Sequence Determination of Sulfated Carrageenan-Derived Oligosaccharides by High-Sensitivity Negative-Ion Electrospray Tandem Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2007

Impact Factor: 5.64 · DOI: 10.1021/ac061416j · Source: PubMed

8 AUTHORS, INCLUDING:

Guangli Yu
Ocean University of China
97 PUBLICATIONS **1,467** CITATIONS
SEE PROFILE

Bo Yang
Rensselaer Polytechnic Institute
43 PUBLICATIONS **566** CITATIONS
SEE PROFILE

Anal. Chem. **2006,** *78,* 8499−8505

Sequence Determination of Sulfated Carrageenan-Derived Oligosaccharides by High-Sensitivity Negative-Ion Electrospray Tandem Mass Spectrometry

Guangli Yu,† Xia Zhao,† Bo Yang,† Sumei Ren,† Huashi Guan,† Yibing Zhang,‡ Alexander M. Lawson,‡ and Wengang Chai*,†,‡

Institute of Marine Drug and Food, Ocean University of China, Qingdao, Shandong 266003, China, and Glycosciences Laboratory, Faculty of Medicine, Imperial College London, Northwick Park & St Mark's Campus, Harrow, Middlesex HA1 3UJ, United Kingdom

Negative-ion electrospray tandem mass spectrometry with collision-induced dissociation is assessed for sequence determination of multiply sulfated oligosaccharide fragments of carrageenan obtained from partial depolymerization of the polysaccharides by either enzymatic digestion or mild acid hydrolysis. Carrageenan oligosaccharides with homogeneous disaccharide compositions were used to establish their fragmentation pattern, which was then applied to sequence determination of unusual oligosaccharides with either "hybrid" biose compositions or odd-numbered residues. As sulfate groups are labile, sulfate loss during collision-induced association was prevented by sodium adduction. The product ion spectra of $[M - Na]^-$ (where M represents the sodium salt of oligosaccharides) feature an extensive series of B- and C-type glycosidic cleavages, whereas the Y-type cleavage occurs mainly at the sulfated residues. The assignment of reducing or nonreducing terminal fragments was assisted by oligosaccharide reduction and the product ion spectra of the derived alditols. Due to the anionic nature of the sulfate present, high-sensitivity detection (1−5 pmol, using a hexasaccharide as an example) was obtained.

Sulfated polysaccharides are widespread in nature, occurring in a great variety of organisms. Sulfated glycosaminoglycans are found in vertebrate tissues, and in recent years, they have attracted considerable attention. Many protein−glycosaminoglycan interactions have been revealed to play important roles in various biological systems.[1] Sulfated galactan and sulfated fucans are present in the lower organisms such as marine algae. The implication of the highly anionic nature of these marine polysaccharides in environmental adaptation and in evolution[2] is not yet completely understood. However, it has been shown that this property is important in the physicochemical events such as cell wall mechanical, osmotic, and ionic regulation[3] of marine plants and also for its involvement in biological signaling activities in plant−microbe interactions.[4,5] There have also been indications that plant cellular defense responses may be analogous to the immune responses of vertebrates and insects.[6] Marine polysaccharides, such as carrageenan, have been widely used in the food industry as important additives, due to their thickening and gelling properties,[7] and recent studies have shown carageenan's antiviral activity against herpes simplex and dengue virus[8,9] and its induction effect on microspore embryogenesis.[10] Knowledge of the polysaccharide sequences is prerequisite for better understanding their biological roles. Due to the hugely complex nature of marine polysaccharides, detailed investigation of their oligosaccharide fragments, including their preparation and sequence determination, are important to assist in the derivation of structure/function relationships.

Carrageenans are highly sulfated galactans occurring in red algae with a linear repeating sequence of alternating 3-linked β-D-galactopyranose (unit G) and 4-linked α-D-galactopyranose (unit D). Classification of carrageenans is based on the presence of the D or the 3,6-anhydro form (unit A) of the α-4-galactose residues and differing sulfate contents and substitutions; e.g., β-, κ-, ι-, and λ-carrageenans have different disaccharide building blocks -$[G-A]_n$-, -$[G4S-A]_n$-, -$[G4S-A2S]_n$-, and -$[G2S-D2S6S]_n$-, respectively. The carrabiose (G−A or G−D, derived

* To whom correspondence should be addressed: (phone) +44-20-8869 3255; (fax) +44-20-8869 3455; (e-mail) w.chai@imperial.ac.uk.
† Ocean University of China.
‡ Imperial College London.

