

Improved methods for isolation of carrageenan from *Hypnea musciformis* and its antioxidant activity

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Abstract Carrageenans are economically important biopolymers used in the pharmaceutical, chemical and food industries. The present study was performed to optimise the extraction of carrageenan from *Hypnea musciformis* collected from Saint Martin Island, Bay of Bengal, Bangladesh, and characterisation of its chemical, rheological properties and antioxidant activities. Aqueous- and alkali-treated carrageenan from *H. musciformis* was extracted using conventional method and ultrasonic-assisted extraction (UAE) because this technique allows rapid extraction, which is important to avoid degradation of labile compounds. The resulting carrageenan was investigated in terms of yield, sulphate, 3,6-anhydrogalactose (AG), galactose (Gal) contents and structural properties using Fourier transform infrared (FT-IR) and ¹H NMR spectroscopy. The carrageenan yield was higher using the novel UAE method and was comparable to that using the conventional technique. In vitro results confirmed a slight variation in sulphate, AG and Gal contents depending on the extraction method used. FT-IR and ¹H NMR spectrum showed that carrageenan extracted using either the conventional method or UAE possesses typical analytical characteristics of κ-carrageenan. The rheological properties of carrageenan were evaluated using differential scanning calorimetry (DSC) and viscosity measurements and found to be similar to those of carrageenan extracted from same species of red alga. The antioxidant activity of carrageenan was measured using the 2,2-diphenyl-1-

picrylhydrazyl (DPPH), OH radical scavenging, reducing power and phosphomolybdenum assays. The results indicated that carrageenan possesses antioxidant activity, the degree of which depends on its structural composition and the extraction method used.

Keywords Extraction · Carrageenan · Characterisation · Rheological properties · Antioxidant

Introduction

The recent identification of various unique carbohydrates in seaweeds has emphasised the importance of further research in this area (Michel et al. 2006). Commercially exploited carbohydrate polymers from seaweed are alginates, agar and carrageenan (De Ruyter and Rudolph 1997). Carrageenan is an economically important galactose-rich carbohydrate polymer extracted from numerous marine red seaweed species of the Rhodophyceae. These polysaccharides are composed of alternating α-(1–3) and β-(1–4) linked D-galactosyl residues. Several types of carrageenan have been identified based on the modification of the disaccharide repeating unit by ester sulphate groups and by the presence of 3,6-anhydrogalactose (AG) as a 4-linked residue, of which three types (λ-, κ- and ι-carrageenan) are commercially available (Stortz and Cerezo 2000). The degree of sulphation of these major carrageenan types affects their properties, and κ-carrageenan is of considerable commercial importance due to its good gel-forming properties (Al-Alawi et al. 2011)

In the food industry, κ-carrageenans are widely utilised for their excellent physical functional properties, such as for thickening, gelling and stabilising (Mabeau 1989). Carrageenans from red seaweed have also been used in various non-food products such as pharmaceuticals, cosmetics,

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printing and textile formulations (Imeson 2000) and as agents for inducing experimental inflammation and inflammatory pain. In addition, several other potential pharmaceutical properties including antitumour, immunomodulatory, antihyperlipidaemic, anticoagulant (Caceres et al. 2000), antiviral (Gonzalez et al. 1987) and antioxidant (Sokolova et al. 2011) activities have been reported. Consumption of natural products rich in antioxidant compounds may help to maintain the balance between reactive oxygen species (ROS) production and endogenous protection when the body undergoes oxidative stress (Rafiquzzaman et al. 2013). However, few reports are available on the antioxidant activity of carrageenan from *Hypnea musciformis*.

The market for carrageenan, and its price, is continuously expanding due to its diversified use (Vázquez-Delfin et al. 2014). Higher prices of carrageenan are associated with the extraction process and availability of seaweed (Navarro and Stortz 2005). Although the functional activities of carrageenans have been established, they are extracted mainly using conventional techniques. These extraction processes are time and energy consuming with a comparatively low efficiency. To overcome these limitations, development of novel extraction techniques is important. Recently, microwave-assisted extraction (MAE) techniques have been evaluated (Vázquez-Delfin et al. 2014). Additionally, ultrasonic-assisted extraction (UAE) has been used to increase the extraction yield of bioactive substances from natural products; this effect is attributed mainly to the disruption of cell walls, particle size reduction and enhanced mass transfer to the cell contents as a result of cavitation bubble collapse (Wang et al. 2009). Despite the benefits of the UAE technique, its application for carrageenan extraction is new and limited. The red seaweed *H. musciformis* contains κ -carrageenan mainly in the cell walls (Arman and Qader 2012) and has been suggested to be an economically important species due to its high yield (Reis et al. 2006).

