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Distribution of hybrid beta/kappa carrageenan from the red alga *Betaphycus gelatinus* in Vietnam

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Abstract

The red alga *Betaphycus gelatinus* showed a monthly little variation in carrageenan yields, 3,6 anhydro galactose and sulfate contents for the native carrageenans (68.2 – 73.1%, 23.1 - 24.8% and 19.5 – 20.3%, respectively) and for alkali modified carrageenans (23.7 – 31.5%, 41.6 – 43.9% and 8.6 – 8.7%, respectively). The gelling, melting temperatures and gel strength of alkali modified carrageenans from April to June showed no change, with the recorded values of 40.8 ^{o}C, 74.5 ^{o}C and 310 g cm^{-2}, respectively. The molecular weight of the native carrageenan was primarily distributed at 876 kDa, with a small fraction at 1.2 kDa. ^{1}H and ^{13}C NMR spectra proved that the carrageenan is composed of a hybrid of beta carrageenan and kappa carrageenan. Calculated from the peak areas of the α-anomeric proton signals in ^{1}H NMR and the signal intensities of the anomeric carbons in ^{13}C NMR spectra indicated that the native carrageenan showed no much difference in components of beta carrageenan (51.8%) and kappa carrageenan (48.2%), but after alkali modified carrageenan, the beta carrageenan component increased significantly (68.6%) compared to the kappa carrageenan component (31.4%). These results will contribute more information of hybrid carrageenan from the red alga *B. gelatinus* in Vietnam.

Keywords: *Betaphycus gelatinus*; Characterization; Hybrid beta/kappa carrageenan; Red alga; Spectroscopy

Introduction

The co-occurrence and distribution of hybrid carrageenans were also reported among some the red algae, such as those from the families Dicranemataceae and Sarcodiaceae (Liao et al. 1993), Tichocarpaceae (Yermak et al. 1999); the algae, *Eucheuma gelatinae* (Greer and Yaphe 1984), *Chondracanthus teedei* var. *lusitanicus* (Pereira and Mesquita 2004), *Sarconema filiforme*, *Acrotylus australis, Amphiplexia hymenocladioides, Antrocentrum nigrescens, Hennedya crispa and Ranavalona duckerae* (Chiovitti et al. 1998, 2008), *Furcellaria lumbricalis* and *Tichocarpus crinitus* (Correc et al. 2012). Recently, due to their novel functional properties, hybrid carrageenans have attracted attention for both studying their structure–property relationships and exploring commercial applications (Yermak et al. 2021).

Hybrid beta/kappa carrageenans from the red alga *Eucheuma gelatinae* (new name: *Betaphycus gelatinus*) collected at the Philippines and Hainan Island of China have been reported (Greer and Yaphe 1984; Santos 1989) and the enzymatic degradation of carrageenan showed the co-occurring kappa- and beta carrabiose units, but with different distributions (Renn et al. 1993). In Vietnam, the red alga *Betaphycus gelatinus* (Esper) Doty ex P.C. Silva, 1996 was checklisted in marine algae from the South China Sea and kept at Herbaria of Institute of Oceanography and Nhatrang Institute of Technology Research and Application, Vietnam Academy of Science and Technology (Phang et al. 2016). The natural distribution of this alga ranges from Quangngai to Ninhthuan provinces and is found on reefs in the subtidal zone of shores exposed to strong wave action. Annually, these algae were mainly harvested from April to June with limited output for application as an ingredient for sweets (Isao et al. 2005). At present, little information is known about carrageenan from this red algal species, except for carrageenans from the genus *Kappaphycus* cultivated in Vietnam (Hung et al. 2019, 2021). Thus, the main objective of this study is to evaluate and compare the characteristics of carrageenan from the red alga *B. gelatinus* in Vietnam to those of carrageenans from *E. galatinae* reported.

Materials and methods

Materials

The red alga, *Betaphycus galatinus* was collected from April to June at Ninhthuan Province (11°35′ N, 109°02′ E), Vietnam. The samples were brought to the laboratory and washed with tap water to eliminate sand, debris and epiphytes. Algal materials were then ground into powder and extracted with cold acetone for 4 h to remove pigments, followed washed with absolute ethanol. The residues were dried by sunlight and kept at -20 °C until used. All chemicals used were analytical grades.