(1) Capila, I.; Linhardt, R. J. *Angew. Chem., Int. Ed.* **2002**, *41*, 391−412.
(2) Aquino, R. S.; Landeira-Fernandez, A. M.; Valente, A. P.; Andrade, L. R.; Mourao, P. A. *Glycobiology* **2005**, *15*, 11−20.
(3) Kloareg, B.; Quatrano, R. S. *Oceanogr. Mar. Biol. Annu. Rev.* **1988**, *26*, 259−315.
(4) Darvill, A.; Augur, C.; Bergmann, C.; Carlson, R. W.; Cheong, J. J.; Eberhard, S.; Hahn, M. G.; Lo, V. M.; Marfa, V.; Meyer, B. *Glycobiology* **1992**, *2*, 181−98.
(5) Bouarab, K.; Potin, P.; Correa, J.; Kloareg, B. *Plant Cell* **1999**, *11*, 1635−50.
(6) Baker, B.; Zambryski, P.; Staskawicz, B.; Dinesh-Kumar, S. P. *Science* **1997**, *276*, 726−33.
(7) De Ruiter, G. A.; Rudolph, B. *Trends Food Sci. Technol.* **1997**, *8*, 389−95.
(8) de Tischer, S. F.; Talarico, P. C.; Noseda, L. B.; Guimarães, M. D.; Damonte, S. M. P. B.; Duarte, E. B.; E. U. R. *Carbohydr. Polym.* **2006**, *63*, 459−65.
(9) Damonte, E. B.; Matulewicz, M. C.; Cerezo, A. S. *Curr. Med. Chem.* **2004**, *11*, 2399−419.
(10) Penhuizic, C. L. L.; Chatelet, C.; Kloareg, B.; Pontin, P. *Plant Sci.* **2001**, *160*, 1211−20.

10.1021/ac061416j CCC: $33.50
Published on Web 11/08/2006

Table 1. Carrageenan Oligosaccharides Used for ES-CID MS/MS

		sequences		nominal molecular masses	
	oligosaccharides	monosaccharide	biose[a]	free acid	Na salt
1	neocarra-tetra-1S	A-G-A-G4S	β'-κ	710	732
2	neocarra-tetra-2S	A-G4S-A-G4S	κ'-κ'	790	834
3	neocarra-hexa-3S	A-G4S-A-G4S-A-G4S	κ'-κ'-κ'	1176	1242
4	neocarra-hexa-4S	A-G4S-A2S-G4S-A-G4S	κ'-ι'-κ'	1256	1344
5	neocarra-octa-4S	A-G4S-A-G4S-A-G4S-A-G4S	κ'-κ'-κ'-κ'	1562	1650
6	carra-tetra-2S	G4S-A-G4S-A	κ'-κ	790	834
7	carra-tri-2S	G4S-A-G4S	κ-G4S	646	690
8	carra-tri-3S	G4S-A2S-G4S	ι-G4S	726	792
9	carra-penta-3S	G4S-A-G4S-A-G4S	κ-κ-G4S	1032	1098
10	carra-hepta-4S	G4S-A-G4S-A-G4S-A-G4S	κ-κ-κ-G4S	1418	1506
11	carra-nona-5S	G4S-A-G4S-A-G4S-A-G4S-A-G4S	κ-κ-κ-κ-G4S	1804	1914

[a] Biose units marked with a Greek letter refer to carrabiose G−A sequence derived from acid hydrolysis whereas the primed biose units refer to the neocarrabiose A−G derived from carrageenase digestion.

from acid hydrolysis) or neocarrabiose (A−G or D−G, derived from carrageenase digestion) units distinguish each class of carrageenans. However, native polysaccharides are rarely in their uniformed or "ideal" form that is similar to other types of polysaccharides, such as the glycosaminoglycan chondroitin sulfates A, B, and C. The structural complexity of carrageenan is attributed to a mixed combination of different biose units or copolymeric chains. The heterogeneity of carrageenan largely depends on the sources of the alga, its developmental stages, and the extraction procedures of the polymers. The most classical copolymers of carrageenan are those found in native κ- and ι-carrageenan chains that usually contain components of the incompletely processed precursors, e.g., γ-, μ-, and ν-carrageenans with the biose units of -[G−D6S]-, -[G4S−D6S]-, and -[G4S−D2S6S]-, respectively.[11] Further complexity occurs when the hydroxyls in D-galactose are substituted by methyl and pyruvate groups.[12,13] The presence of hybrid or "nonideal" biose sequences introduces structural heterogeneity, which confers a wide range of biological and physiochemical properties.