Although the extraction and characterisation of carrageenan isolated from *H. musciformis* from various regions have been described (Table 1), the isolation and characterisation of carrageenan from *H. musciformis* collected in Bangladesh have not been reported. The composition and yield vary according to season, age, species and geographic location (Graham and Wilcox 2000). In addition, the structure and functional activity of algal sulphated polysaccharides vary according to algal species, environmental conditions and life stage of seaweed (Rinaudo 2007). Therefore, isolation and characterisation of the structural and biofunctional activities of carrageenan extracted from *H. musciformis* collected from Saint Martin coast, Bangladesh, are important. Thus, in the present study, we investigated and compared carrageenan extracted from

H. musciformis using both conventional and UAE methods in terms of yield, AG, sulphate contents and structural properties using Fourier transform infrared (FT-IR) and ^1H NMR spectroscopy. Additionally, the rheological properties and antioxidant activity were evaluated.

Materials and methods

Hypnea musciformis was collected in June, 2013 from the Saint Martin coast (92° 19' 21.28" E and 20° 37' 38.12" N, Bay of Bengal, Bangladesh, during mature stage. The seaweed was harvested manually by cutting the fronds, washed with clean seawater to remove impurities and transported in ice box (4 °C) to the laboratory. Then, the fresh plants were thoroughly washed under running tap water to remove seawater, sand and any other impurities. The cleaned seaweed was left to dry in sunlight for 2 days, sealed in small quantities in plastic bags and stored in a refrigerator at 4 °C until use.

Extraction procedure

During the extraction process, 4 g of the sun-dried seaweed were hydrated with 100 mL deionised water and kept at room temperature (RT) for 12 h followed by depigmentation using 100 mL of methanol-acetone (1:1) mixture to eliminate the organic soluble fraction. The depigmented seaweed was treated in an alkaline solution of 3 % KOH (Pereira et al. 2003) for the alkali treatment or in deionised water for the aqueous treatment ($\sim 150 \text{ mL g}^{-1}$) (Reis et al. 2008) then heated at 80 °C for 4 h. The extract was filtered and washed several times with deionised water to eliminate excess KOH salt. In a subsequent step, the retained extract was redissolved in 1 L of deionised water, heated for 4 h at 90 °C and coarse-filtered using a cotton cloth followed by glass microfibre filter (Whatman GF/D). Then, the resulting concentrated extract was precipitated with three volumes of 95 % ethanol (1:3, v/v), centrifuged, dried and milled into fine powder to pass through a 500- μm mesh.

UAE method

UAE was performed using an ultrasonicator (Sonifier, Branson 250). In this method, hydration and depigmentation of seaweed was performed following the conventional method, as described above (Pereira et al. 2003; Reis et al. 2008). After depigmentation, the pretreated samples were separated from the organic solvent using gauze cloth. UAE was then performed using ultrasonic times of 10 and 20 min and ultrasonic powers of 400 and 500 W. During ultrasonic extraction, temperature was maintained using ice to prevent degradation of carrageenan. Carrageenan was extracted following the conventional method as described above.

Table 1 Yield (%) of carrageenan extracted from *Hypnea musciformis* of different localities using different methods

Extraction conditions	Yield (%)	Locality	Reference
Aqueous	34	Israel	Friedlander and Zelikovitch (1984)
Aqueous, HCl pretreated	0.4–25	Italy	Knutsen et al. (1995)
Alkali (0.1 M NaOH)	20–36	Tanzania	Mtolera and Buriyo (2004)
Aqueous (HCl pretreated)	34–44	Pakistan	Bi et al. (2006)
Alkali	40	Pakistan	Bi et al. (2006)
Aqueous	13.5–41	Morocco	Aziza et al. (2008)
Aqueous	21–48	Brazil	Reis et al. (2008)
Aqueous	30.2	Pakistan	Arman and Qader (2012)
Alkali (0.1 N NaOH)	39.8	Pakistan	Arman and Qader (2012)
Acid (0.1 N HCl)	4.3	Pakistan	Arman and Qader (2012)
Aqueous	37.8	Mexico	Vázquez-Delfin et al. (2014)
Aqueous (MAE)	21.5	Mexico	Vázquez-Delfin et al. (2014)
Alkali (3 % KOH)	18.7	Mexico	Vázquez-Delfin et al. (2014)
Alkali (3 % KOH; MAE)	16.6	Mexico	Vázquez-Delfin et al. (2014)
Aqueous; conventional	39.78	Bangladesh	This study
Aqueous; UAE	49.01	Bangladesh	This study
Alkali (3 % KOH; conventional)	32.34	Bangladesh	This study
Alkali (3 % KOH; UAE)	38.04	Bangladesh	This study

Carrageenan analysis

Determination of sulphate content Sulphate content of carrageenan extracted from *H. musciformis* was analysed according to the simple turbidometric method of Jackson and McCandless (1978). Initially, barium-agarose reagent was freshly prepared by mixing 0.02 % agarose and 0.5 % barium chloride (BaCl₂). Next, 1 mL of carrageenan sample was added to 1.2 mL of 8 % trichloroacetic acid (TCA), and then, 600 µL of barium-agarose reagent were added to this mixture, followed by incubation at RT for 30 min. After incubation, the absorbance at 500 nm was measured against a blank. Sodium sulphate was used as the standard.

