ELSEVIER

Carbohydrate Research 337 (2002) 433–440

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

Structural studies on κ-carrageenan derived oligosaccharides

Guangli Yu,[a] Huashi Guan,[a] Alexandra S. Ioanoviciu,[b] Sulthan A. Sikkander,[b] Charuwan Thanawiroon,[b] Joanne K. Tobacman,[c] Toshihiko Toida,[d] Robert J. Linhardt[b,*]

[a]*Marine Drug and Food Institute, Ocean University of Qingdao, Qingdao 266003, People's Republic of China*
[b]*Department of Medicinal and Natural Products Chemistry, Chemistry, and Chemical and Biochemical Engineering, College of Pharmacy, University of Iowa, Iowa City, IA 52242, USA*
[c]*Department of Internal Medicine, University of Iowa Health Care, Iowa City, IA 52242, USA*
[d]*Department of Bioanalytical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan*

Received 3 September 2001; received in revised form 7 January 2002; accepted 8 January 2002

Abstract

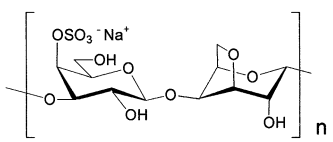
Oligosaccharides were prepared through mild hydrochloric acid hydrolysis of κ-carrageenan from *Kappaphycus striatum* carrageenan. Three oligosaccharides were purified by strong-anion exchange high-performance chromatography. Their structure was elucidated using mass spectral and NMR data. Negative-ion electrospray ionization (ESI) mass spectra at different fragmentor voltages provided the molecular weight of the compounds and unraveled the fragmentation pattern of the κ-carrageenan oligosaccharides. 2D NMR techniques, including $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ TOCSY and $^{13}C-^{1}H$ HMQC, were performed to determine the structure of a trisulfated pentasaccharide. 1D NMR and ESIMS were used to determine the structures of a κ-carrageenan-derived pentasaccharide, heptasaccharide, and an undecasaccharide. All the oligosaccharides characterized have a 4-*O*-sulfo-D-galactopyranose residue at both the reducing and nonreducing ends. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: κ-Carrageenan; *Kappaphycus striatum*; NMR data; Oligosaccharides

1. Introduction

Carrageenans are a family of anionic polymers extracted from certain marine red algae. These polysaccharides are used as additives to improve food texture, gelation, stability, and viscosity[1] and are generally regarded as safe (GRAS) by the Food and Drug Administration in the US.[2] Breakdown products of molecular weight $<40,000$, called poligeenans, have been implicated in gastrointestinal malignancy in animal models.[3,4] Thus, additional biological evaluation of poligeenans is warranted.[4–7]

Carrageenans consist of alternating (1→3)-linked β-D-galactopyranose (Gal*p*) and (1→4)-linked α-D-galactopyranose. The commercially important κ-carrageenan (Scheme 1) contains a 3,6-anhydro-α-D-galactopyranose (AnGal*p*) in place of α-D-galactopyranose, giving it gelling properties.[2] The addition of a 2-*O*-sulfo group to the κ-carrageenan sequence [→3)-β-D-Gal*p*4S-(1→4)-α-D-AnGal*p*-(1→]$_n$ affords another commercially important carrageenan, ι-carrageenan [→3)-β-D-Gal*p*4S-(1→4)-α-D-AnGal*p*2S-(1→]$_n$.[8] Structural studies on these intact polymers have primarily relied on ^{1}H and ^{13}C NMR spectroscopy.[9] Other studies have prepared κ-carrageenan oligosaccharides through depolymerization by acid hydrolysis,[10,11] methanolysis,[12] reductive hydrolysis,[13,14] and using κ-



Scheme 1.

* Corresponding author. Tel.: +1-319-3358834; fax: +1-319-3356634.
E-mail address: robert-linhardt@uiowa.edu (R.J. Linhardt).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00009-5

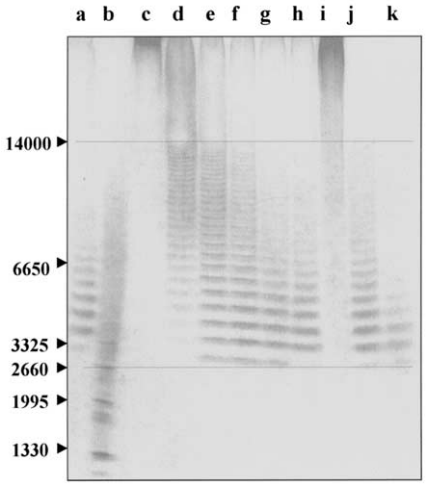


Fig. 1. Gradient PAGE analysis to follow the acid hydrolysis of κ-carrageenan. Lanes a and b contain oligosaccharide standards prepared in our laboratory. The heparin oligosaccharide bands in lane b are labeled on the left of the gel with their molecular weights.[24] Lanes c and i contain κ-carrageenan. Lanes d, e, f, g, and h contain κ-carrageenan hydrolyzed with 0.1 M HCl at 37 °C for 24, 36, 48, 60, and 72 h, respectively. Lanes j and k contain κ-carrageenan hydrolyzed with 0.1 M HCl at 60 °C for 2 and 4 h, respectively. The gel was visualized by Alcian blue staining. This cationic dye binds to polyanions with affinity that increases with increased charge, thus oligosaccharides having a low-molecular weight and/or a low level of charge are not detected well using this method.