Mass spectrometry has become increasingly important for analysis of carbohydrates, including fast screening[14,15] and detailed sequence analysis,[16−22] due to its high sensitivity, high accuracy, and fast processing compared with NMR and various chromatographic methods. Similar to glycosaminoglycans, most marine polysaccharides are highly acidic and rich in uronic acid residues, sulfate groups, or both. The anionic nature, particularly the lability of the sulfate group, poses problems for sequence analysis by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) although this is not generally a major problem for molecular mass determination. Matrix-assisted laser-desorption/ionization mass spectrometry[23,24] and electrospray mass spectrometry (ESMS)[23,25−27] have been useful for detection of carrageenan oligosaccharides. Negative-ion ESMS/MS has been used for analysis of structural heterogeneity of κ-carrageenan oligosaccharides[28] and has been linked on-line with ion-pairing HPLC for characterization of κ-, ι-, and hybrid ι-/ν-carrageenans following specific carrageenase digestions.[29] Recently, it has been concluded that negative-ion detection for MS/MS is hindered with sulfate being lost by expulsion as HSO_4^-, $NaSO_3$, or $NaHSO_4$, and positive-ion nanoES-FTICR-MS/MS was attempted to prevent the sulfate loss for sequence analysis of κ-carrageenan oligosaccharides.[30]

In the present report, we demonstrate high-sensitivity negative-ion ES CID MS/MS for sequence determination of carrageenan oligosaccharides derived by carrageenase digestion and mild acid hydrolysis, with even- and odd-numbered monosaccharide residues of either "ideal" or "hybrid" biose compositions. The principles established here should be readily applicable to other types of carrageenan oligosaccharides.

EXPERIMENTAL SECTION

Materials. Neocarraoligosaccharides 1−5 (Table 1), including mono- and disulfated neocarratetraose, tri- and tetrasulfated neocarrahexaose and tetrasulfated neocarraoctaose, were purchased from Sigma (Poole, U.K.) which were derived from carrageenase digestion. Gel filtration media Bio-Gel P6 (fine, 45

(11) Craigie, J. S.; Wong, K. F. *Proc. Int. Seaweed Symp.* **1979**, *9,* 369−77.
(12) Mendoza, W. G.; Montaño, N. E.; Ganzon-Fortes, E. T.; Villanueva, R. D. *J. Appl. Phycol.* **2002**, *14*, 409−18.
(13) Di Ninno, V.; McCandless, E. L.; Bell, R. A. *Carbohydr. Res.* **1979**, *71*, C1−C4.
(14) Dell, A.; Morris, H. R. *Science* **2001**, *291*, 2351−56.
(15) Chai, W.; Luo, J.; Lim, C. K.; Lawson, A. M. *Anal. Chem.* **1998**, *70*, 2060−66.
(16) Chai, W.; Piskarev, V.; Lawson, A. M. *Anal. Chem.* **2001**, *73*, 651−57.
(17) Chai, W.; Piskarev, V.; Lawson, A. M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 670−79.
(18) Chai, W.; Piskarev, V. E.; Zhang, Y.; Lawson, A. M.; Kogelberg, H. *Arch. Biochem. Biophys.* **2005**, *434*, 116−27.
(19) Chai, W.; Piskarev, V. E.; Mulloy, B.; Liu, Y.; Evans, P.; Osborn, H. M. I.; Lawson, A. M. *Anal. Chem.* **2006**, *78*, 1581−92.
(20) Kogelberg, H.; Piskarev, V. E.; Zhang, Y.; Lawson, A. M.; Chai, W. *Eur. J. Biochem.* **2004**, *271*, 1172−86.
(21) Harvey, D. J. *Proteomics* **2005**, *5*, 1774−86.
(22) Zhang, Z.; Yu, G.; Zhao, X.; Liu, H.; Guan, H.; Lawson, A. M.; Chai, W. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 621−30.
(23) Ackloo, S.; Terlouw, J. K.; Ruttink, P. J.; Burgers, P. C. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1152−59.
(24) Fukuyama, Y.; Ciancia, M.; Nonami, H.; Cerezo, A. S.; Erra-Balsells, R.; Matulewicz, M. C. *Carbohydr. Res.* **2002**, *337*, 1553−62.
(25) Yu, G.; Guan, H.; Ioanoviciu, A. S.; Sikkander, S. A.; Thanawiroon, C.; Tobacman, J. K.; Toida, T.; Linhardt, R. J. *Carbohydr. Res.* **2002**, *337*, 433−40.
(26) Antonopoulos, A.; Favetta, P.; Helbert, W.; Lafosse, M. *Carbohydr. Res.* **2004**, *339*, 1301−09.
(27) Yuan, H.; Song, B. H. *J. Appl. Phycol.* **2005**, *17*, 7−13.
(28) Ekeberg, D.; Knutsen, S. H.; Sletmoen, M. *Carbohydr. Res.* **2001**, *334*, 49−59.
(29) Antonopoulos, A.; Favetta, P.; Helbert, W.; Lafosse, M. *Anal. Chem.* **2005**, *77*, 4125−36.
(30) Aguilan, J. T.; Dayrit, F. M.; Zhang, J.; Niñ0nuevo, M. R.; Lebrilla, C. B. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 96−103.