Determination of AG content A stock solution was prepared by dissolving 130 mg of resorcinol reagent in 100 mL of absolute ethyl alcohol. Next, 10 mL of the stock solution were added to 100 mL of 12 M hydrochloric acid. This solution was freshly prepared and stored in a brown bottle for further use. The AG content of carrageenan was analysed according to the method of Yaphe (1960). Carrageenan solution (2 mL) with freshly prepared resorcinol reagent (10 mL) was transferred to the boiling tube and placed in an ice bath for 5 min. The tube was then heated for 10 min at 80 °C and cooled in an ice bath. The absorbance at 500 nm was recorded.

Determination of galactose content The galactose content of carrageenan was determined by colorimetric method

using anthrone reagent according to slight modified method of Yaphe (1960), and galactose was used as a reference. Briefly, stock solution of anthrone reagent was prepared by dissolving 200 mg of reagent in 100 mL of 83.6 % sulphuric acid and stored at 4 °C for further use. Carrageenan solution (1 mL) with freshly prepared anthrone reagent (10 mL) was transferred to the boiling tube and heat at boiling water bath for 11 min. The tube was then cooled rapidly in an ice bath. The absorbance at 630 nm was recorded.

FT-IR spectroscopy The freeze-dried extracted carrageenan was used for FT-IR measurements in the 4000–650 cm⁻¹ frequency range using PerkinElmer (USA), Spectrum X. The extracted carrageenan was placed on the surface of the ATR crystal, and a pressure was applied to expel air from the powder particles. The spectrum of the sample was collected by averaging 128 scans at four resolutions.

¹H NMR spectroscopy The ¹H NMR spectra were recorded at 80 °C on JEOL (Japan) JNM ECP (400 MHz) using D₂O as solvent. The carrageenan concentration was 1 % (w/w).

Differential scanning calorimetry The thermal analysis was implemented using a modulated differential scanning calorimeter (model Q100, TA instruments, USA). The solution–gel transitions of single gels and powders were scanned at a rate of 5 °C min⁻¹ from 25 to 100 °C for gels and 25 to 200 °C for powders. The gels were prepared with 1.5 % κ-carrageenan

concentration and an ionic strength of 30 mM KCl in an aqueous environment.

Determination of viscosity The viscosity of gels obtained from carrageenan was measured in a Brookfield Model DV-III Programmable Rheometer (USA), using spindle SC4-18 and the Brookfield Rheolac for Windows software.

Antioxidant activity

DPPH radical scavenging activity The antioxidant activity of the carrageenan was analysed by investigating its ability to scavenge DPPH free radicals according to the method of Blois (1958). Briefly, a solution of 0.15 mM DPPH in ethanol (100 mL) was prepared and mixed with 100 mL of deionised water. To evaluate DPPH activity, the DPPH solution was mixed with carrageenan at various concentrations. The reaction mixture was kept in the dark at RT for 30 min. Ascorbic acid was used as a positive control. The ability to scavenge DPPH radicals was calculated using the following equation: DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$, where $Abs_{control}$ is the absorbance of DPPH radicals + methanol and Abs_{sample} is the absorbance of DPPH radicals + sample extract/standard.

Evaluation of antioxidant capacity using the phosphomolybdenum method The total antioxidant capacity of carrageenan was evaluated using the method of Prieto et al. (1999). An aliquot of 0.1 mL extract at various concentrations was mixed with 900 μ L of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample tubes were sealed and incubated in a boiling water bath for 90 min. After incubation, the absorbance of each aqueous solution was measured at 695 nm against a blank. A typical blank sample contained 1 mL of reagent solution; the appropriate volume of the solvent used was incubated under the same conditions as the other samples. For samples of unknown composition, water-soluble antioxidant activity was expressed as ascorbic acid (AA) equivalent.

Reducing power assay The reducing power of carrageenan was evaluated using the method of Oyaizu (1986). Briefly, 750 μ L of carrageenan at various concentrations were mixed with 750 μ L of phosphate buffer (0.2 M, pH 6.6) and 750 μ L of potassium hexacyanoferrate (1 %, w/v), followed by incubation at 50 °C in a water bath for 20 min. The reaction was terminated by adding 750 μ L TCA solution (10 %) and then centrifugation at $800 \times g$ for 10 min. The supernatant was collected and mixed with 1.5 mL deionised water and 100 μ L ferric chloride solution (0.1 %, w/v) and incubated at RT for 10 min. The absorbance at 700 nm of the reaction mixture was determined; higher absorbance indicated greater reducing power.