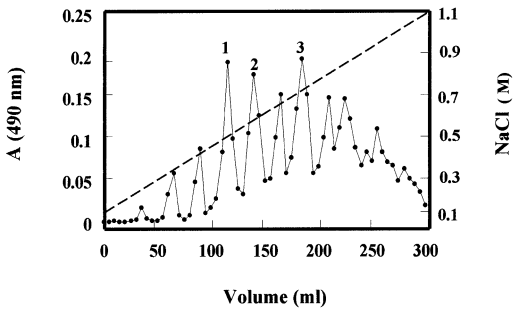


Fig. 2. SAX-HPLC fractionation of carrageenan oligosaccharides. The major peaks labeled **1**–**3** eluting between 100 and 200 mL were characterized.

carrageenanase.[15–18] The structures of the resulting oligosaccharides have been determined using 1D and 2D ^{1}H and ^{13}C spectroscopy[12,15,18] and electrospray ionization–mass spectrometry (ESIMS).[19]

There is interest in the possibility that κ-carrageenans in foods might be hydrolyzed in the acidic environment of the stomach producing poligeenans. Strong mineral acids are known to hydrolyze carrageenans and have been useful in preparing monosaccharide components for derivatization and composition analysis by GC–MS.[20] Earlier work[21,22] showed that κ-carrageenan treated with simulated gastric fluid (pH 1.0) showed significant breakdown, as measured by viscosimetry and gel-permeation chromatography. On the basis of these studies, we decided to undertake a detailed examination of controlled acid hydrolysis under relatively mild conditions to examine the stability of κ-carrageenan, to prepare oligosaccharide standards, and to test their biological activity. This paper reports the structure determination of three major oligosaccharides prepared from κ-carrageenan by mild-acid hydrolysis, using NMR spectroscopy, and ESIMS.

2. Results and discussion

Carrageenan from *Kappaphycus striatum* was fractionated[23] to obtain pure κ-carrageenan in 66% yield. Mild-acid hydrolytic depolymerization of κ-carrageenan affords poligeenan, a mixture of lower molecular-weight polysaccharides and oligosaccharide products. Depolymerization reactions using 0.1 M hydrochloric acid at 37 and 60 °C were monitored by discontinuous gradient polyacrylamide gel electrophoresis,[24] and visualized by staining with Alcian blue dye (Fig. 1). At 60 °C, the 2 and 4 h time points (Fig. 1, lanes j and k) both showed a good distribution of low-molecular weight products. Thus, a 3-h timepoint was selected for a larger scale (5 g) preparation of carrageenan oligosaccharides of a size suitable for structural characterization. The poligeenan–oligosaccharide mixture was pressure filtered to remove poligeenan components $M_W > 5000$ from the desired carrageenan oligosaccharides. The filtrate was then desalted and fractionated by semi-preparative SAX-HPLC (Fig. 2). The early peaks eluting at low-salt concentration between 0 and 100 mL, corresponding to low-molecular weight oligosaccharides (monosaccharide to tetrasaccharides), were difficult to desalt and were not characterized. Gradient PAGE analysis (not shown) indicated that the late peaks eluting at high-salt concentrations between 200 and 300 mL contained inseparable, complex mixtures of higher oligosaccharides and thus, were not further characterized. The major peaks eluting between 100 and 200 mL (labeled **1**–**3** in Fig. 2) were collected, re-fractionated on SAX-HPLC, desalted, and analyzed.

Gradient PAGE analysis using Alcian blue staining suggested that fractions **2** and **3** were sufficiently pure to undertake their analyses (Fig. 3(A)). Alcian blue staining visualizes highly sulfated oligosaccharides, but is relatively insensitive to oligosaccharides having a low content of sulfo groups.[25] Our failure to observe oligosaccharide **1** suggested that it contained a relatively small number of sulfo groups. To further assess the purity of oligosaccharides **1**–**3**, each was labeled by reductive amination with ANDS, a charged fluorescent tag, fractionated by PAGE, and visualized by

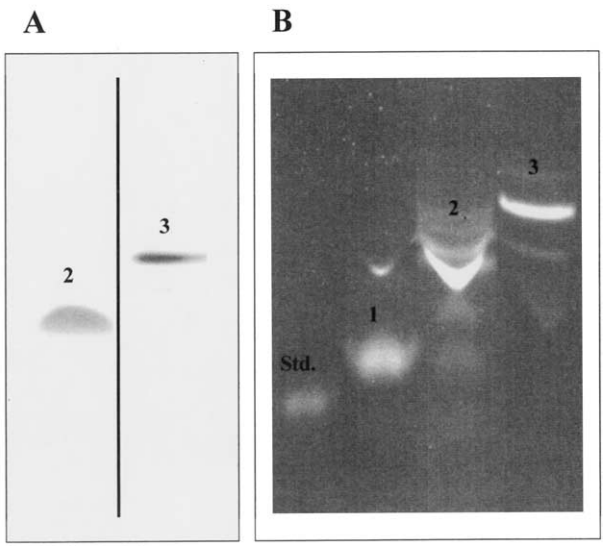


Fig. 3. PAGE analysis of carrageenan oligosaccharides **1**–**3**. (A) Gradient PAGE analysis of fractions **2**–**3** using Alcian blue staining. (B) Gradient PAGE analysis of ANDS-labeled oligosaccharides **1**, **2**, and **3** with visualization by UV transillumination. The lane on the left marked std. corresponds to an ANDS-labeled dermatan sulfate hexasaccharide standard.[29]

transillumination[26] (Fig. 3(B)). The results of this analysis showed that oligosaccharides **1**, **2**, and **3** each contained a major band in addition to minor contaminating bands. High sensitivity, quantitative analysis of oligosaccharide purity, relying on reversed-polarity capillary electrophoresis using fluorescence detection[27] (data not shown) indicated **1**, **2**, and **3** were 73, 89, and 88% pure. The impurities observed corresponded to multiple, very minor peaks, suggesting that all three samples were suitably pure for spectral analysis.