μm) was from BioRad (Richmond, CA) and Sephadex G-10 from Amersham Biosciences (Uppsala, Sweden). Strong anion-exchange (SAX)-HPLC was performed on Spherisorb S5 SAX column (20 × 250 mm, Clwyd, U.K.).

Preparation of Carrageenan Oligosaccharides of the Carra-oligosaccharide Series by Mild Acid Hydrolysis. Odd-numbered carraoligosaccharides (7 and 9−11, Table 1) were prepared from polysaccharide κ-carrageenan, extracted from *Kappaphycus striatum*, and fractionated with KCl.[31] The polysaccharide, as its sodium salt, was dialyzed and lyophilized before IR, and ^{1}H and ^{13}C NMR analyses for confirmation of κ-carrageenan. Mild acid hydrolysis of κ-carrageenan polysaccharide (1 g) was carried out with 0.1 M HCl (100 mL) at 60 °C for 3 h. The partial hydrolysis was stopped by neutralization with 2 M NaOH, and large molecules were removed by ultrafiltration (MWCO 8000). The filtrate was concentrated by rotary evaporation and desalted on a Sephadex G10 column (1.6 × 60 cm) with elution by double-distilled water and monitoring by a refractive index (RI) detector. The oligosaccharide mixture was then fractionated based on size by FPLC using a Superdex30 column (XK16/60, 30 μm, Amersham Biosciences, Uppsala, Sweden) with elution by 0.2 M NH_4HCO_3 at a flow rate of 0.5 mL/min and detection by RI. The size-uniform oligosaccharides (5−30 mg) were further purified by semipreparative SAX-HPLC with a gradient of 0−1 mol/L NaCl in 3 h at a flow rate of 3 mL/min.

Carra-tetra-2S (6, Table 1) and carra-tri-3S (8, Table 1) were prepared from ι-carrageenan (*Eucheuma spinosa*, Sigma) essentially as described above, and the details will be described elsewhere.

The purity of samples prepared was checked by gradient discontinuous polyacrylamide gel electrophoresis with Alician staining for the penta-, hepta-, and nonasaccharides or by capillary electrophoresis following fluorescent labeling with monopotassium 7-amino-1,3-naphthalenedisulfonic acid for the tri- and tetrasaccharides as described.[25] The purities were >95% for the homooligosaccharides and ~80% for the hybrid oligosaccharides.

NMR Spectroscopy. Confirmation of the presence of 4-*O*-sulfate on the 3-linked galactose of the κ-carrageenan, 4-*O*-sulfate on the 3-linked galactose, and 2-*O*-sulfate on the 4-linked 3,6-anhydrogalactose of ι-carrageenan was by ^{1}H NMR as described.[32]

Oligosaccharide Reduction. $NaBD_4$ reagent (20 μL, 0.05 M $NaBD_4$ in 0.01 M NaOH) was added to the freeze-dried oligosaccharide (typically 20 μg), and the reduction carried out at 4 °C overnight as described.[20] The reaction solution was then neutralized to pH 7 with a solution of AcOH/H_2O (1:1) to destroy borohydride before passing through a minicolumn of cation exchange (AG50W-X8, H form, Bio-Rad). Boric acid was removed by repeated coevaporation with MeOH.

Electrospray Mass Spectrometry. ESMS was carried out on a Micromass Q-Tof or Q-Tof Ultima instruments (Waters, Manchester, U.K.) in the negative-ion mode. Nitrogen was used as the desolvation and nebulizer gas at a flow rate of 250 and 15 L/h, respectively. Source temperature was 80 °C, and the desolvation temperature was 150 °C. Samples were dissolved in CH_3CN/2 mM NH_4HCO_3 (1:1, v/v), typically at a concentration of 5−10 pmol/μL, of which 5 μL was loop-injected. Mobile phase (CH_3CN/2 mM NH_4HCO_3 1:1, v/v) was delivered by a syringe pump at a flow rate of 5 μL/min. Capillary voltage was maintained at 3 kV while cone voltage was 50−120 V, depending on the size of the oligosaccharides and the type of precursor ions selected. Generally, longer chain length required higher voltage, while the sodiated precursor required higher energy than the free acid precursor. For CID MS/MS product ion scanning, argon was used as the collision gas at a pressure of 1.7 bar and the collision energy was adjusted between 17 and 100 eV for optimal sequence information. The high-resolution product ion spectrum of disulfated neocarratetraose was acquired by the Q-Tof Ultima instrument at a resolution of 10 000. $[M - H]^-$ and $[M - H - 120]^-$ ions were observed are m/z 811.0885 and 691.1398, respectively. The 120 Da difference (119.9487) is equivalent to $NaHSO_4$ (theoretical value 119.9493; deviation 5 ppm).