Determination of OH scavenging activity Hydroxyl radical scavenging activity was determined according to Beara et al. (2009) with some modifications. Carrageenan solution (500 μ L) at various concentrations was mixed with 250 μ L orthophenanthroline (7.5 mM), 1.25 mL phosphate buffer (0.2 M, pH 6.6), 250 μ L ferrous sulphate (7.5 mM) and 250 μ L H₂O₂ (0.5 %) and diluted with deionised water to a final volume of 6.25 mL. The solution was mixed vigorously and incubated at RT for 30 min. After incubation, the absorbance at 490 nm was measured in a 96-well microplate using a microplate reader. The scavenging percentage (P%) was calculated using the following formula:

$$P\% = \frac{(A-A1)}{A2-A1} \times 100,$$

where A is the absorbance value of all solutions including H₂O₂, $A1$ is the absorbance value without the sample and $A2$ is the absorbance value without H₂O₂ and the sample.

Statistical analysis

All experiments were performed in triplicate, and data are presented as means \pm standard deviation. Analysis of variance (ANOVA) was performed, and comparisons of means were conducted using Turkey's multiple comparison tests using the SPSS program (version 16.0 for windows, SPSS Inc.). Values were considered to differ significantly if the p value was <0.05 .

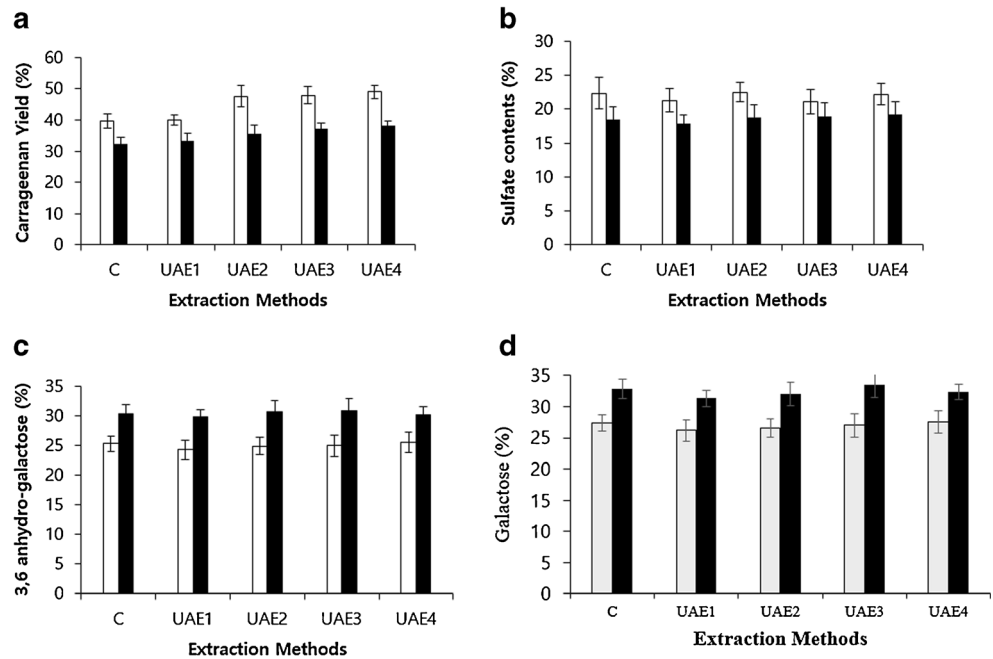
Results

Extraction yield, sulphate and AG content

Previously, several extraction methods were used to quantify and optimise the yield of carrageenan from *H. musciformis* collected from various locations worldwide (Table 1). In this study, we used a novel UAE method and the conventional method, which resulted in yields of 32.34–49.01 % (Fig. 1a). The maximum carrageenan yield (49.01 %) was obtained using the aqueous UAE method. The lowest yield was obtained after alkali treatment. In general, the yield was increased significantly ($p < 0.05$) by all UAE conditions for both aqueous and alkali extraction methods. Figure 1a shows that UAE (500 W, 20 min) significantly improved the carrageenan yield. However, minimal variation in terms of yield was observed among the various UAE conditions tested.

The sulphate content of carrageenan extracted using the conventional and UAE methods under various time and ultrasonic power conditions are shown in Fig. 1b. Sulphate content was slightly higher for aqueous

Fig. 1 Yield (a), sulfate content (b), anhydrogalactose content (c) and galactose (d) of carrageenan extracted from *H. musciformis* with conventional (c) and UAE methods (UAE1: 400 W, 10 min; UAE2: 400 W, 20 min; UAE3 500 W, 10 min; UAE4: 500 W, 20 min). *White bars* indicate aqueous, and *black bars* indicate alkali-treated carrageenan. The results are from three separate experiments, and data are presented as mean±SD. Values were considered to differ significantly if the *p* value was less than 0.05



conventional extraction (22.34 %) compared with alkali conventional extraction (18.45 %). The sulphate content did not differ significantly between the conventional and UAE methods.