The structure of oligosaccharides **1**–**3** was next investigated using mass spectrometry and NMR spectroscopy. ESIMS gave molecular ions for the sodium salt forms of all three sulfated oligosaccharides. ESIMS analysis of compound **1** (Table 1) showed a molecular ion $[M-Na^+]^-$ at m/z 1075 corresponding to a mass of 1098 suggesting that it was a pentasaccharide containing three galactopyranose (Gal*p*) residues and two 3,6-anhydrogalactopyranose (AnGal*p*) residues and had three *O*-sulfo groups. Fragmentation was consistent with each of the three Gal residues carrying one *O*-sulfo group. Next, full structural characterization was undertaken using ^1H and ^{13}C NMR spectroscopy (Fig. 4) and was compared to published values for carrageenan and carrageenen oligosaccharides (Tables 2 and 3).

The $^1H-^1H$ COSY spectrum of **1** demonstrated the presence of several spin systems. The first spin system describes the reducing end (re) α-4-*O*-sulfogalactopyranose anomer (α-Gal*p*4S$_{re}$). The correlations present in the COSY spectrum allowed the assignment of H-1, H-2, H-3, and H-4 of this galactopyranose unit (Table 2). The coupling constant between H-4 and H-5 protons in this galactopyranose residue was too small to give a correlation. The signal of H-1 of the AnGal*p* residue, directly linked to the α-Gal*p*4S$_{re}$ unit, was slightly shifted downfield. A distinct signal could be

Table 1
Assignment of ESIMS of carrageenan oligosaccharides **1**–**3**

Ion	Carrageenan oligosaccharide [a]		
	1	**2**	**3**
$[M-Na^+]^{-1}$	1075 (0, 70, 140, 210)	1483 (210)	
$[M-2Na^+]^{-2}$	526 (0, 70, 140, 210)	730 (70, 210)	1138 (70, 210)
$[M-3Na^+]^{-3}$	343 (0, 70)	479 (70, 210)	751 (70, 210)
$[M-4Na^+]^{-4}$		353.5 (70)	557.5 (70, 210)
$[M-6Na^+]^{-6}$			364 (70)
$[M-NaSO_3^- +H^+-Na^+]^{-1}$	973 (70)		
$[M-NaSO_3^- +H^+-2Na^+]^{-2}$	475 (0, 70)		
$[M-Galp4S-Na^+]^{-1}$	811 (140, 210)		
$[M-2Galp4S-Na^+]^{-1}$	547 (140, 210)		
$[M-Galp4SAnGalp-4Na^+]^{-4}$			455.5 (70)
$[M-Galp4SAnGalpGalp4S-3Na^+]^{-3}$			527 (70)
$[M-Galp4SAnGalpGalp4SAnGalpGalp4S-H_2O-Na^+]^{-1}$			1201 (210)
$[Galp4SAnGalpGalp4S-Na^+]^{-1}$	667 (0)	667 (210)	
$[Galp4SAnGalp2SAnGalp-Na^+]^{-1}$		811 (210)	
$[Galp4SAnGalp-Na^+]^{-1}$	403 (210)	403 (210)	403 (210)
$[Galp4SAnGalp-H_2O-Na^+]^{-1}$	385 (210)	385 (210)	385 (210)
$[Galp4S-H_2O-Na^+]^{-1}$	241 (210)	241 (210)	241 (210)

[a] Fragmentor voltages are shown in parentheses.