(31) Smith, D. B.; Cook, W. H.; Neal, J. L. *Arch. Biochem.* **1954**, *53*, 192−204.
(32) van de Velde, F.; Peppelman, H. A.; Rollema, H. S.; Tromp, R. H. *Carbohydr. Res.* **2001**, *331*, 271−83.

RESULTS AND DISCUSSION

Negative-ion ES-CID MS/MS was initially assessed with variously charged molecular ions, including singly, doubly, and triply charged, as precursors for optimal sequence information. Product ion spectra of singly charged ions were chosen for the study as other precursors did not produce fragments that were more structurally informative (see Figures S-1 and S-2 in Supporting Information for representative spectra of doubly charged ions). Neocarra- and carraoligosaccharides of ideal disaccharide compositions obtained from enzyme digestion or mild acid hydrolysis of carrageenans were used to investigate the fragmentation pattern, and the principles established were then applied to sequence determination of unusual oligosaccharides with either odd-numbered residues or hybrid biose compositions.

Stablization Effect on Sulfate by Sodium Salt Formation and Sequence Determination of Neocarratetrasaccharides. CID MS/MS of $[M_a - H]^-$ of the free acid and $[M - Na]^-$ of the sodiated form of tetrasaccharide neocarra-tetra-2S (2, Table 1) containing two sulfates were compared for sequence information derived from glycosidic bond cleavage, where M_a represents the free acid and M the fully sodiated molecule. As shown in Figure 1a, the product ion spectrum of the deprotonated molecule ion $[M_a - H]^-$ (m/z 789) of the free acid is dominated by desulfation with the loss of SO_3 (−80 Da, m/z 709) while glycosidic fragments are absent. In contrast, the sodiated molecular ion species (m/z 811, Figure 1b) is stable and using as the precursor $[M - Na]^-$ produces extensive glycosidic bond cleavage. Only minor desulfation occurs as indicated by the presence of a weak ion at m/z 691 (−120 Da, equivalent to the loss of $NaHSO_4$, measured m/z 119.9487 and theoretical m/z 119.9493; see Experimental Section), and the stabilization effect on the *O*-sulfate by sodiation is apparent. Hence, the sodiated ions were selected as precursors for CID MS/MS of carrageenan oligosaccharides throughout the work.

In order to differentiate fragment ions with identical masses arising from glycosidic cleavages at both termini, neocarra-tetra-2S was reduced and the product ion spectrum of the alditol (Figure 1c) was obtained, in which the reducing terminal fragment ions should show an increase of 2 Da from the reduction. Apparently only two glycosidic ions, m/z 259 and 667, have the 2 Da shift (Figure 1b and c) and these were assigned as Y_1 and Y_3, respectively (nomenclature used to define the fragmentation is

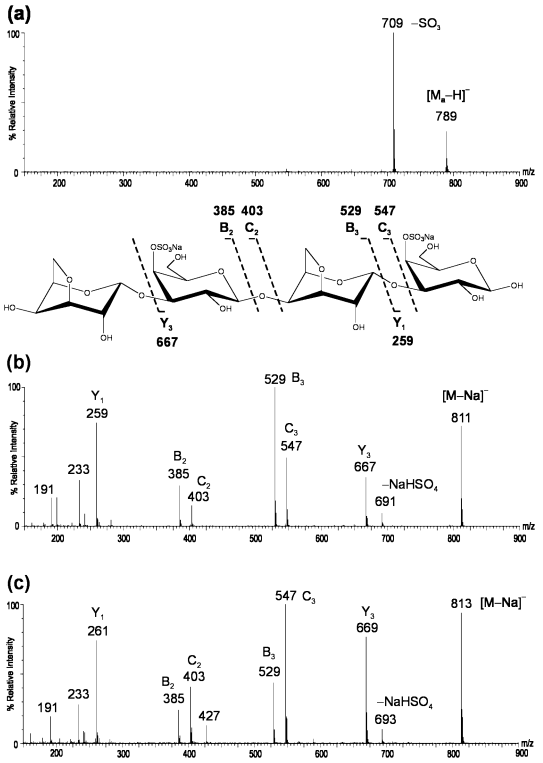

Figure 1. Negative-ion ES CID MS/MS product ion spectra of neocarra-tetra-2S. (a) $[M_a - H]^-$ of free acid, (b) $[M - Na]^-$ of the sodium salt, and (c) $[M - Na]^-$ of the reduced alditol. M_a represents the free acid and M the fully sodiated molecule. The sequence together with the glycosidic oxygens is shown to indicate the proposed fragmentation.