Significant variation ($p < 0.05$) in AG content was found between aqueous and alkali treatments. The AG content of carrageenan extracted using the conventional and UAE methods under various time and ultrasonic power conditions is shown in Fig. 1c. The highest AG content was detected in the carrageenan extracted after alkali treatment and was similar to that obtained under all UAE conditions.

The galactose content of carrageenan extracted using different methods is shown in Fig. 1d. Galactose content was higher for alkali extraction (32.88 %) compared with aqueous extraction (27.36 %). The galactose content did not differ significantly between different UAE conditions

FT-IR analysis

To investigate the carrageenan composition, we performed FT-IR of aqueous- and alkali-treated carrageenan extracted from *H. musciformis* (Fig. 2). The spectra of aqueous- and alkali-treated carrageenan were similar to those of the κ -standards, independently of the extraction condition (UAE) used. All spectra showed absorption bands at 1220–1226 cm^{-1} , corresponding to sulphate esters (Chopin et al. 1999). The presence of strong bands at 926–930 cm^{-1} in the FT-IR spectra indicated the presence of AG in all samples (Chopin et al. 1999; Pereira et al. 2003). The intensity of this band was not increased in aqueous- or alkali-treated carrageenan, implying

either absence or undetectable levels of the precursor 1,4-linked galactose-6-sulphate (D6S). All spectra also showed a band at 845 cm^{-1} , which was assigned to D-galactose-4-sulphate (G4S).

¹H NMR spectroscopy

Quantification of carrageenan by ¹H NMR spectroscopy is based on the position and intensity of the resonance of the

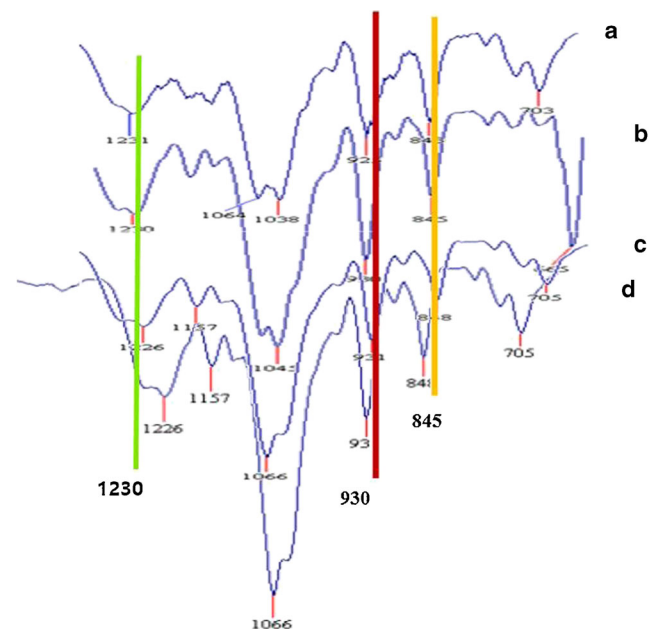


Fig. 2 FTIR spectra of sigma κ -carrageenan (a), conventional aqueous extracted (b), conventional alkali extracted (c) and ultrasonic-assisted extracted carrageenan: 500 W, 20 min (d)

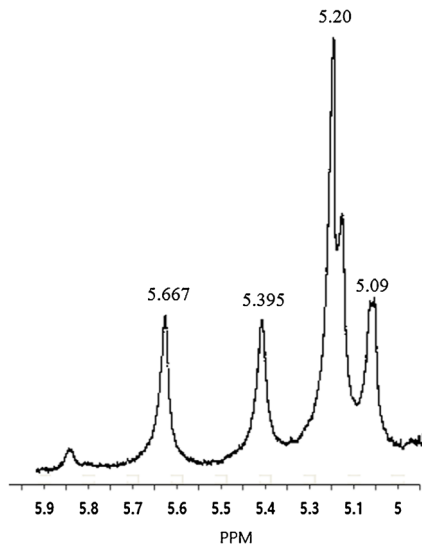


Fig. 3 ^1H NMR spectra of carrageenan in deuterium oxide (D_2O). The analysis was performed on JEOL JNMECP-400 MHz spectrometer at 80°C

α -anomeric hydrogen of the repeating D and DA—units in the region from 5.1 to 5.7 ppm. The peak at 5.09 show the presence of κ -monomers (Knutsen et al. 1994), and the peak at 5.6 shows the presence of α -D-galactose 2,6-disulfate (Fig. 3) (Van de Velde and De Ruiter 2002). No peak could be observed at 5.26 ppm, which refers to the presence of μ -monomers (precursor of κ -carrageenan) (Van de Velde and De Ruiter 2002)

Rheological parameters

Differential scanning calorimetry (DSC) To investigate the thermal stability of carrageenan, a gel was prepared using 1.5 % carrageenan containing 30 mM KCl in an aqueous environment. The heating transition obtained from DSC measurements for extracted κ -carrageenan occurred a single endothermic peak corresponding to the gel melting which was at $\sim 70.45^\circ\text{C}$ (Fig. 4a).