Extraction of native and alkali-modified carrageenan

Native carrageenan extraction was performed according to the method described (Hellebust and Craige 1978). Dry algal powder was extracted in 0.5 M $NaHCO_3$ at 90 °C for 2 h with vigorous stirring. The mixture was hot filtered with Celite aid by vacuum. Filtrates were precipitated by aqueous cetyltrimethylammonium bromide. The precipitate was recovered by centrifugation. The pellet was washed three times with a near-saturated solution of sodium acetate in 95% ethanol to remove Cetavlon residues, and three additional times with 95% ethanol to remove sodium acetate residues. The carrageenan was recovered and dried at 60 °C for 24 h.

Alkali-modified carrageenan extraction was carried out according to the method described (Santos 1989; Renn et al. 1993). Dry algal powder was treated in 20% KOH solution at 80 °C for 2 h. Then the algae were washed and extracted with distilled water at 90 °C for 2 h. Thereafter, the mixture was filtered and gelled, frozen overnight and then thawed, washed with fresh water and ethanol at least twice, dried at 60 °C and milled into powder.

Prior to determine chemical composition and structures, carrageenan samples were repurified by dialysis using a Spectrapor Membrane with Mw cut-off 12,000–14,000 Da, against milli-Q water for 24 h with frequent changes of water. After dialysis, the sample was filtrated by 0.45-µm filter and precipitated by absolute ethanol, dried at 60 ^{o}C and milled into powder.

Determine 3,6-anhydrogalactose and sulfate contents

3,6-anhydro galactose content in carrageenan samples was determined according to the method described (Yaphe and Arsenault 1965) using D-fructose as a standard. Sulfate content was determined according to the method described (Terho and Hartiala 1972) using Na_2SO_4 as a standard. Prior to determining sulfate content, all samples were hydrolyzed in 0.1 N HCL for 6 h.

Determine gelling, melting temperatures and gel strength

Gelling and melting temperatures were determined according to the method described (Craigie and Leigh 1978), using 1.5% carrageenan solutions and RW-0525G Refrigerated and Heating Bath Circulators (Jeiotech, Korea) to control the temperature. The gelling temperature was measured in a test tube containing 10 mL of the hot carrageenan solution with a thermometer (0.1^{o}C divisions) inserted and placed in the water bath of RW-0525G. Glass beads (3.0 mm) were periodically introduced onto the surface of the solution and gelling temperature was recorded when the bead failed to sink through the gel. The melting temperature was measured by placing a glass bead on the surface of the gel in water bath of RW-0525G and the melting temperature was recorded when the glass bead sank to the bottom.

Gel strength was measured using a Rheometer (CR-500DX; Sun Scientific, Tokyo, Japan) with a plunger diameter of 12 mm and a gel thickness is 10 mm, using 1.5% carrageenan solutions.

Determine molecular weight

The molecular weight of sample was determined by Gel Permeation Chromatography (GPC) (Shimadzu LC-20AD, Japan), using a refractive index detector and GPC/SEC Suprema Ultrahigh column (8.0 mm × 300 mm, Germany). A 20 µl aliquot of 5 mg/mL solution of sample was injected on the column and eluted by 0.1 M $LiNO_3$ with a flow rate of 1.0 mL min^{-1} at 40 ^{o}C. Dextran with different molecular weights was used as standards.

FT-IR spectroscopy

Carrageenan samples were recorded in the range of 2000 to 500 cm^{-1} on a Fourier transform infrared spectrophotometer (Shimadzu, Japan).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies

Carrageenan samples were dissolved in 1 mL of D_2O containing 10 mM of 2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate (DSS) as an internal standard. The ^{1}H NMR spectrum was recorded at 70 ^{o}C and operated at 600.4 MHz. The ^{13}C NMR spectrum was measured at 70 ^{o}C and operated at 150.97 MHz. All samples were measured on a Bruker Avance Neo 600 MHz spectrometer (Switzerland). Chemical shifts were corrected relative to the internal standard of DSS (δ = 0.000 ppm for both ^{1}H and ^{13}C NMR) (Van de Velde et al. 2004).

