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Synthesis and characterization of carboxymethyl derivatives of kappa-carrageenan

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ABSTRACT

Carboxymethyl derivatives of kappa-carrageenan (KC) with varying degrees of substitution were obtained by multi-step carboxymethylation process. The structural and chemical profiles of the derivatives were determined by FT-IR, ^{13}C NMR, gel permeation chromatography (GPC), elemental analysis, potentiometric titration and visual estimation of gelation temperature. The derivatives exhibited high degrees of substitution (DS) and acidity. However, sulfur content, molecular weight (Mw) and gelation temperature were lower compared to the parent KC polymer. FT-IR confirmed the presence of carboxymethyl (CM) groups in the polymer backbone while ^{13}C NMR revealed that functionalization occurred at the three hydroxyl groups in kappa-carrageenan disaccharide unit.

1. Introduction

Chemical modification of polysaccharides is considered as one of the most important route to enhance the properties of these biopolymers. Recent trends and strategies in research are geared towards functionalization of known materials and carboxymethylation of polysaccharides is one of the widely studied conversions that lead to development of new biomaterials with very promising applications. Carboxymethylation reactions have been studied in natural polymers, such as cellulose (Heinze, Pfeiffer, Liebert, & Heinze, 1999), starch (Stojanovic, Jeremic, & Jovanovic, 2000; Tijsen, Kolk, Stamius, & Beenackers, 2001), chitin (Kurita, 2001), chitosan (Abreu de & Campana-Filho, 2005), dextran (Huynh, Chaubet, & Jozefonvicz, 1998), gellan (Miyamoto, Tsuji, Nakamura, Tokita, & Komai, 1996), galactomannan (Parvathy, Susheelamma, Tharanathan, & Gaonkar, 2005) and xylan (Petzold, Schwikal, & Heinze, 2006), as starting materials. The derivates obtained are polyelectrolytes that can be applied in the chemical, pharmaceutical, food and cosmetic industries (Silva et al., 2004). Recently, novel materials based on carboxymethylated kappa-carrageenan were successfully developed (Yagi, Nagasawa, Hiroki, Tamada, & Aranilla, 2010) as suitable carriers for intestinal-targeted delivery of bioactive molecules (Leong et al., 2011) and wound healing matrix possessing anticoagulant and antimicrobial properties with moisture absorbability and retention capacity (Fan et al., 2011). The degree of substitution of polysaccharides can be increased by a multi-step reaction, i.e., the carboxymethylated polysaccharide synthesized is isolated and subsequently carboxymethylated again under comparable conditions. Multi-step carboxymethylation was applied as an alternative path of polysaccharide etherification leading to new functionalization patterns and derivatives with valuable features (Lawal, Lechner, & Kulicke, 2008a, 2008b; Heinze & Koschella, 2005; Lazik, Pfeiffer, Albrecht, & Mischnick, 2002).

Carrageenans are naturally occurring, commercially important family of hydrophilic polysaccharides extracted from certain species of marine red algae (Velde van de, Pereira, & Rollema, 2004; Therkelsen, 1993). More specifically, carrageenans are high molecular weight, highly sulfated, linear molecules with galactose backbones. Kappa-carrageenan is one of the three most important commercial forms of carrageenans and is widely used in biomedical, food and non-food applications (Aguilan, Dayrit, Lebrilla, Ninonuevo, & Zhang, 2006; Mendoza, Montaño, Ganzon-Fortes, & Villanueva, 2002). The idealized disaccharide repeating unit is consist of alternating α-(1,4)-D-galactose-4-sulfate (G4S) and β-(1,3)-3,6-anhydro-D-galactose (DA) residues (Abad et al., 2011; Jiang, Guo, & Tian, 2005).

This paper describes the functionalization of a commercial-grade kappa-carrageenan to produce derivatives with high degree of substitution using multi-step carboxymethylation process. The

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chemical and structural characteristics of the derivatives are described and compared to the untreated polymer. The work is part of the study on preparation and characterization of carboxymethyl-kappa carrageenan based hydrogels crosslinked by gamma radiation and evaluation of these biomaterials for potential applications as wound dressing, metal adsorbent and water absorbent gel.