Carrageenan viscosity is shown in Fig. 4b. Alkali-treated carrageenan showed higher viscosity compared to that obtained using the conventional method. The carrageenan viscosity

did not vary significantly among the various UAE methods tested.

Antioxidant activity

We evaluated the antioxidant activity using different in vitro antioxidant assay (phosphomolybdenum, DPPH scavenging, hydroxyl radical scavenging and reducing power assay) since different methods works via different mechanisms. In case of these assay, the antioxidant activity of carrageenan did not vary significantly among the various UAE methods tested.

DPPH scavenging activity The DPPH radical is used to measure the free radical scavenging activity of antioxidants. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH free radical scavenging activity of carrageenan varied between 15.01 and 45.09 % (Fig. 5a), depending on the extraction method used whereas ascorbic acid (AA) showed 97.86 % scavenging activity. Aqueous-extracted carrageenan displayed greater (45.09 %) DPPH scavenging ability compared to that extracted using the alkali method.

Hydroxyl radical scavenging The hydroxyl radical is a highly reactive oxidising species that can react with most biomolecules. Carrageenan showed dose-dependent hydroxyl radical scavenging activity ranging from 7.8 to 50.7 % depending on the extraction method used (Fig. 5b). The maximum scavenging activity (50.7 %) was detected in carrageenan extracted using the alkali method.

Total antioxidant assay Quantitative antioxidant determination was performed using the phosphomolybdenum method, which is based on the reduction of molybdenum (Mo VI to Mo V) by the sample. In this assay, higher absorbance indicates higher antioxidant activity. The highest total antioxidant activity (ascorbic acid equivalent) was found for the alkali-treated carrageenan at 2 mg mL^{-1} , and the value was 3.8 mg AA equivalent (Fig. 5c). A slight variation in total antioxidant activity was observed between alkali- and aqueous-treated carrageenan.

Fig. 4 DSC thermographs (a) and viscosity (b) of extracted carrageenan

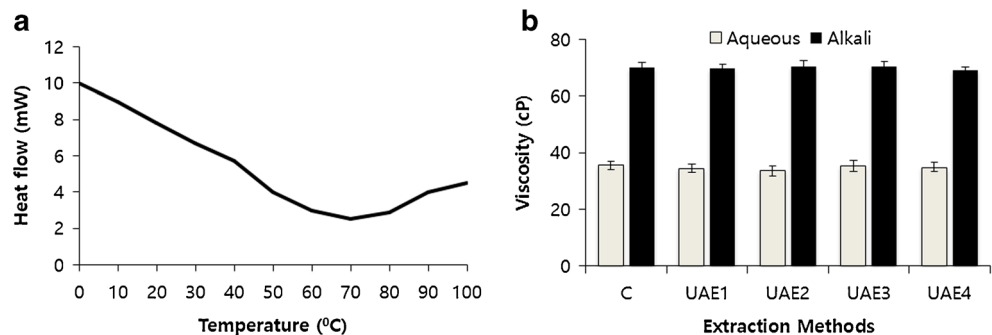
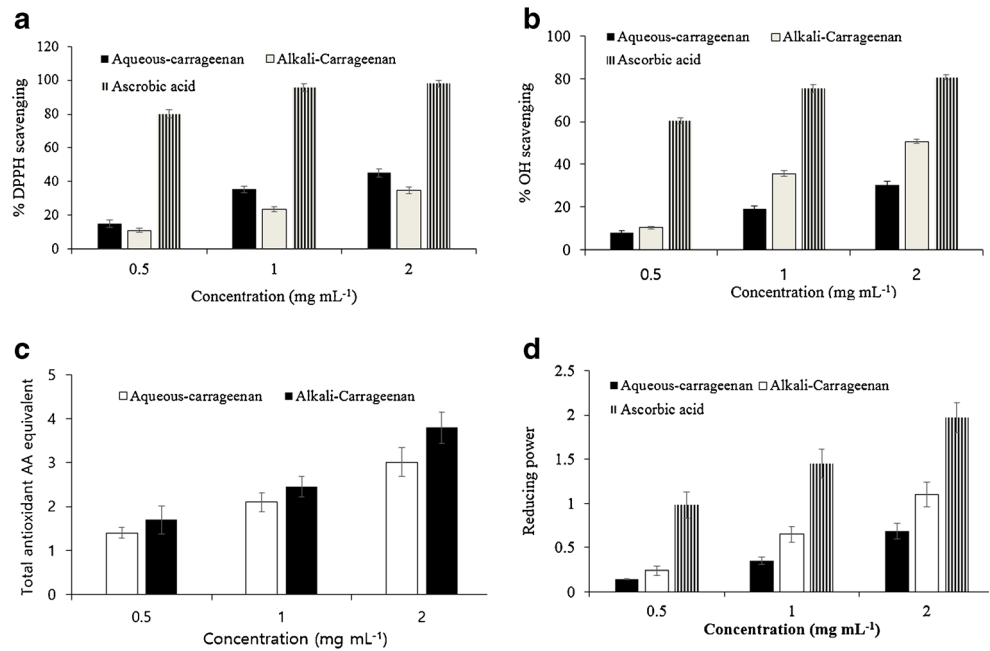