Results and discussion

Chemical composition and physicochemical properties

The red alga *B. gelatinus* showed a minimal monthly variation in carrageenan yields and qualities with the highest values of yield, 3,6 anhydro galactose and sulfate recorded in May for the native carrageenan (73.1%, 24.8% and 20.3%, respectively) and for alkali modified carrageenan (31.5%, 43.9% and 8.6%, respectively) (Table 1). The native carrageenan samples collected from April to June were non-gelling and thus were not recorded. The gelling and melting temperatures, as well as the gel strength of alkali modified carrageenans from April to June showed no significant difference, with the recorded values of 40.8 ± 0.8 ^{o}C, 74.5 ± 0.7 ^{o}C and 310 ± 15 g cm^{-2}, respectively. Carrageenan yield from the red alga *Eucheuma gelatinae* collected at Hainan Island of China was also reported with a total of 61% of the dry weight of the algae and 32% of 3,6 anhydro galactose per total carbohydrate from hot extract and 48% of 3,6 anhydro galactose from alkali modification (Greer and Yaphe 1984). Similarly, beta carrageenans isolated and purified from *E. gelatinae* obtained from both the Philippines and Hainan Island of China were also reported for 3,6 anhydro galactose contents of 35.98% and 38.24%, respectively and the gelling and melting temperatures ranged from 31 to 33 °C and 63 to 70 °C (Renn et al. 1993). Such differences can be attributed to the methodology and samples used in each study to evaluate physicochemical properties.

Among the carrageenan samples collected monthly, the sample in May, which recorded for the highest content of 3,6 anhydrogalactose and carrageenan yield in both the native and alkali modified forms, was selected to evaluate structural characteristics and molecular weight by FT-IR, ^{1}H and ^{13}C NMR, and GPC.

FT-IR analysis of carrageenans

The FT-IR spectral profile of the native and alkali modified carrageenans revealed the typical absorption bands associated with sulfated polysaccharide of carrageenan (Fig. 1). The typical absorption bands at approximately 929, 848 and 1254 cm^{-1} of the native (Fig. 1a) and alkali

modified carrageenan (Fig. 1b) confirmed the presence of C–O–C of 3,6-anhydro-D-galactose, C_4–O–S stretching and O=S=O symmetric vibrations of β-D-galactose-4-sulfate, respectively, for kappa carrageenans (Greer and Yaphe 1984; Santos 1989; Mendoza et al. 2002; Aguilan et al. 2003; Chiovitti et al. 2008). The bands at 891 cm^{-1} observed in both carrageenan types, indicated unsulfated 3-linked β-D-galactose, suggesting the polysaccharides also contained a component of beta carrageenan (Liao et al. 1993; Chiovitti et al. 2008).

^{1}H and ^{13}C NMR spectroscopies

The complexity in assignment of chemical shifts in the ^{1}H NMR spectra reflected the polysaccharide heterogeneity. By comparing to the major signals reported in the literature, kappa carrageenan and beta carrageenan were distinguished in the α-anomeric protons of 4-linked 3,6-anhydro-α-D-galactopyranose (DA) by signals at 5.093 ppm for kappa carrageenan and 5.074 ppm for beta carrageenan (Van de Velde et al. 2004). The ^{1}H NMR spectra of both native and alkali modified carrageenans also displayed the major signals for the α-anomeric protons of DA at 5.094 ppm for kappa carrageenan and 5.074 ppm for beta carrageenan (Fig. 2a and 2b), similar to the reported data in literature (Van de Velde et al. 2004). The native and alkali-modified carrageenans from three *Kappaphycus* species showed the major signal observed at 5.10 ppm, corresponding to the anomeric protons of the 4-linked 3,6-anhydro-α-D-galactopyranosyl residues of kappa carrageenan repeat units (Usov 1984; Aguilan et al. 2003). The hybrid kappa-/beta carrageenans from *Furcellaria lumbricalis* and *Tichocarpus crinitus* have also been differentiated by the chemical shift of the anomeric signals of the α-linked galactose DA-H1 at 5.12 ppm for kappa carrageenan and at 5.10 ppm for beta carrageenan (Correc et al. 2012). The ^{1}H NMR chemical shifts of the α-anomeric protons of carrageenans were only slightly different from those reported (Aguilan et al. 2003; Van de Velde et al. 2004; Correc et al. 2012).