2. Experimental

2.1. Materials

Refined commercial grade kappa-carrageenan (WG-2000) was purchased from Shemberg Marketing Corporation, Philippines and used “as received” in the carboxymethylation reactions. All chemicals were analytical grade and used without further purification. Distilled or purified water was utilized for all reagent preparations.

2.2. Carboxymethylation of κ-carrageenan (CMKC)

Kappa-carrageenan (80 g) was suspended in 80% (w/v) 2-propanol (720 mL) in a 1 L 3-necked glass reactor vessel equipped with a reflux cooler and attached to a speed adjustable impeller. While stirring vigorously at room temperature, 40% (w/v) NaOH (93.5 mL) was added dropwise with the aid of a buret. Stirring was continued for another 1 h at 40 °C and after alkaline activation, monochloroacetic acid (MCA; 49.1 g) was added. The reaction was allowed to proceed for 3 h at 40 °C. After carboxymethylation, the mixture was filtered, suspended in 80% (w/v) 2-propanol and neutralized with acetic acid. The products were collected after filtration, washed 3 times with 80% (w/v) 2-propanol followed by pure 2-propanol and dried in vacuum oven at 40 °C. The subsequent carboxymethylation steps were run in a similar procedure.

Derivatives were coded as CMKC-1s (1× conversion), CMKC-2s (2× conversion) and CMKC-3s (3× conversion). Blanks were prepared for each conversion step by treating κ-carrageenan samples in the same reaction conditions devoid but of MCA.

2.3. Chemical and structural characterization

Prior to characterization, all samples were purified 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, samples were precipitated in pure 2-propanol, vacuum dried at 40 °C and milled into powder under nitrogen using a SPEX 6700 freezer mill.

The degree of substitution and degree of acidity were estimated by potentiometric-back titration. All samples were first transformed into acid form to ensure that all carboxymethyl and sulfate groups will be titrated. Aqueous solutions (2.5%, w/v) were passed through an ion-exchange resin (Amberlite IR-120H^+) and freeze dried subsequently. Titration was accomplished using a KEM AT-510 automatic potentiometric titrator, where solutions of acidified samples (10 mg/mL) were titrated with standardized 0.0185 M HCl after addition of known amount of standardized 0.020 M NaOH. The alkali-treated κ-carrageenan samples served as blanks to account for sulfate acids present. The DS was calculated as the molar ratio of carboxymethyl acid groups to kappa-carrageenan disaccharide unit minus the blank. The results were expressed as mean of three trials.

Table 1
Chemical properties of untreated, alkali-treated and carboxymethylated κ-carrageenan.

Sample	DS	Degree of acidity	Sulfur (%)	Mw (kDa)
κ-Carr[a]	–	1.06	7.26	3271
κ-Carr Alk 1s[b]	–	0.82	6.89 (95[d])	1173
κ-Carr Alk 2s	–	0.81	6.50 (89)	1053
κ-Carr Alk 3s	–	0.77	3.83 (71)	701
CMKC-1s[c]	1.20	2.02	6.55 (90)	2318
CMKC-2s	1.44	2.25	5.07 (70)	1526
CMKC-3s	1.66	2.43	3.71 (51)	1421

[a] Untreated.
[b] Alkali- treated (blanks).
[c] Carboxymethylated.
[d] Percent remaining sulfur in the samples.

Weight average molecular weight (Mw) was measured using a Tosoh gel permeation chromatograph Model CO-8020 equipped with three TSKgel PWL_{XL} columns in series. Elution was at 40 °C with 0.2 M $NaNO_3$ solution at a flow rate of 0.5 mL/min and monitored by RI-8020 detector. The Mw was determined from a calibration curve constructed from eight pullulan standards with Mw range values from 5.9 kDa to 790 kDa.

Analysis of elemental sulfur was performed using Perkin Elmer CHNS Analyzer 2400 series II using glycine as standard.

Visual estimation value for gelation temperature (T_{gel}) was arbitrarily chosen as the temperature at which a gel would initially form. Ten milliliter (10 mL) aqueous samples at concentrations 0.1 g/mL, 0.25 g/mL, 0.5 g/mL and 1.0 g/mL were prepared in tubes and heated for 30 min at 80 °C to dissolve completely. After complete dissolution, the tubes were covered with fitted thermometer and cooled down at room temperature for observation of gel formation. Potassium chloride solution was also used to determine the effect of potassium ion (K^+) on the gelation of the samples.