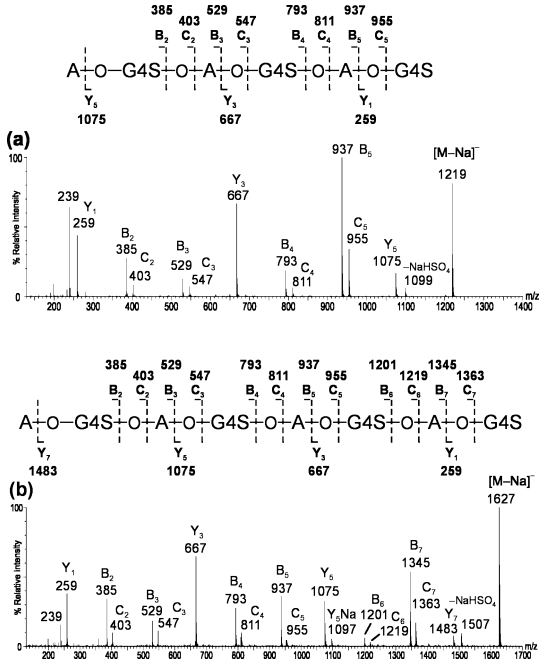

Figure 2. Negative-ion ES CID MS/MS product ion spectra of neocarra-hexa-3S (a) and neocarra-octa-4S (b). The sequences together with the glycosidic oxygens are shown to indicate the proposed fragmentations.

based on that introduced by Domon and Costello[33]). The ion at m/z 691, $[M - Na - 120]^-$, is also a reducing terminal fragment as it shifts to m/z 693 in the spectrum of the alditol. The loss of 120 Da is frequently observed for a reducing terminal hexose as a loss of $C_4H_8O_4$ to give an $^{2,4}A$ ion.[19,34] However, this cross-ring A-type cleavage ion could not form after reduction due to the saccharide ring opening, and therefore, this is in further support of the above assignment of the sulfate loss in the form of $NaHSO_4$.

Clearly the sequence of neocarra-tetra-2S can be readily derived from the product ion spectrum of $[M - Na]^-$ (Figure 1b), with the B-/C-ion doublets formed at both sulfated and nonsulfated residues, except the nonreducing terminal, and the Y-ions at the nonreducing side of the sulfated residues.

Fragmentation of Neocarrahexa- and Octasaccharides. Neocarra-hexa-3S and neocarra-octa-4S are ideal κ-oligosaccharides containing three and four neo-κ-carrabiose units (Table 1), respectively, and their product ion spectra of $[M - Na]^-$ (Figure 2) are similar to that of the neocarra-tetra-2S (Figure 1b). The spectra feature extensive B- and C-ions together with some prominent Y-ions (Figure 2 and Table 2) from which their sequences can be readily deduced. B- and C-type cleavages occur at every glycosidic bond, except the nonreducing terminal ones, as the nonreducing terminal residue A does not contain a sulfate to site a negative charge. The regular ion pattern of B-/C-ion doublets, 18 mass units apart with B-ions of higher intensity than C-ions, is unique to the oligosaccharide with ideal biose composi-

tions. Y-Ions are prominent at each of the sulfated G4S residues, e.g., Y_1, Y_3, and Y_5 in the spectrum of hexasaccharide 3 (Figure 2a) and Y_1, Y_3, Y_5, and Y_7 in that of octasaccharide 5 (Figure 2b). Although the sulfate content is higher than that of tetrasaccharide 2, sulfate loss is at similar levels and the relative intensities of the derived ions m/z 1099 and 1507 in the spectra of Figure 2a and b, respectively, are ~10%.