In the ^{13}C NMR spectrum, the chemical shifts of DSS are larger (average 2.1ppm) than those of dimethyl sulfoxide (DMSO) previously reported (Usov et al. 1980; Van de Velde et al. 2002, 2004). Referring to the chemical shifts of carbon atoms in the ^{13}C NMR relative to DSS as internal standard (Van de Velde et al. 2004), the chemical shift signals of carbon atoms of carrageenan in this study (Fig. 3a and 3b. Table 2) are similar to those reported (Van de Velde et al. 2004). The ^{13}C NMR spectra of both the native and alkali modified carrageenans displayed the major signals of the anomeric carbons at C-1 of β-D-galactose (G) (104.81 ppm) and 3,6 anhydro-α-D-galactose (DA) (96.78 ppm) for beta carrageenan and at C-1 of β-D-galactose-4-sulfate (G4S) (104.68 ppm) and 3,6 anhydro-α-D-galactose (DA) (97.33 ppm) for kappa carrageenans as reported (Liao et al. 1993; Mendoza et al. 2002; Villanueva and Montano 2003; Aguilan et al. 2003; Van de Velde et al. 2004; Chiovitti et al. 2008; Cosenza et al. 2014; Hung et al. 2021). The chemical shifts of the anomeric carbons measured in DMSO of the kappa/beta hybrid carrageenans have been reported, such as from the hot extract of *E. gelatinae* for beta carrageenan (102.5 ppm and 94.6 ppm) and kappa carrageenan (102.5 ppm and 95.3 ppm) (Usov et al. 1980; Greer and Yaphe 1984); from the alkali-modified polysaccharide of *Tylotus obtusatus* for beta carrageenan (102.7 ppm and 94.7 ppm) and kappa carrageenan (102.7 ppm and 95.3 ppm) (Liao et al. 1993); from *Hennedya crispa* for beta carrageenan (102.6 ppm and 94.7 ppm) and kappa carrageenan (102.6 ppm and 95.3 ppm) (Chiovitti et al. 2008). Furthermore, the carbon chemical shifts at C-2, C-3, C-4, C-5 and C-6 of β-D-galactose (G) and 3,6-anhydro-D-galactose (DA) of beta carrageenan and those of β-D-galactose-4-sulfate (G4S) and 3,6-anhydro-D-galactose (DA) of kappa carrageenan in this study corresponded to those of C-2, C-3, C-4, C-5 and C-6 of beta carrageenan (G and DA) and kappa carrageenan (G4S and DA) from *T. obtusatus* (Liao et al. 1993), *Acrotylus australis*, *Amphiplexia hymenocladioides*, *Antrocentrum nigrescens* and *H. crispa* reported (Chiovitti et al. 2008).

Moreover, a weak signal at 5.179 ppm (Fig. 2a) was attributed to the anomeric proton of 4-linked α-D-galactose-6-sulfate residues of carrageenans (Greer and Yaphe 1984; Mendoza et

al. 2002; Aguilan et al. 2003; Hung et al. 2021). The absence of this signal in ^{1}H NMR spectrum of the alkali modified carrageenan sample (Fig. 2b) reflects the conversion of 6-sulfated galactose residues into 3,6-anhydrogalactose units upon alkali modification. In comparison, the peak areas of α-anomeric protons of DA for kappa carrageenan (5.094 ppm) in both the native and alkali modified carrageenan spectra showed no significant change (Fig. 2a and 2b), indicating that 4-linked α-D-galactose-6-sulfate residues are primarily present in the beta carrageenan of native carrageenan and defined as 6-sulfated beta carrageenan precursor (Greer and Yaphe 1984).

The distribution of hybrid beta/kappa carrageenan from the red alga *E. gelatinae* was reported through the enzymatic degradation of carrageenan (Greer and Yaphe 1984). In this study, the distribution of beta/kappa carrageenan in carrageenan from the red alga *B. gelatinus* estimated was based on calculation of the peak areas of the α-anomeric proton signals of DA (5.094 ppm for kappa carrageenan and 5.074 ppm for beta carrageenan) in ^{1}H NMR spectra (Fig. 2a and 2b) and the signal intensities of the anomeric carbons at C-1 (104.81 ppm and 96.78 ppm for beta carrageenan; 104.68 ppm and 97.33 ppm for kappa carrageenan) in ^{13}C NMR spectra (Fig. 3a and 3b). The results indicates that the native carrageenan contains no much difference in components of beta carrageenan (51.8%) and kappa carrageenan (48.2%), however after alkali modified carrageenan, the beta carrageenan component increased significantly (68.6%) compared to kappa carrageenan component (31.4%). A similar result was reported for carrageenan from *E. gelatinae*, where after complete hydrolysis with kappa/iota carrageenase, a beta carrageenan component of 66% was obtained (Greer and Yaphe 1984). The differences in the distribution of beta/kappa carrageenan components in carrageenan from the red algae *E. gelatinae* and *B. gelatinus* can be attributed to the methodology used as samples collected in each study.