The FT-IR spectra were recorded on films using Spectrum One Perkin Elmer Spectrophotometer. Films were prepared by drying 1 mL (3 mg/mL) sample solutions on a polyethylene surface at 60 °C.

^{13}C NMR spectra were recorded on a Bruker DRB-300WB spectrometer operating at 75.48 MHz at 90 °C. Intact samples (50 mg/mL) were dissolved in 1:1 D_2O:H_2O and transferred into 5 mm o.d. NMR tubes. Deuterated dimethylsulfoxide (DMSO-d_6) was added as an internal reference. Chemical shifts (δ ppm) were relative to internal standard DMSO-d_6 ($\delta = 39.4$ ppm).

3. Results and discussion

The conditions used for the carboxymethylation process had been optimized previously by studying the effect of reaction parameters such as 2-propanol/H_2O ratio, NaOH, MCA, temperature and reaction time. The optimum conditions guaranteed that the reaction mixture could be stirred for the duration of the reaction and no gelation would occur (Aranilla, 2008). Carboxymethylation was carried out three times and the yield for each step was more than 95%. The etherification reaction is depicted in Fig. 1.

3.1. Chemical properties

The chemical properties of the untreated, alkali-treated and carboxymethylated KC are summarized in Table 1.

Multi-step carboxymethylation of kappa-carrageenan lead to the synthesis of derivatives with varying DS, acidity, sulfation and molecular weight. The DS and degree of acidity of the samples increased with increase in functionalization steps. The acidity of kappa-carrageenan is attributed to the presence of half-ester sulfate moieties in its backbone. The results indicate the successful introduction of carboxymethyl groups in the KC polymer chain.

However, desulfation occurred significantly for the alkali-treated and carboxymethylated samples, the latter treatment causing higher reduction effect. The high alkaline condition in the reaction induced hydrolysis of SO_3^- in C-4 of the β-D-galactose unit. In the industrial processing of carrageenans, alkaline treatment results in desulfation by removal of sulfate group *syn* to a

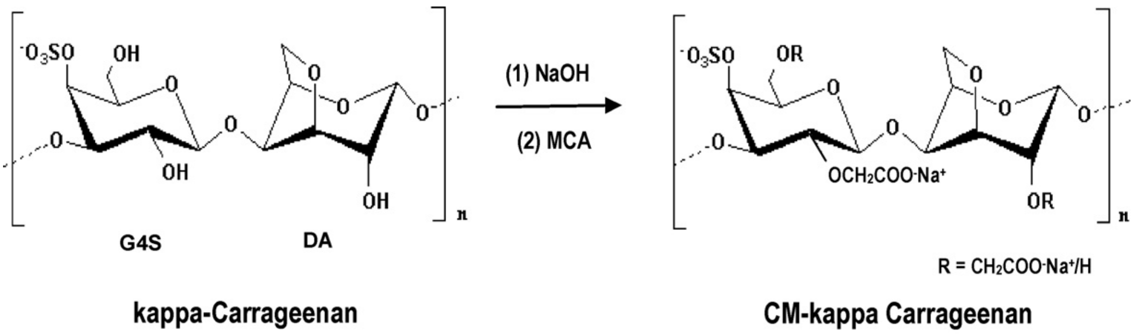

Fig. 1. Carboxymethylation of kappa-carrageenan.

free hydroxyl group. Also, in the presence of base, intramolecular nucleophilic displacement occurs resulting in anhydro-ring formation (Aguilan, 2005). For this study, desulfation at C-4 of the β-D-galactose residue possibly transformed the carbon into a hydroxylated form.

Depolymerization was also concomitant with the modification process as indicated by the decrease in Mw of the samples. The reduction was more significant in alkali-treated KC while it was evident only after the first-step carboxymethylation of KC. With subsequent steps, decrease in Mw became minimal and the effect of CM group substitution became apparent. Molecular weight degradation during etherification process was also observed in high molecular weight cellulose (Schult & Moe, 1997) and other polysaccharides (Xu et al., 2009; Ren, Sun, & Peng, 2008).