Fragmentation of Carratetrasaccharide. Carra-tetra-2S (6, Table 1) contains two κ-carrabiose units, derived from acid hydrolysis that is similar to neocarra-tetra-2S (2, Table 1) in monosaccharide composition while the latter contains two neo-κ-carrabiose units derived from carrageenase digestion. The product ion spectra of the two tetraoses are different, reflecting their differing sequences. The spectrum of carra-tetra-2S (Figure 3) has two major fragment ions B_2 (m/z 385) and Y_2 (m/z 403), and the latter assignment was confirmed by the product ion spectrum of its alditol after reduction. In the spectrum of neocarra-tetra-2S, the Y_1 and Y_3 are prominent (Figure 1b) as they are derived from cleavages at the nonreducing side of the glycosidic bonds of sulfated GS. In the spectrum of carra-tetra-2S, Y_2 ion (m/z 403) is most intense as it is derived from fission at a GS residue (Figure 3). Unexpectedly, there are also differences in the stability of sulfate. Although the loss of $NaHSO_4$ (m/z 691) is at a similar extent (<10% of the precursor), the loss of SO_3 (−80 Da) or its sodiated form (−102 Da) from the B- and C-ions at GS residues are apparent, e.g., $B_1 - SO_3$ (m/z 161), $C_1 - SO_3$ (m/z 179), $B_3 - NaSO_3 + H$ (m/z 547), and $C_3 - NaSO_3 + H$ (m/z 565). The apparent reduced intensity of B- and C-ions is due to the larger distribution of the ion signals between intact and desulfated fragments. The origin of the ion at m/z 505 is unknown, but it is a reducing terminal fragment indicated by the spectrum of its alditol. It may well arise from an impurity with an isomeric

(33) Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397−409.
(34) Konig, S.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1125−34.

Table 2. Fragmentation Ions Observed in the Product Ion Spectra of [M − Na]⁻ as Precursors[a]

samples	[M−Na]⁻	B_1	C_1	B_2	C_2	B_3	C_3	B_4	C_4	B_5	C_5	B_6	C_6	B_7	C_7	B_8	C_8	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	$-NaHSO_4$
neocarra-tetra-1S	709	–	–	–	–	–	–											259	403	565					
neocarra-tetra-2S	811	–	–	385	403	529	547											259	403	667					691
neocarra-hexa-3S	1219	–	–	385	403	529	547	793	811	937	955							259	403	667	811	1075			1099
neocarra-hexa-4S	1321	–	–	385	403	631	649	895	913	1039	1057							259	403	–	913	1177			1201
neocarra-octa-4S	1627	–	–	385	403	529	547	793	811	937	955	1201	1219					259	403	667	811	1075	1219	1483	1507
carra-tetra-2S	811	241	259	385	403	649	667											–	403						691
carra-tri-2S	667	241	259	385	403													259	–						547
carra-tri-3S	769	241	259	487	505													259	–						649
Carra-penta-3S	1075	–	–	385	403	649	667	793	811									259	–	667	–				955
carra-hepta-4S	1483	–	–	385	403	649	667	793	811	1057	1075	1201	1219					259	–	667	–	1075	–		1363
carra-nona-5S	1891	–	–	385	403	649	667	793	811	1057	1075	1201	1219	1465	1483	1609	1627	259	–	667	–	1075	–	1483	1771

[a] Note: M refers to the sodium salts of oligosacchrides investigated and − refers to not detected.

Figure 3. Negative-ion ES CID MS/MS product ion spectrum of carra-tetra-2S. The sequence together with the glycosidic oxygens is shown to indicate the proposed fragmentation. Desulfated B- and C-ions are designated as B_S and C_S, respectively, and a loss of 80 Da is $-SO_3$ whereas a loss of 102 Da is $-NaSO_3 + H$.

Figure 4. Negative-ion ES CID MS/MS product ion spectrum of carra-hepta-4S. The sequence together with the glycosidic oxygens is shown to indicate the proposed fragmentations; desulfated Y-ions designated as Y_S (loss of 120 Da, $-NaHSO_4$) while some minor desulfated B- and C-ions are not marked.

sequence containing G4S−A2S as this tetrasaccharide was isolated from ι-carrageenan.

Sequence Determination of Unusual Odd-Numbered Carraoligosaccharides Derived from Mild Acid Hydrolysis of κ-Carrageenan. Odd-numbered carraoligosaccharides (7 and 9−11, Table 1) were obtained from mild HCl treatment of κ-carrageenan. Negative-ion ESMS unambiguously identifies their odd-number monosaccharide compositions to be GS-$(A-GS)_n$. Their sequences can be readily determined by CID MS/MS. The fragmentation pattern is similar to that of carra-tetra-2S (Table 2), and representative is the product ion spectrum of carra-hepta-4S (9, Table 1) shown in Figure 4. Due to the symmetrical nature of the sequence, Y- and C-ions derived from the sulfated GS residues are at the same m/z values, e.g., Y_3 and C_3 at m/z 667 and Y_5 and C_5 at m/z 1201 in the spectrum of carra-hepta-4S (Figure 4). The nonreducing terminal fragment ions were dominated by the B-/C-ion doublets at nonsulfated A residues, e.g., B_2 and C_2 (m/z 385 and 403), B_4 and C_4 (m/z 793 and 811), and B_6 and C_6 (m/z 1201 and 1219). The B_3/C_3 and B_5/C_5 ions are extremely weak. The ions at m/z 259, 667, and 1075 have major contributions from the Y_1, Y_3, and Y_5 ions, respectively, based on the analysis of the spectrum of its reduced alditol (spectrum not shown). Again sulfate loss from the main sequence ions (e.g., Y_3 and Y_5) in the form of −120 ($-NaHSO_4$) or −102 Da ($-NaSO_3 + H$) was observed and these contribute to the minor peaks in the spectrum.