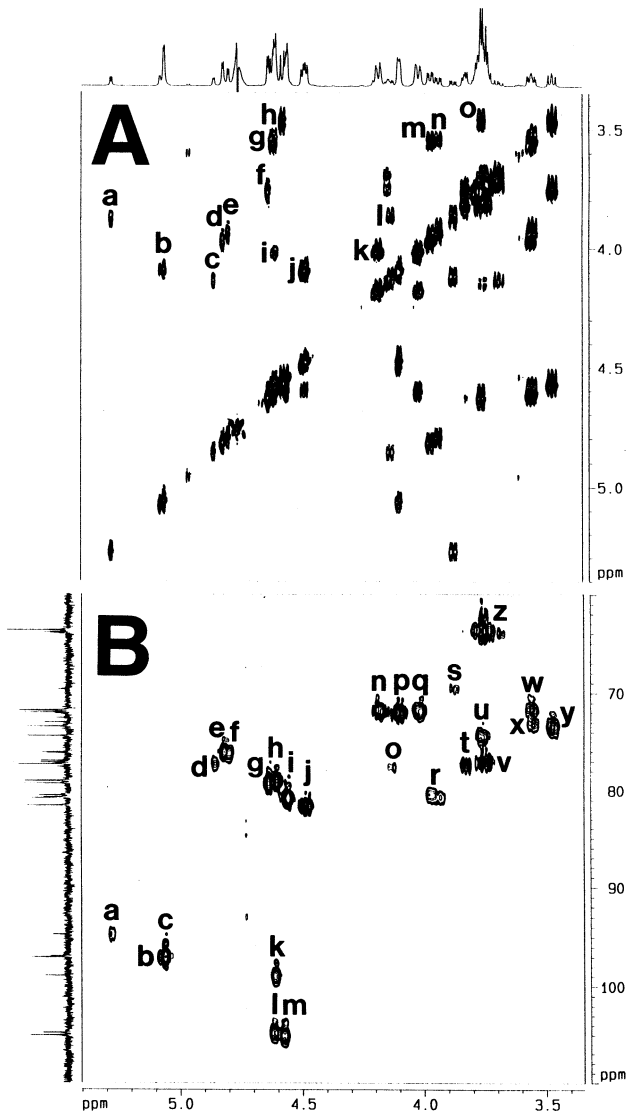


Fig. 4. NMR characterization of oligosaccharide **1** using two-dimensional spectroscopy. (A) COSY spectrum with cross-peaks labeled: (a), αGalp_{re}4S H-1/H-2; (b) AnGal$p$ H-1/H-2; (c) αGalp_{re}4S H-3/H-4; (d) βGalp_{int}4S H-3/H-4; (e) βGalp_{re}4S H-3/H-4; (f) βGalp_{nre} H-3/H-4; (g) βGalp_{re}4S and βGalp_{int}4S H-1/H-2; (h) βGalp_{nre} H-1/H-2; (i) AnGal$p$ H-5/H-6a; (j) AnGal$p$ H-2/H-3; (k) AnGal$p$ H-6a/H-6b; (l) αGalp_{re}4S H-2/H-3; (m) βGalp_{int}4S H-2/H-3; (n) βGalp_{re}4S H-2/H-3; (o) βGalp_{nre} H-2/H-3. (B) HMQC spectrum with cross-peaks labeled: (a) αGalp_{re}4S H-1/C-1; (b) AnGal$p$ adjacent to αGalp_{re}4S H-1/C-1; (c) AnGal$p$ H-1/C-1; (d) αGalp_{re}4S H-4/C-4; (e) βGalp_{int}4S H-4/C-4; (f) βGalp_{re}4S H-4/C-4; (g) βGalp_{nre} H-4/C-4; (h) AnGal$p$ H-5/C-5; (i) AnGal$p$ H-4/C-4; (j) AnGal$p$ H-3/C-3; (k) βGalp_{re}4S H-1/C-1; (l) βGalp_{nre}4S H-1/C-1; (m) βGalp_{int}4S H-1/C-1; (n) AnGal$p$ H-6b/C-6; (o) αGalp_{re}4S H-3/C-3; (p) AnGal$p$ H-2/C-2; (q) AnGal$p$ H-6a/C-6; (r) βGalp_{re}4S and βGalp_{int}4S H-3/C-3; (s) αGalp_{re}4S H-2/C-2; (t, u and v) unidentified (βGalp_{nre} H-3/C-3 and β, αGal$p$ H-5/C-5); (w) βGalp_{int}4S H-2/C-2; (x) βGalp_{re}4S H-2/C-2; (y) βGalp_{nre} H-2/C-2; (z) Gal$p$4S H-6/C-6.

observed downfield of the AnGal$p$ H-1, having the same small coupling constant. The integral for this other AnGal$p$ H-1 at 5.10 ppm is equal to the integral of the anomeric proton signal α-Gal$p$4S$_{re}$ at 5.29 ppm, and from the comparison of the value of this integral to that of the other AnGal$p$ H-1, the α,β mutarotation equilibrium in ^2H_2O at 25 °C was estimated 35–40% α and 60–65% β. The anomeric proton at 5.29 ppm has a coupling constant of 3.8 Hz, typical of a Gal$p$4S$_{re}$ H-1 coupling with Gal$p$4S$_{re}$ H-2 and clearly indicates that a galactopyranose residue is present at the reducing end. The α-Gal$p$4S$_{re}$ H-2 proton is a doublet of doublets centered at 3.89 ppm having a small $J_{1,2}$ (3.8 Hz) and a large $J_{2,3}$ value (10 Hz). The α-Gal$p$4S$_{re}$ H-3, which resonates at 4.15 ppm, shows a correlation to H-4 with a $J_{3,4}$ of 3.0 Hz and this proton in turn appears as a doublet at 4.87 ppm. These coupling constants correspond to a reducing-end unit galactose in which $J_{1,2}$ and $J_{3,4}$ are small due to axial–equatorial coupling and $J_{2,3}$ is large reflecting the diaxial coupling.

The most upfield signal at 3.49 ppm is the entry point for the nonreducing end (nre) residue. The H-2 signal, a doublet of doublets centered at 3.49 ppm, shows correlations to H-1, a signal at 4.60 ppm and to H-3, a signal centered at 3.71 ppm. The correlation between H-3 and H-4 allows the identification of the Gal$p$4S$_{nre}$ H-4 as a signal centered at 4.56 ppm. The coupling constants for $J_{1,2}$ and $J_{2,3}$ are 8.0 and 10 Hz, respectively.

The analysis of the next spin system begins with the next upfield signal centered at 3.56 ppm, corresponding to the H-2 of both the internal (int) Gal$p$4S$_{int}$ residue and the H-2 of the β anomer of the Gal$p$4S$_{re}$ residue. This signal shows an additional splitting due to the overlap of these three different hydrogen atoms. The H-2 signal corresponding to β-Gal4S$_{re}$ and Gal$p$4S$_{int}$ shows correlations to an H-1 proton at 4.63 ppm. In addition, this also correlates to the H-3 proton by showing two doublet of doublets at 3.95 and 3.99 ppm. The H-3 coupling with H-4 leads to the identification of the H-4 at 4.82 and 4.84 ppm. The coupling constant of the protons in β-Gal$p$4S$_{re}$ and Gal$p$4S$_{int}$ are identical to those of β-Gal$p$4S$_{nre}$ (described above).

The final spin system belongs to the two AnGalp_{int} residues whose signals overlap completely. AnGalp_{int} H-1 at 5.09 ppm correlates to H-2, a doublet at 4.12 ppm, which correlates to the doublet of doublets for H-3 at 4.51 ppm. The connectivity from H-3 to H-4 could not be established, due to the small $J_{3,4}$. The two protons, H-6a at 4.05 ppm and H-6b at 4.21 ppm, were easily identified due to their strong correlation resulting from their geminal coupling of -10 Hz. The H-6b signal is downfield of H-6a, probably due to anisotropic effects of the pyranose ring oxygen atom. Only H-6a shows a correlation to H-5 signal at 4.63 ppm, which overlaps with others located in the same region.