3.2. *Physical gelation properties*

Physical gel formation is one of the most important features of carrageenans. In aqueous solutions and in the presence of cations, both kappa- and iota-carrageenans easily form thermoreversible gels, which melt on heating and form into gel again upon cooling (Velde van de and Ruiter de, 2002). One mechanism universally accepted on the gelation of carrageenans describes that at moderate polymer concentration, ion-induced conformational transition takes place from a disordered (coil) to an ordered (helix) form followed by aggregation and network formation when the polymer concentration and/or the ionic strength are increased and/or temperature is decreased (Reynaers, 2003). Several investigators favor coaxial double helix as the fundamental ordered conformation: upon reducing the temperature or increasing the salt concentration, double-helical stretches will be formed in a thermoreversible process. Afterwards, these double helices will pair and form the physical junctions responsible for the gelling behavior of carrageenans (Mangione, Giacomazza, Bulone, Martorana, & San Biagio, 2003; Meunier, Nicolai, & Durand, 2001; Morris, Rees, & Robinson, 1980). Other groups of researchers presented evidences of a single helix as the fundamental ordered state of the carrageenan prior to association and gel formation. In the latter context, it is accepted that single-helical regions along the polymer chain associate into larger stretches upon increasing salt concentration, polymer concentration and/or decreasing temperature (Bosco, Segre, Miertus, Cesaro, & Paoletti, 2005; Ciancia, Milas, & Rinaudo, 1997; Ueda, Itoh, Matsuzaki, Ochiai, & Imanura, 1998; Smidsrod, Andresen, Grasdalen, Larsen, & Painter, 1980).

The estimated gelation temperature and formation of physical gel (in the presence of 1% KCl) of the different samples are presented in Table 2. In alkali-treated KC, gelation occurred at a higher concentration but at a lower temperature as compared to untreated KC. In the presence of K^+ ion, gel formation was positive for all concentrations. The observed difference in the gelation behavior of the two samples can be attributed mainly to difference in Mw. At the same concentration but lower Mw, shorter polymer chains would associate easily resulting to lower gelation temperature. The gelation of alkali-treated KC was still exhibited with and without the presence of K^+ ion. In carboxymethylated samples, only CMKC-1s and CMKC-2s formed gels while CMKC-3s formed no gels for all concentrations studied. In the presence of K^+ ion, gelation occurred only at 5–10% polymer concentration for CMKC-2s and CMKC-3s. With increase in CM groups, gelation ceased to occur. These observations show that carboxymethyl groups affect the gelation behavior of KC by imparting steric hindrance to the regular chain of KC and interfering with the conformational transition and aggregation of the polymer to form gel. In the presence of K^+ ion, gelation may have occurred as a result of aggregation of polymer chains through bridging effect of potassium between CM groups.

3.3. *FT-IR analyses*

The FT-IR spectra of KC and carboxymethylated KC are presented in Fig. 2. In the parent KC, the band at $\nu = 928\ \mathrm{cm}^{-1}$ was attributed to the C–O–C vibration of the 3,6-anhydro-D-galactose residue.

Table 2
The gelation properties of κ-carrageenan (untreated, alkali-treated and carboxymethylated).

Sample	Gelation Temperature (°C) Concentration (%)				Gelation in the presence of 1% KCl			
	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0
κ-Carr	31	43	56	83	+	+	+	+
κ-Carr Alk 1s	–	28	45	63	+	+	+	+
κ-Carr Alk 3s	–	–	18	33	+	+	+	+
CMKC-1s	–	26	42	65	–	+	+	+
CMKC-2s	–	–	23	40	–	–	+	+
CMKC-3s	–	–	–	–	–	–	+	+