Molecular weight of carrageenan

The molecular weight of the native carrageenan was distributed mainly at 876 kDa and with a small fraction at 1.2 kDa (Fig. 4). This small fraction was suggested as the presence of trace amounts of iota carrageenan within the polymer (Greer and Yaphe 1984). The molecular weight of the alkali modified carrageenan was not recorded, due to gel formation even at a concentration of 2 mg/mL.

The carrageenan from the red alga *B. gelatinus* was identified as a hybrid carrageenan, composed predominantly of beta carrageenan and kappa carrageenan (Fig. 5). The physicochemical properties and structural characteristics of hybrid beta/kappa carrageenan from the red alga *B. gelatinus* in Vietnam were similar well to those of hybrid carrageenan from the red alga *E. gelatinae*. This similarity indicated that the distribution of the red alga *B. gelatinus* was not only beyond in marine algae of Philippines and Hainan Island of China, but also extended to Vietnam as well.

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Authors' contributions

LDH supervised all the assays, writing –reviewed and edited the manuscript, project administration, methodology and investigation. HTTN, VTDT, LTN, DTT and TTTT made the experiments. VTDT prepared the figures and tables and wrote the original draft of the manuscript. All authors reviewed the manuscript and agreed to the published version of the manuscript.

Data availability

The data supporting the findings of this study are available from the corresponding author, upon reasonable request.

Ethics Declarations

Competing interests

The authors declare no competing interests.

Supplementary files

Fig. 1

Fig. 4

Fig. 5

Table 2

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Legend to Figures

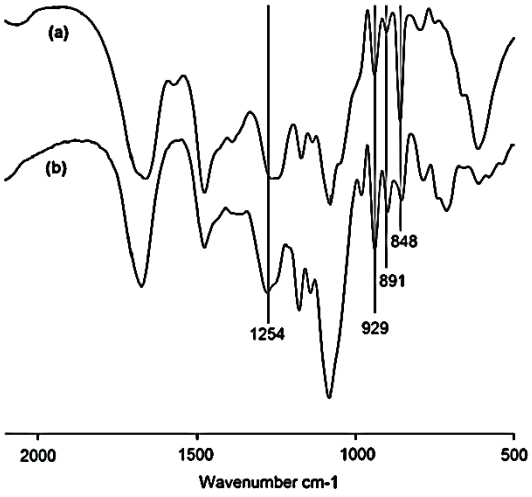


Fig. 1 FT-IR spectra of carrageenans extracted from *B. gelatinus*. (a) Native and (b) alkali-modified carrageenan