The $^1H-^1H$ TOCSY spectrum (not shown) confirmed all the assignments made using $^1H-^1H$ COSY. The $^1H-^{13}C$ HMQC spectrum allowed the transfer of the proton assignments to the corresponding carbon atoms and the carbon assignments of oligosaccharide **1** are given in Table 3. The α,β equilibrium of the Gal*p*4S$_{re}$ residue affected not only the carbon atoms of the terminal residue, but also some of the carbon atoms of

Table 2
^1H NMR assignments for κ-carrageenan oligosaccharides **1**–**3**

Residue	Proton	Chemical shift (ppm) [a]			
		1	**2**	**3**	Lit. values [b]
α-Gal*p*4S$_{re}$	H-1	5.29	5.21	5.21	5.32
	H-2	3.89	3.82	3.82	3.92
	H-3	4.15	4.07	4.07	4.16
	H-4	4.87	4.80	4.80	4.86
β-Gal*p*4S$_{int}$ and β-Gal*p*4S$_{re}$	H-1	4.63	4.56	4.56	4.65–4.66
	H-2	3.56	3.50	3.49	3.59–3.60
	H-3	3.95, 3.99	3.92	3.91	3.98–4.01
	H-4	4.82, 4.84	4.76	4.76	4.83–4.86
	H-5	3.67–3.88	3.60–3.80	3.60–3.78	3.77–3.81
	H-6a,6b	3.67–3.88	3.60–3.80	3.60–3.78	3.78–3.80
β-Gal*p*4S$_{nre}$	H-1	4.60	4.52	4.49	
	H-2	3.49	3.42	3.41	
	H-3	3.78	3.60–3.80	3.60–3.78	
	H-4	4.65	4.56	4.57	
	H-5	3.67–3.88	3.60–3.80	3.60–3.78	
	H-6a,6b	3.67–3.88	3.60–3.80	3.60–3.78	
AnGal*p* adjacent to α-Gal*p*4S$_{re}$	H-1	5.10	5.01	5.01	5.12
AnGal*p*	H-1	5.09	5.00	5.00	5.10
	H-2	4.12	4.04	4.03	4.14
	H-3	4.51	4.43	4.43	4.53
	H-4	4.60–465	4.56	4.56	4.61
	H-5	4.63	4.54	4.55	4.65
	H-6a	4.05	3.96	3.96	4.06
	H-6b	4.21	4.13	4.13	4.22

[a] Chemical shift value of 4.76 ppm for HO^{2}H was used in calculating the chemical shifts.
[b] Literature values were taken from Ref. 12.

Table 3
^{13}C NMR assignments for κ-carrageenan oligosaccharide **1**

Residue	Chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Gal*p*4S$_{re}$	94.7 (92.8) [a]	69.5 (67.6)	77.5 (75.6)	77.1(75.3)		63.6 (61.9)
β-Gal*p*4S$_{re}$	98.9 (96.9)	72.9 (71.0)	80.6 (78.6)	76.1 (74.2)		63.8 (61.8)
Gal*p*4S$_{int}$	104.8 (102.8)	71.7 (69.8)	76.9 (78.5)		77.2 (75.1)	
β-Gal*p*4S$_{nre}$	105.0		76.0	79.2	74.3	63.8
AnGal*p* Adjacent to α re	96.9 (94.9)					
AnGal*p* Adjacent to β re	97.0 (95.0)					
AnGal*p*	97.0 (95.0)	71.9 (69.9)	81.5 (81.3)	80.7 (78.8)	78.9 (77.8)	71.7 (69.4)

[a] Literature values shown in parentheses are taken from Ref. 12.

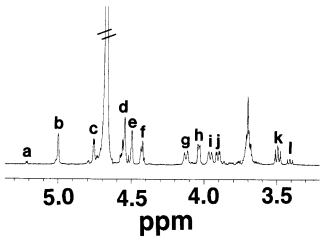


Fig. 5. One-dimensional ^{1}H NMR spectrum of oligosaccharide **3**. Signals: (a) αGalp_{re}4S H-1; (b) AnGal$p$ H-1; (c) βGalp_{int}4S H-4; (d) βGalp_{int}4S H-1, βGalp_{re}4S H-1, AnGal$p$ H-4 and βGalp_{nre} H-4; (e) AnGal$p$ H-5; (f) AnGal$p$ H-3; (g) AnGal$p$ H-6b; (h) AnGal$p$ H-2; (i) AnGal$p$ H-6a; (j) βGalp_{int}4S H-3; (k) βGalp_{int}4S H-2; (l) βGalp_{nre} H-2.

the adjacent AnGal$p$ residue. This accounts for the unexpectedly large number of carbon peaks observed. Although the relative intensities of the peaks in the decoupled ^{13}C spectrum do not provide an accurate quantitative assessment in the absence of quaternary carbon atoms (which would give low-intensity peaks), it was possible to identify some lower intensity signals as peaks arising from carbon atoms of the αGal$p$4S$_{re}$ residue. No peaks are present at 66.4 ppm corresponding to C-4 of unsulfated Gal$p$ residues; thus, the ^{13}C and the HMQC spectra confirm that all Gal$p$ residues contained a 4-*O*-sulfo group. NMR and MS analysis definitively establish the structure of oligosaccharide **1** to be a trisulfated pentasaccharide of the structure: β-D-Gal$p$4S-(1→4)-α-D-AnGal$p$-(1→3)-β-D-Gal$p$4S-(1→4)-α-D-AnGal$p$-(1→3)-α,β-D-Gal4S.

