and that shorter pre-treatment duration along with lower concentrations are needed to obtain similar correlation with NaOH treatment.

4. Conclusions

Tailoring the alkali concentration and pre-treatment duration allows the extraction of KI from *M. stellatus* with a palette of gel elasticity and gel setting temperature in NaCl and KCl. For KOH, the correlation between the gel thermoelastic properties and the molar fraction in nu-carrageenan in the KI rationalizes the tuning of the gel properties through the adequate choice of the alkali pre-treatment parameters. Higher KOH concentrations and longer pre-treatment durations favour the conversion of nu-carrageenan into kappa-carrageenan, and consequently favour the formation of stronger gels. However, excessive KOH concentration promotes the depolymerisation of KI as inferred from the decrease in the intrinsic viscosity. As a result, the gel elasticity is depressed. Thus, optimum alkali pre-treatment parameters which enable the recovery of KI with maximum gel elasticity are found. Namely, treating *M. stellatus* seaweeds with 5% w/v KOH during 5 h leads to the recovery of a KI with 68 mol% kappa-carrageenan and 32 mol% iota-carrageenan. At a concentration of 1% w/v in 0.1 M KCl, this KI gels at 60 °C and the corresponding elasticity is 8 kPa at 20 °C. KI with similar gel properties are obtained if NaOH is used instead of KOH. Indeed, lower concentration and less pre-treatment duration are needed to isolate KI with maximized gel properties, thus indicating that NaOH is more efficient than KOH to convert the nu-carrageenan precursor to iota-carrageenan. However, no direct correlations between the gel properties and the chemical structure of KI obtained with NaOH could be identified. Overall, this study delivered a set of KI showing a suite of gelling properties for a wide range of applications, resulting from different molecular mass distributions and nu-carrageenan molar fractions.

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