Fig. 5 Antioxidant activity of carrageenan measured by DPPH (a), hydroxyl radical scavenging (b), phosphomolybdenum (c) and reducing power (d) assay. Data are expressed as mean \pm SD, $n=3$. Difference letter superscripts indicate significant difference ($p\leq 0.05$)



Reducing power assay The reducing power assay is used to evaluate the ability of an antioxidant to donate electrons or hydrogen. In this assay, the presence of reductants in carrageenan reduces the ferric cyanide complex to a ferrous form. An increase in absorbance indicates an increased reducing power. Carrageenan showed dose-dependent reducing power, which varied depending on the extraction method used. The reducing power of 2 mg mL⁻¹ carrageenan was 0.687–1.1 (Fig. 5d). The maximum reducing power of alkali-extracted carrageenan was 1.1 (A_{700nm}) at 2 mg mL⁻¹. The absorbance of the control was 0.05 (A_{700nm}).

Discussion

Carrageenans are widely used in the food industry and may be a valuable and low-cost component of new drugs in the near future (Campo et al. 2009). Among the various types, κ -carrageenan is the major polysaccharide obtained from *H. musciformis*. Carrageenan has been extracted from *H. musciformis* collected from various regions worldwide (Table 1). These reports showed varying yields, chemical compositions, rheological properties and biofunctional activities, which were attributed to several factors such as species type, habitats, maturity, region and environmental conditions (Wong and Cheung 2000). However, no report of the isolation and characterisation of carrageenan extracted from *H. musciformis* in Bangladesh is extant. Therefore, in the present study, we isolated and characterised the chemical composition, rheological properties and antioxidant activity of carrageenan extracted from *H. musciformis* of Bangladeshi origin.

The market for carrageenan continues to expand and is relatively stable; however, its price is increasing due to the energy consumed during the extraction process and availability of raw materials. Thus, development of cost-effective carrageenan extraction methods and identification of new sources of seaweed are important. Although to date, diverse methodologies have been employed for carrageenan extraction including microwave-assisted extraction and UAE has not to our knowledge been utilised. The UAE method has been demonstrated to be a new extraction method for the recovery of a variety of bioactive compounds (Yang et al. 2011; Samaram et al. 2015). Therefore, we hypothesised that UAE could increase the carrageenan yield. In this study, carrageenan was extracted using the conventional and UAE methods. The influence of ultrasonic power (400 and 500 W) and time (10 and 20 min) was investigated in terms of yield and sulphate and AG contents. The yield was higher using the aqueous UAE (500 W, 20 min) method compared with the alkali UAE, aqueous- and alkali-extracted conventional methods (Fig. 2a). This result is in agreement with the report by Yang et al. (2011) showing that use of a hot alkaline condition resulted in some degradation of the polysaccharide due to extreme heat and alkaline processing. Conversely, the higher content of carrageenan obtained using the aqueous UAE method compared with the conventional method could be attributed to disruption of cell walls, reduction in particle size and increase in the mass transfer to the cell contents (Wang et al. 2009). In addition, the intensity of ultrasonic power increased the vibration of sample molecules and so facilitated the recovery of target compounds from solid material to the liquid solvent phase.

The sulphate and AG contents varied depending on the carrageenan extraction method used. Alkali pretreatment reduced carrageenan sulphate content by 17.41 % and increased that of AG by 20.16 %. Specifically, the chemical composition of carrageenan was altered due to alkali treatment, which is in agreement with Freile-Pelegrin et al. (2006). To analyse the ratio of AG with Gal, we determined galactose content of carrageenan obtained from different methods. The AG/Gal ratio deduced from colorimetric method is 1:1.08 which is close to that expected from a κ -carrageenan (Navarro and Stortz 2003). However, the significant increase in AG content after alkali treatment suggests the presence of undetectable levels of the μ -carrageenan precursor. Vázquez-Delfin et al. (2014) also stated that AG content was increased after alkali treatment indicated the presence of μ -carrageenan precursor.