MS and NMR analysis (Tables 1 and 2) of oligosaccharide **2** showed it to be homologous to oligosaccharide **1**, containing one additional repeat unit. Oligosaccharide **2** is a tetrasulfated heptasaccharide of molecular weight 1506. Its structure is β-D-Gal$p$4S-(1[→4)α-D-AnGal$p$-(1→3)-β-D-Gal$p$4S-(1→]$_2$4)-α-D-AnGal$p$-(1→3)-α,β-D-Gal4S.

Oligosaccharide **3** was the largest and was among the purest of the three oligosaccharides prepared. One-dimensional ^{1}H NMR confirmed the high level of purity of oligosaccharide **3** (Fig. 5). Comparisons of the area for the H-2 protons of the Gal$p$4S residues were made to the area for H-1 protons of the AnGal$p$ residues afforded a ratio consistent with an oligomer containing 11 saccharide units. Structural assignment of oligosaccharide **3** also relied on ESIMS fragmentation (Figs. 6 and 7). ESIMS analysis had been previously used to successfully analyze κ-carrageenan oligosaccharides, including a dodecasaccharide prepared using κ-carrageenanase.[19] Oligosaccharide **3** is a hexasulfated undecasaccharide of molecular weight 2322 and is homologous in its structure to oligosaccharides **1** and **2**. It has the structure: β-D-Gal$p$4S-(1[→4)-α-D-AnGal$p$-(1→3)-β-D-Gal$p$4S-(1]$_4$→4)-α-D-AnGal$p$-(1→3)-α,β-D-Gal4S.

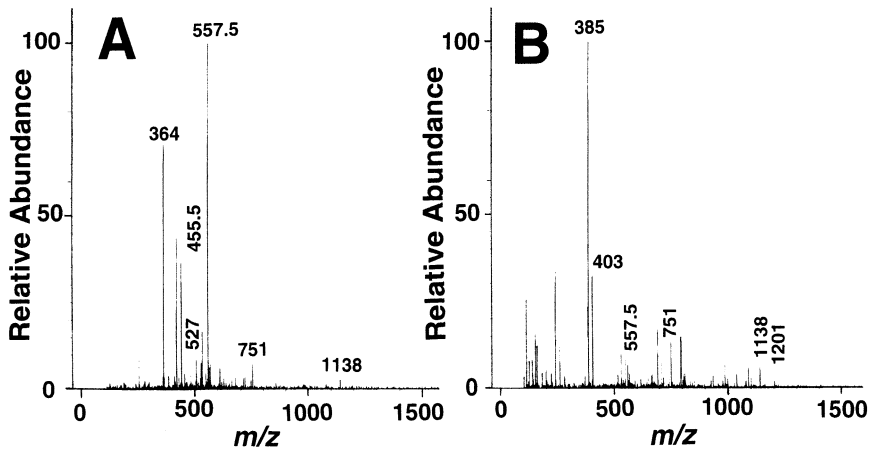


Fig. 6. ESIMS analysis of oligosaccharide **3**. (A) ESIMS spectrum obtained at a fragmentor voltage of 70 V. (B) ESIMS spectrum obtained at a fragmentor voltage of 210 V.

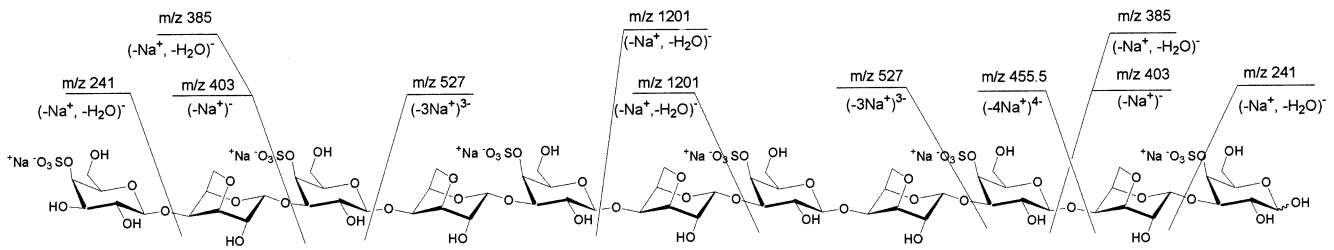


Fig. 7. Fragmentation analysis of oligosaccharide **3**.

Work is currently underway to prepare and characterize larger molecular-weight oligosaccharides for biological evaluation.

3. Experimental

Materials.—Carrageenan from *K. striatum* (Philippines) was purchased from Yantai Algae Industries (Shandong, China). Bio-Gel P2 (superfine) was from Bio-Rad (Richmond, CA). SAX-HPLC was performed on a POROS HQ50 column (10 μm, 22 × 100 mm) column. Acrylamide (ultrapure) and Tris (ultrapure) were from Life Technologies Inc. (Gaithersburg, MD). Alcian blue dye, bromophenol blue dye, and ammonium persulfate were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glycine, sodium EDTA, boric acid, sucrose, *N*,*N*-methylene bisarylamide, and TEMED were from Fisher Chemical Co. (Fair Lawn, NJ). ^2H_2O (99.96, atm.%) was from Aldrich Chemical Co. (Milwaukee, WI).

Purification and characterization of κ-carrageenan.—The yield of carrageenan from *K. striatum* algae was approximately 30%. The carrageenan was treated with NaOH and KCl to increase the content of AnGal*p* residues to enhance gel strength. Properties provided by the manufacturer include: a gel strength of 800 g/cm^2 at 1.5% water; mp 50 °C, and condensing point, 41 °C. The polymer is a random coil at temperatures of >50 °C and is a double helix at rt.