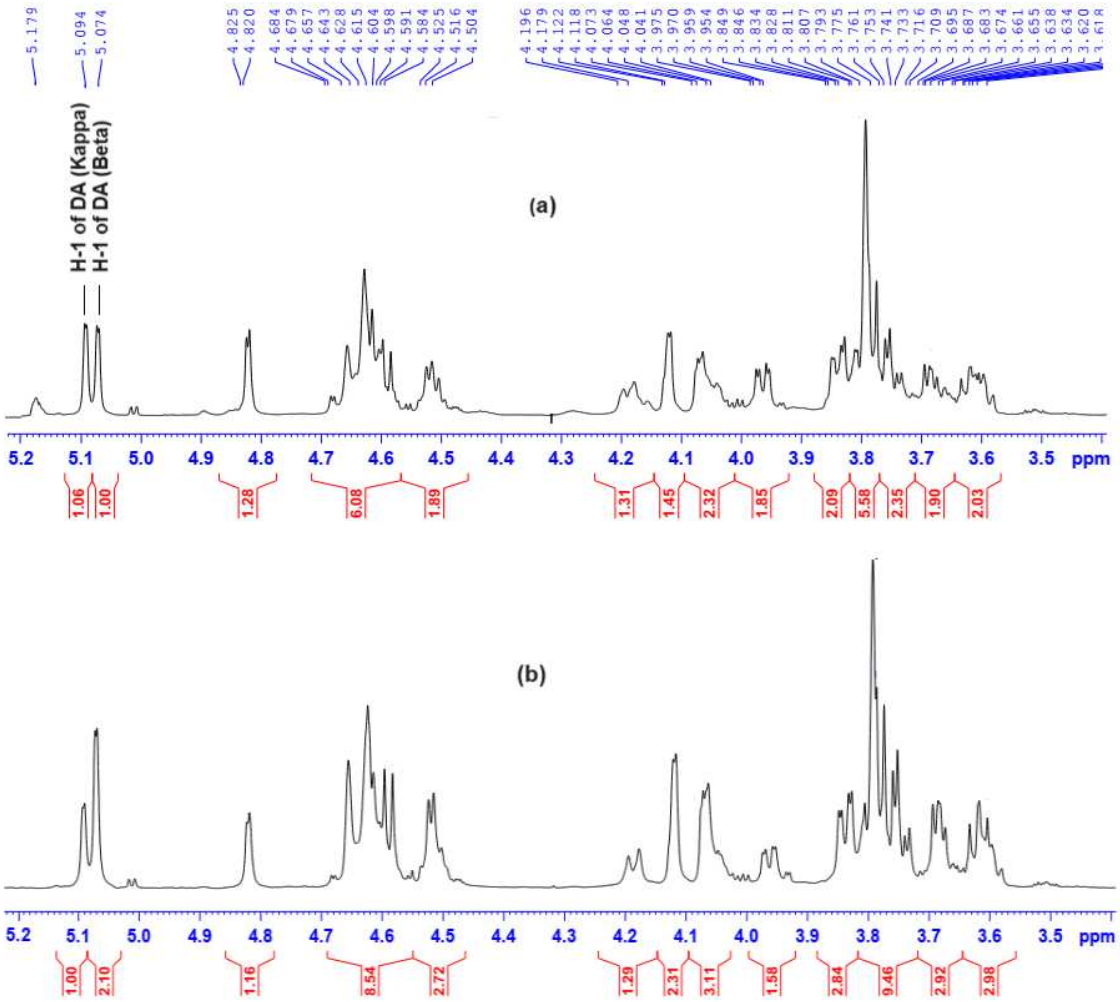


Fig. 2 ^{1}H NMR spectra of carrageenans extracted from *B. gelatinus*. (a) Native and (b) alkali-modified carrageenan. H-1 of DA (Kappa): the α-anomeric proton of 3,6 anhydro-α-D-galactose of kappa carrageenan; H-1 of DA (Beta): the α-anomeric proton of 3,6 anhydro-α-D-galactose of beta carrageenan