To confirm this, we analysed the FT-IR and ^1H NMR spectra and found no evidence of the μ - and ν - precursors. Spectra analysis showed no variation in peak patterns between the aqueous- and alkali-treated carrageenan. However, the significant increase in AG content after alkali treatment suggests the presence of undetectable levels of the μ -carrageenan precursor. Confirming this fact, we performed ^1H NMR spectroscopy and no peak was observed at 5.26 ppm, which refers to the presence of μ -monomers (precursor of κ -carrageenan) (Van de Velde and De Ruyter 2002). Similarly, Vázquez-Delfin et al. (2014) reported that although AG contents increased after alkali treatment, no changes were found in the FT-IR peak pattern between aqueous- and alkali-treated carrageenan. Moreover, no band at 805 cm^{-1} was detected, indicating the absence of the ι -carrageenan precursor. The spectra of all carrageenans showed strong characteristic bands for G4S at $845\text{--}848\text{ cm}^{-1}$; AG at $930\text{--}931\text{ cm}^{-1}$ and sulphate ester (S) at $1220\text{--}1226\text{ cm}^{-1}$, which was confirmed as κ -carrageenan.

To assess rheological properties, thermal analysis was performed using DSC. To investigate their thermal properties, κ -carrageenan gels were prepared using 1.5 % alkali-treated κ -carrageenan with an ionic strength of 30 mM KCl in an aqueous environment. The heating transition of κ -carrageenan gel occurred over a temperature range with single endothermic peaks and a midpoint temperature of $\sim 70.45\text{ }^\circ\text{C}$. These findings are compatible with a previous report that the melting transition of κ -carrageenan occurred at $70\text{--}73\text{ }^\circ\text{C}$, even at higher salt concentrations (Hilliou et al. 2006). Similar results were also reported by Al-Alawi et al. (2011). In addition, alkali-treated κ -carrageenan showed higher viscosity compared to the aqueous κ -carrageenan. This result is in agreement with Freile-Pelegrin et al. (2006) who reported that alkali treatment prior to extraction leads to less sulphated carrageenan with better viscosity. Freile-Pelegrin et al. (2006) also reported that carrageenan viscosity is associated with the sulphate content.

Carrageenan is reportedly useful for preventing oxidative damage by scavenging free radicals produced in the human

body (Sokolova et al. 2011; Alves et al. 2012). In this study, we evaluated the antioxidant activity of carrageenan as measured using DPPH, hydroxyl radical scavenging, phosphomolybdenum and reducing power assays. The antioxidant activity of carrageenan extracted using the conventional and UAE methods was similar in all assays (data not shown). The stable DPPH radical is used widely to evaluate antioxidant activity and is less time-consuming than other methods. Both aqueous- and alkali-extracted carrageenans showed varying degrees of DPPH radical scavenging activity. Aqueous-extracted carrageenan showed superior scavenging activity than alkali-treated carrageenan (Fig. 5a), likely due to its hydrogen-donating ability, which is dependent on the polysaccharide structure, sulphate (Alves et al. 2012) and protein content. Confirming this fact, we measured protein content by Bradford method (Bradford 1976) and found that aqueous-extracted carrageenan contain higher amount of protein compared with alkali-treated carrageenan (data not shown). The higher amount of protein might be one of the reasons for showing higher DPPH scavenging activity by aqueous-extracted carrageenan since protein has the electron-donating properties (Kim et al. 2012). Similarly, aqueous-extracted carrageenan samples exhibited lower hydroxyl radical scavenging activity compared with alkali-treated carrageenan. This result is in agreement with Yuan et al. (2006) who reported that hydroxyl radical scavenging activities are likely associated with specific chelating groups (sulphate groups). This hypothesis was supported by our finding that the sulphate content was slightly higher in the aqueous than the alkali-treated carrageenan.

Conversely, alkali-treated carrageenan—which had a lower sulphate content—showed higher reduction capacity, as measured using phosphomolybdenum and reducing power assays. In addition, the reduction capacity is not only dependent on the presence of functional groups but also the structure of the polysaccharide itself (Sokolova et al. 2011). The authors found that κ -carrageenan, the least sulphated polysaccharide, showed the highest reduction capacity among all commercial carrageenans tested. Similarly, in our study, the aqueous-extracted carrageenan—which had a higher sulphate content—exhibited lower reducing activity than the alkali-treated carrageenan. Therefore, the reduction capacity depends not only on the structure of the polysaccharide but also its sulphate content.

Conclusion

This is the first study to assess the potential commercial exploitation of marine biopolymer as a raw material for κ -carrageenan extraction in Bangladesh. Carrageenan was extracted from *H. musciformis* using conventional and UAE methods, and its chemical composition and antioxidant

activities were analysed using a battery of chemical and spectroscopic methods. UAE improved the yield significantly compared with the conventional method and represented a good source of relatively pure κ -carrageenan. The FT-IR and ^1H NMR spectrum of carrageenan was identical to that of the reference sigma κ -carrageenan. The rheological properties were comparable with those of carrageenan isolated from similar species of red algae, in terms of showing moderate anti-oxidant activity in several in vitro systems. Therefore, *H. musciformis* from Bangladesh represents a potential raw material for carrageenan in the food industry as an additive and for pharmacological applications. Further studies are necessary to elucidate the structure of these phycocolloids.

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