κ-Carrageenan was purified by the method of Smith et al.[23] The polysaccharide was converted to the sodium salt, dialyzed, lyophilized, and analyzed as C, 30.25; H, 5.53; S, 7.35%. IR analysis shows an absorbance at 848 cm^{-1} indicative of the presence of 4-*O*-sulfo esters. The ^{1}H and ^{13}C NMR spectra were consistent with the structure of κ-carrageenan. GPC–HLPC performed on PL Aquagel-OH-mixed and PL Aquagel-OH-30 (8 μm, 7.5 × 300 cm) columns (Polymer Laboratories Ltd., Amherst, MA, USA) in series at a flow rate of 1.0 mL/min (0.1 M Na_2SO_4, pH 7.0) at 35 °C utilized RI detection. Dextran and dextran sulfate standards of molecular weights 10,000, 21,400, 41,000, 84,000, 133,800, and 500,000 gave a standard curve of $\log M_n = -0.435x + 10.59$ ($r^2 = 0.997$; x, min). Based on this equation, κ-carrageenan gave an $M_n = 310{,}400$, $M_w = 431{,}320$, $M_z = 553{,}400$, and a polydispersity of 1.19.

Mild-acid hydrolysis of κ-carrageenan.—κ-Carrageenan (5 g, 10 mg/mL) was dissolved at 60 °C in 0.1 M hydrochloric acid and kept for 3 h. The degradation was terminated by neutralization with 0.1 M NaOH, then filtered by Millipore membrane (GS, 0.45 μm), and then the supernatant was pressure filtered by a 5000 MWCO membrane using a 600 mL, stirred pressure-filtration cell with a nitrogen gas pressure of 1.0 kg/cm^2. The filtered sample was concentrated by rotary evaporation.

Desalting.—All desalting was performed on Bio-Gel-P2 columns (2.6 × 80 cm or 1.5 × 60 cm) monitored using a refractive index detector (Gilson 132 RI, France).

Separation and purification of κ-carrageenan oligosaccharides.—The oligosaccharide mixture was separated on a semi-preparative SAX-HPLC (POROS HQ50, 10 μm, PE Perspective Biosystems Inc.) column. The column was pre-equilibrated with 0.1 M NaCl (pH 7.0) at 5.0 mL/min, then in a linear gradient of NaCl from 0.1 to 2.0 M in 120 min. By using fraction collector (RediFrac, Pharmacia Biotech.), each fraction (5 mL/tube) was automatically collected and the content of each fraction was tested by colorimetric assay.[28] The purity of each fraction was monitored by PAGE analysis.[24] The major peaks, were pooled, desalted on a Bio-Gel-P2 column, freeze-dried and again applied to the semi-preparative SAX-HPLC column (using a newly optimized gradient), and the fractionation and desalting were repeated. Finally, pure oligosaccharides were obtained.

PAGE analysis of κ-carrageenan oligosaccharide.—Gradient (12–22%) discontinuous polyacrylamide gel electrophoresis (PAGE) analysis was performed on a vertical slab (0.1 × 16 × 20 cm) gel system. The gel was loaded with 20–50 μg of sample and subjected to electrophoresis for 4 h at 400 V while cooling at 5–10 °C with a circulating bath. The gel was visualized with Alcian blue (0.5% in 2% AcOH) staining.

Fluorescent labeling of oligosaccharides.—Monopotassium 7-amino-1,3-napthalenedisulfonic acid (ANDS) (Aldrich) was recrystallized from deionized water for the fluorescent labeling of the oligosaccharide. Compounds **1**, **2**, and **3** (100 μg of each) were dissolved in a solution containing ANDS (5 mg in 100 μL of 15% AcOH) and incubated for 1 h at rt after which 100 μL of 1.0 M sodium cyanoborohydride in water was added, and the mixture was incubated for 12 h at 45 °C. Excess ANDS was removed by gel-permeation chromatography on a Sephadex G-25 column (45 × 1.5 cm i.d.) with detection at 247 nm. The fluorescently labeled oligosaccharide fractions (eluting prior to the excess ANDS) were collected, pooled, and freeze-dried. Electrophoresis was performed in a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hercules, CA). The gel was loaded with 2–5 μg of sample and subjected to electrophoresis for about 1 h at 200 V (constant voltage). The gel was visualized in a UV–light chamber and photographed.

Capillary electrophoresis of fluorescently labeled oligosaccharides.—The labeled oligosaccharides were analyzed with a capillary electrophoresis system (Dionex, Sunnyvale, CA) at 25 kV by fluorescence detection (λ_{ex} of 250 nm and λ_{em} of 450 nm). Separation

and analysis were carried out in a reversed-polarity mode using a fused silica (external coated except where the tube passed through the detector) capillary tube (55-cm long × 50 μm i.d.). The separation buffer contained 20 mM of sodium phosphate, pH 3.5. The sample was pressure injected (5 s, 5 psi) resulting in an injection volume of 0.5 μL.

NMR analysis.—Each sample was dissolved in ^2H_2O (approximately 0.5–1.1 mM) and freeze-dried twice to replace all exchangeable protons with deuterium. The ^1H NMR and ^1H–^1H 2D NMR were all acquired at 25 °C using a VARIAN INOVA 500 instrument and VNMR 6.1C software. The water peak served as a reference (HO^2H, 4.76 ppm). Shift values were confirmed using acetone as an internal standard to give a signal at 2.22 ppm. In the ^1H–^1H COSY and ^1H–^1H TOCSY spectra, a 12-ppm spectral width was used in both dimensions. In F^2, 1024 complex points were collected while the resolution in the F^1 dimension was 512 complex points using States Habercorn phase cycling. The ^{13}C spectrum was recorded on a Bruker DPX 400 spectrometer; DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) served as an external reference. The ^1H–^{13}C HMQC was run on a Bruker AMX 600, using a 1 K × 1 K matrix (80 scans per block, an acquisition time of 0.213 s).