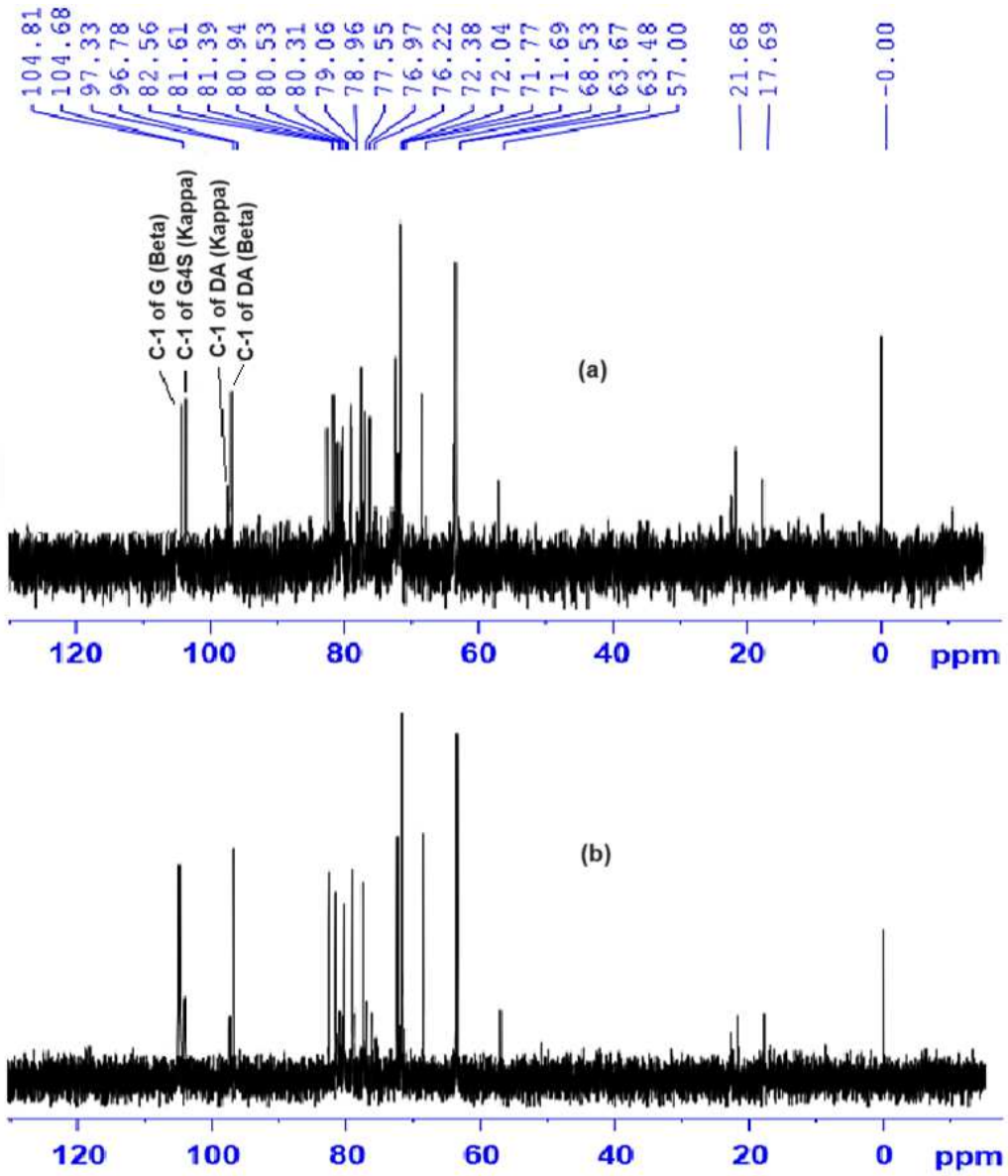


Fig. 3 ^{13}C NMR spectra of carrageenans extracted from *B. gelatinus*. (a) Native and (b) alkali-modified carrageenan. C-1 of G and DA (Beta): the anomeric carbons at C-1 of β-D-galactose and 3,6 anhydro-α-D-galactose of beta carrageenan, respectively; C-1 of G4S and DA (Kappa): the anomeric carbons at C-1 of β-D-galactose-4-sulfate and 3,6 anhydro-α-D-galactose of kappa carrageenan, respectively

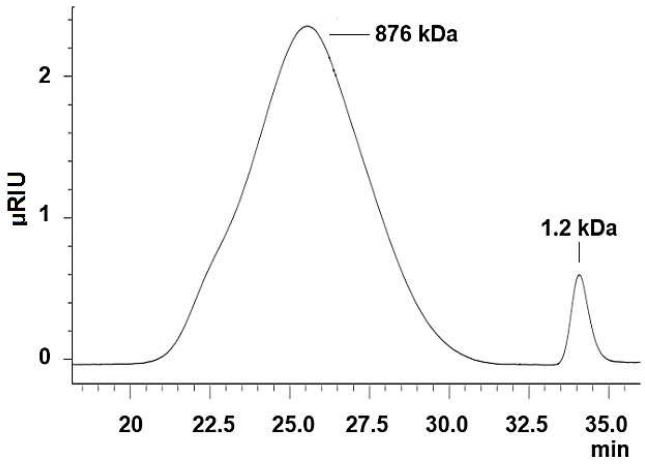


Fig. 4 Distribution of molecular weight of the native carrageenan from *B. gelatinus*