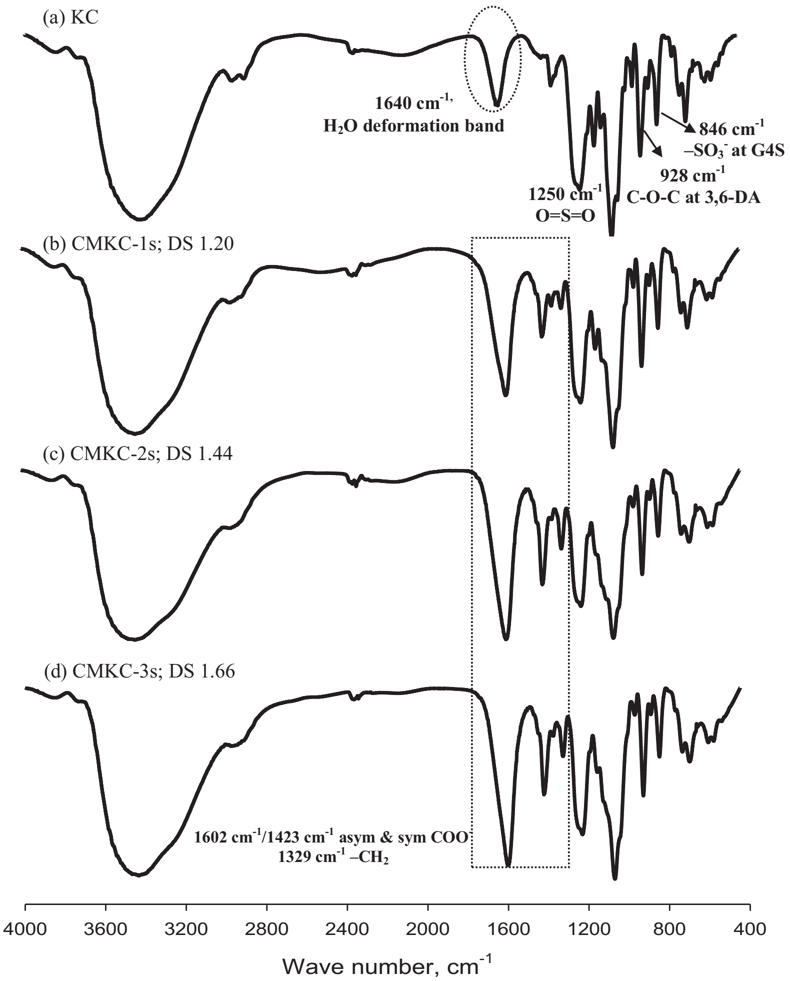

Fig. 2. FT-IR spectra of kappa-carrageenan and carboxymethylated kappa-carrageenan.

The presence of signals at 1250 cm^{-1} and 846 cm^{-1} were assigned respectively to O═S═O symmetric vibration and to $-O-SO_3$ stretching vibration at C-4 of β-D-galactose residue (Roberts & Quemener, 1999; Turquois, Ascquistapace, Arce Vera, & Welti, 1996). The intense band at 1640 cm^{-1} was related to the structural water deformation band (Kacurakova & Wilson, 2001; Usov, 1984). In the spectra of CMKC samples, the new bands at $\nu = 1602\ cm^{-1}$ and 1423 cm^{-1} were assigned to the asymmetrical and symmetrical stretching vibrations of carboxylate anions ($-COO^-$) while the band at $\nu = 1329\ cm^{-1}$ was a characteristic of the $-CH_2$ scissoring in the carboxymethyl group (Tien-Le, Milette, Mateescu, & Lacroix, 2004; Wang, Zhang, & Ruan, 2004). The three new bands confirm the successful carboxymethylation of κ-carrageenan.

3.4. ^{13}C NMR analyses

The proton decoupled ^{13}C NMR analyses gave more significant information on the structural properties of CMKC derivatives. The characteristic signals are depicted in Fig. 3.

Fig. 3a corresponds to the spectrum of untreated KC showing strong intensity signals of the twelve carbon atoms in the disaccharide repeating unit. The chemical shifts are given in Table 3 and agree closely with reported values (Kolender & Matulewicz, 2004; Aguilan et al., 2003).

New signals were present in the CMKC spectra (Fig. 3b–d) affirming more the results of FT-IR analyses. The prominent peak at $\delta = 178.1$ ppm was assigned to the carboxylate anion ($-COO^-$) of the carboxymethyl group. The observed splitting of the peak may be attributed to different possible positions of CM substituents on the KC polymer chain (Lawal et al., 2008a, 2008b). The methylene ($-CH_2$) carbon atom of the CM substituents were assigned with peaks at $\delta = 73.3$ ppm and 73.5 ppm. These assignments agree with published values (Xu et al., 2009; Lawal et al., 2008a, 2008b).