ESIMS analysis.—The low-resolution ESI mass spectra of compounds **1**–**3** were acquired on a 1100 mass selective detector (Agilent Technologies) equipped with a single quadrapole, at different fragmentor voltages using a 1:1 mixture of 1% NH_4OH–MeCN. The fragmentor voltage was changed from 70 to 2100 V in 70 V intervals to modify the degree of fragmentation. Nitrogen gas was used as a nebulizer gas at 4.2 kg/cm^2 and kept at 350 °C. The flow rate was 0.2 mL/min and samples of 50 ng were injected in a 5 μL loop.

Acknowledgements

The authors thank John Snyder of the NMR Central Research Facility, William Kerney of the College of Medicine NMR facility, and Lynn Teesch of the High-Resolution Mass-Spectrometry Facility at the University of Iowa. The authors are also grateful to Professor D. Loganathan of IIT Madras and Nur Sibel Gunay of the University of Iowa for their assistance in assigning spectra. This research was funded by a grant (GM38060) from the National Institutes of Health.

References

1. Therkelsen, G. H. In *Carrageenan*; Whistler, R. L.; BeMiller, J. N., Eds. Industrial Gums: Polysaccharides and Their Derivatives, third ed.; Academic Press: San Diego, CA, 1993; pp. 145–180.
2. Substances that are generally regarded as safe. Fed. Reg. 21,9368–9370.
3. Tache, S.; Peiffer, G.; Millet, A.-S.; Corpet, D. E. *Nutr. Cancer* **2000**, *37*, 193–198.
4. IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Human. *Carrageenan. IARC Monogr. Eval. Carcinog. Risk. Hum.* **1983**, *31*, 79–94.
5. Tobachman, J. K. *Environ. Health Perspect.* **2001**, *109*, 983–994.
6. Gold, L. S.; Manley, N. B.; Slone, T. H. In *Handbook of Carcinogenic Potency and Genotoxicity Databases*; Gold, L. S.; Zeiger, E., Eds. Summary of Carcinogenic Potency Database by Chemicals; CRC: New York, 1997; p. 116 Chapter 3.
7. National Research Council. *Carcinogens and Anti-Carcinogens in the Human Diet*; Washington, DC: National Academy Press, 1996; p. 398.
8. Knutsen, S. H.; Myslabodski, D. E.; Larsen, B.; Usov, A. I. *Bot. Mar.* **1994**, *37*, 163–169.
9. van de Velde, F.; Peppelman, H. A.; Rollema, H. S.; Tromp, R. H. *Carbohydr. Res.* **2001**, *331*, 271–283.
10. Caram-Lelham, N.; Sundelöf, L.-O.; Andersson, T. *Carbohydr. Res.* **1995**, *273*, 71–76.
11. Roches, C.; Heyraud, A. *Polym. Bull.* **1981**, *5*, 81–86.
12. Knutsen, S. H.; Grasdalen, H. *Carbohydr. Res.* **1992**, *229*, 233–244.
13. Usov, A.; Klochkova, N. G. *Bot. Mar.* **1992**, *35*, 371–378.
14. Stevenson, T. T.; Furneaux, R. H. *Carbohydr. Res.* **1991**, *210*, 277–298.
15. Bellion, C.; Brigand, G.; Prome, J. C.; Welti, D.; Bociek, S. *Carbohydr. Res.* **1983**, *119*, 31–48.
16. Greer, C. W.; Shomer, I.; Goldstein, M. E.; Yaphe, W. *Carbohydr. Res.* **1984**, *129*, 189–196.
17. Barbeyron, T.; Michel, G.; Potin, P.; Henrissat, B.; Kloareg, B. *J. Biol. Chem.* **2000**, *275*, 35499–35505.
18. Knutsen, S. H.; Sletmoen, M.; Kristensen, T.; Barbeyron, T.; Kloareg, B.; Potin, P. *Carbohydr. Res.* **2001**, *331*, 101–106.
19. Ekeberg, D.; Knutsen, S. H.; Sletmoen, M. *Carbohydr. Res.* **2001**, *334*, 49–59.
20. Cerezo, A. S. *Carbohydr. Res.* **1973**, *26*, 335–340.
21. Ekström, L.-G. *Carbohydr. Res.* **1985**, *135*, 283–289.
22. Ekström, L.-G.; Kuivinen, J. *Carbohydr. Res.* **1983**, *116*, 83–94.
23. Smith, D. W.; Cook, W. H.; Neal, J. L. *Arch. Biophys. Biochem.* **1954**, *53*, 192–204.
24. Edens, R. E.; Al-Hakim, A.; Weiler, J. M.; Rethwisch, D. G.; Fareed, J.; Linhardt, R. J. *J. Pharm. Sci.* **1992**, *81*, 823–827.
25. Mao, W.-J.; Thanawiroon, C.; Linhardt, R. J. In *Analytical Chemistry*; Volpi, N., Ed. Analytical Techniques to Evaluate Structure and Function of Natural Polysaccharides and Glycosaminoglycans; Research Sign Post: Kerala, India, 2001.
26. Lee, K. B.; Al-Hakim, A.; Loganathan, D.; Linhardt, R. J. *Carbohydr. Res.* **1991**, *224*, 155–168.
27. Park, Y.; Cho, S.; Linhardt, R. J. *Biochem. Biophys. Acta* **1997**, *1337*, 216–226.
28. Bitter, J.; Muir, H. M. *Anal. Biochem.* **1962**, *4*, 330–334.
29. Yang, H. O.; Gunay, N. S.; Toida, T.; Kuberan, B.; Yu, G.; Kim, Y. S.; Linhardt, R. J. *Glycobiology* **2000**, *10*, 1033–1040.