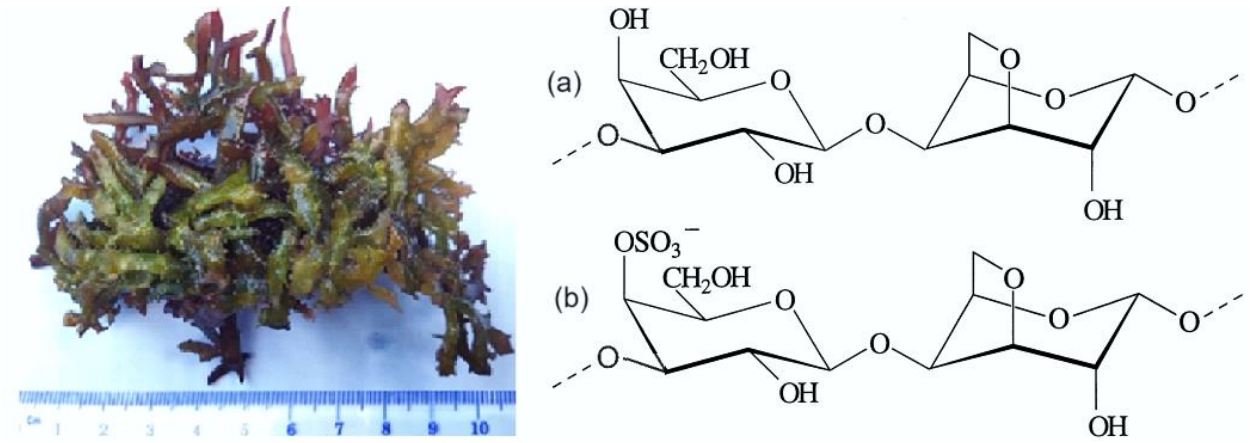


Fig. 5 The red alga *B. gelatinus* and proposed repeating disaccharide units in the carrageenan: (a) Beta carrageenan: (O-3,6-anhydro-α-D-galactopyranosyl (l-3)-O-β-D-galactopyranosyl (l-4)); (b) Kappa carrageenan: (O- 3,6-anhydro-α-D-galactopyranosyl (l-3)-O-β-D-galactopyranosyI-4-sulfate (l-4))

Table 1 Chemical properties of the monthly native and alkali modified carrageenans from the red alga *B. gelatinus*.

	Native carrageenans			Alkali modified carrageenans		
Months	Yield[a]	DA[b]	Sulfate[b]	Yield[a]	DA[b]	Sulfate[b]
April	68.2 ± 2.1	23.1 ± 0.5	17.9 ± 0.7	24.2 ± 1.7	42.2 ± 0.6	8.7 ± 0.4
May	73.1 ± 2.3	24.8 ± 0.6	20.3 ± 0.6	31.5 ± 1.8	43.9 ± 0.7	8.6 ± 0.5
June	68.4 ± 2.2	23.4 ± 0.4	20.0 ± 0.7	23.7 ± 1.6	41.6 ± 0.6	8.8 ± 0.5

[a] Calculated on % of dry weight of alga

[b] Calculated on % of the carrageenan

DA, 4-linked 3,6-anhydro-α-D-galactose

Table 2 Chemical shifts in the ^{13}C NMR spectra of the carrageenan from the red alga *B. gelatinus*

Carrageenan	Unit	C-1	C-2	C-3	C-4	C-5	C-6
Beta/Beta[a]	G/G[a]	104.81/104.81	71.69/71.72	82.56/82.58	68.53/68.56	77.55/77.55	63.67/63.49
	DA/DA[a]	96.78/96.81	72.38/72.40	81.61/81.64	80.31/80.33	79.06/79.26	71.77/71.72
Kappa/Kappa[a]	G4S/G4S[a]	104.68/104.70	71.77/71.72	80.94/80.98	76.22/76.25	76.97/77.00	63.48/63.49
	DA/DA[a]	97.33/97.34	72.04/72.11	81.39/81.41	80.53/80.54	78.96/79.07	71.69/71.72

G, 3-linked β-D-galactopyranose; DA, 4-linked 3,6-anhydro-α-D-galactopyranose;

G4S, 3-linked β-D-galactopyranose 4-sulfate

[a] Referred to the value of chemical shifts in ppm to DSS at 0.000 ppm in the literature (Van de Velde et al. 2004)