Carboxymethyl substitution caused a downfield shift of ~10 ppm on C-6 as indicated by the resonance at $\delta = 71.4$ ppm which was assigned to the functionalized C-6 of the G4S residue. Substantial downfield shift on C-2 was also observed on signals at $\delta = 77.5$ ppm and $\delta = 78.3$ ppm assigned to the modified C-2 of G4S and DA residues, respectively. Generally, functionalization results in specific modifications of the ^{13}C NMR spectrum of the parent polysaccharide (Liao, Kraft, Munro, Craik, & Bacic, 1993). The substituents give rise to a substantial downfield shift of the nearest carbon atom resonance and to moderate upfield shifts of the signals of the neighboring carbon atoms (Roberts & Quemener, 1999; Turquois et al., 1996). Such a downfield shift helps to define the location and content of functional groups in the polymer. In the case of methylation, glycosylation or carboxymethylation of a hydroxyl group, a downfield shift by about 8–11 ppm in the resonance of the attached carbon is observed (Lawal et al., 2008a, 2008b; Aguilan, 2005).

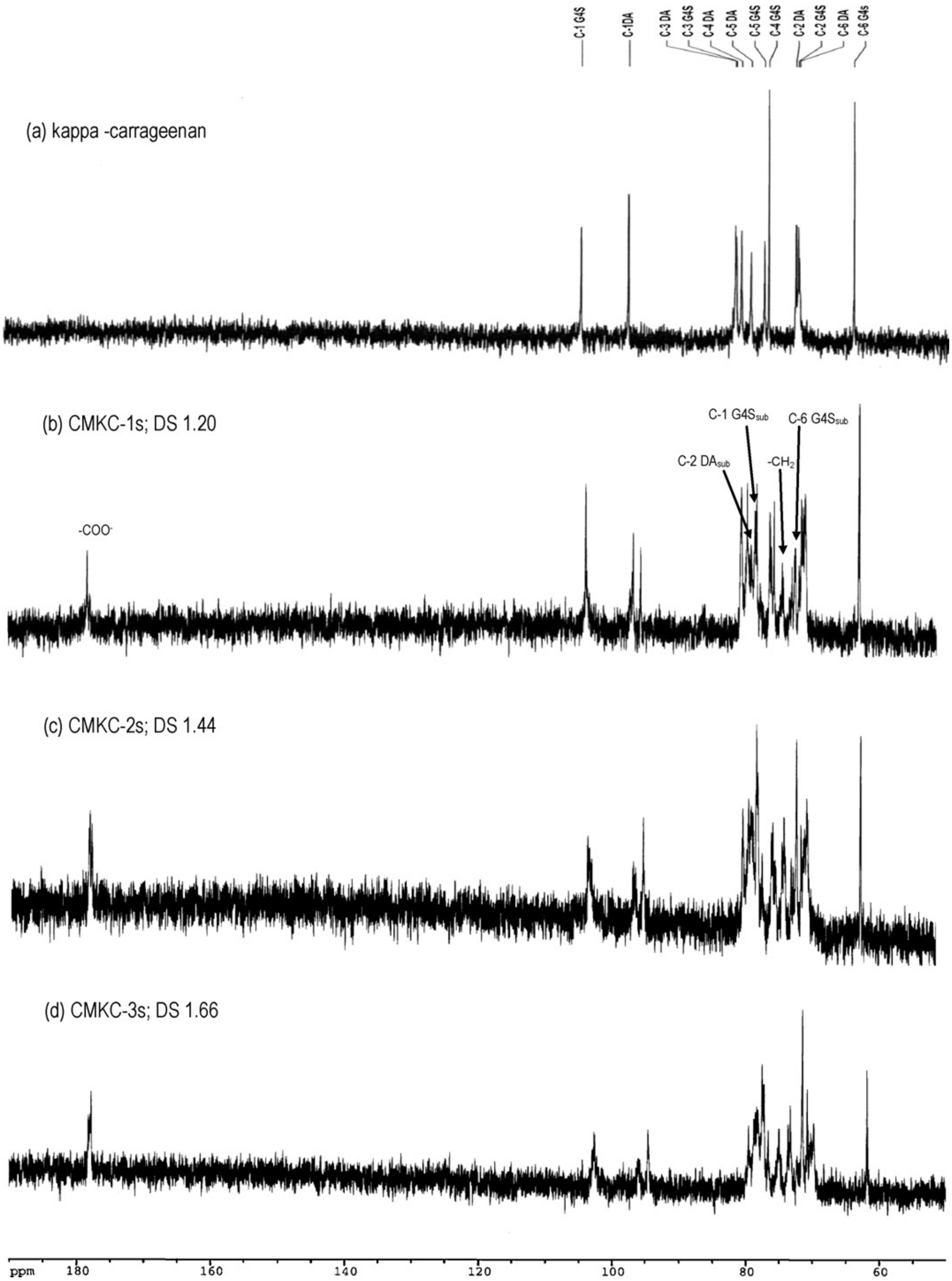

Fig. 3. ^{13}C NMR spectra of kappa-carrageenan and carboxymethylated kappa-carrageenan.

No signals were present at $\delta = 66.4$–66.5 ppm in the spectra of carboxymethylated samples, which was reported to correspond to C-4 of an unsulfated β-D-galactose residue (Falshaw, Furneaux, & Wong, 2003; Yu et al., 2002; Liao et al., 1993). However, desulfation was indicated by the appearance of a peak at $\delta = 94.5$ ppm assigned to the anomeric carbon (C-1) of the 3,6-anhydro-D-galactose residue. The upfield shift in resonance of the anomeric carbon was said to be due to attachment of the residue to a desulfated β-D-galactose unit (Falshaw et al., 2003). In the NMR study of β-carrageenan (a desulfated κ-carrageenan), C-1 in the DA residue is shown to resonate at 94.7 ppm (Kolender & Matulewicz, 2004; Falshaw et al., 2003; Liao et al., 1993).

The ^{13}C NMR analysis suggested the relative reactivity for carboxymethylation of the hydroxyl groups in kappa-carrageenan. From the ratio of the peak intensity of the substituted carbon to the unsubstituted carbon, –OH reactivity was found to be in the order of O-C2$_{G4S}$ > O-C6$_{G4S}$ > O-C2$_{DA}$. This relative order was also observed in the study on succinylation of low-molecular weight kappa-carrageenan (Jiang et al., 2005).

Table 3
^{13}C chemical shifts (δ ppm) of κ-carrageenan and CM-κ-carrageenan.

Unit	Carbon nuclei	KC[a]/Ref[b]	CMKC-3s
D-Galactose-4-sulfate (G4S)	C-1	102.9/102.7	102.9
	C-2	70.1/69.9	69.9
	C-3	79.5/79.1	79.5
	C-4	74.5/74.3	74.5
	C-5	75.2/75.0	75.2
	C-6	61.7/61.5	61.7
3,6-Anhydro-D-galactose (DA)	C-1	95.7/95.4	95.7
	C-2	70.4/70.1	70.4
	C-3	79.6/79.4	79.4
	C-4	78.7/78.5	78.5
	C-5	77.2/77.0	77.0
	C-6	69.9/69.7	69.7
Carboxymethyl group	$-COO^-$	–	178.1,177.9,177.7
	$-CH_2$	–	73.3, 73.5
Functionalized carbon	C-6 G4S	–	71.4
	C-2 G4S	–	77.5
	C-2 DA	–	78.3

[a] 50 mg/mL in 1:1 H_2O/D_2O recorded at 90 °C, shifts relative to DMSO-d_6 (δ = 39.4 ppm).
[b] 20–40 mg/mL in 1:1 H_2O/D_2O recorded at 80 °C, shifts relative to DMSO, δ = 39.6 ppm (Kolender & Matulewicz, 2004).

4. Conclusions

Functionalization of a commercial grade kappa-carrageenan was successfully achieved via multi-step carboxymethylation process. The derivatives exhibited high DS values ranging from 1.20 to 1.66 and increased degree of acidity. However, depolymerization and desulfation were concomitant with the modification process. The carboxymethyl derivatives also showed different gelling behavior compared to the parent polymer. FT-IR analyses confirmed the introduction of carboxymethyl groups into the kappa-carrageenan backbone. The ^{13}C NMR analyses revealed the functionalization and relative reactivity of the hydroxyl groups of kappa-carrageenan disaccharide unit. The relative reactivity proceed in the order $O\text{-}C2_{G4S} > O\text{-}C6_{G4S} > O\text{-}C2_{DA}$.

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