Sequence Determination of Carrageenan Oligosaccharides with Hybrid Disaccharide Compositions. Sequence

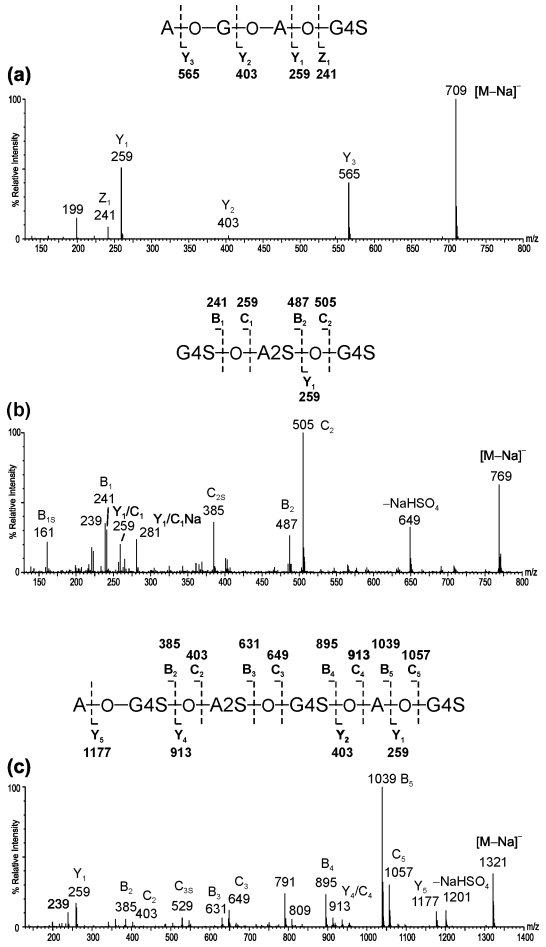

Figure 5. Negative-ion ES CID MS/MS product ion spectra of carrageenan oligosaccharides with hybrid compositions: (a) neocarra-tetra-1S; (b) carra-tri-3S; and (c) neocarra-hexa-4S. The sequences together with the glycosidic oxygens are shown to indicate the proposed fragmentations. Different forms of desulfated B- and C-ions are all designated as B_S and C_S, respectively; loss of 80 Da, $-SO_3$; loss of 102 Da, $-NaSO_3 + H$; loss of 120 Da, $-NaHSO_4$.

determination of nonideal oligosaccharides is challenging due to the irregularity of the fragmentation pattern and potential presence of impurities from incomplete purification.

The product ion spectrum of neocarra-tetra-1S (1, Table 1) using $[M - Na]^-$ as the precursor shows exclusively Y-type ions (Y_1 at m/z 259, Y_2 at m/z 403, and Y_3 at m/z 565, Figure 5a) and lack of B- and C-type ions, due to the reducing terminal location of the sulfate group, and clearly identifies a nonsulfated residue G at an internal location, with a neocarrabiose sequence of β'-κ' (Table 1).

Trisulfated trisaccharide carra-tri-3S (8, Table 1) was derived from acid hydrolysis of ι-carrageenan with sulfate at each residue. Similar to carra-tetra-2S and odd-numbered κ-carraoligosaccharides, presumably due to the presence of sulfate at the nonreducing terminal residues, its product ion spectrum contains mainly nonreducing terminal B- and C-ions (Figure 5b and Table 2). In addition, the 2-*O*-sulfate at the anhydrogalactose residue (A) is more labile than the 4-*O*-sulfate, and the loss of sulfate from the precursor $[M - Na]^-$ and fragment ion C_2 are apparent (see the high intensities of desulfated ions m/z 649 and 385).

Neocarra-hexa-4S (4, Table 1) contains two neo-κ- and one neo-ι-carrabiose units. Compared with the spectrum of trisulfated

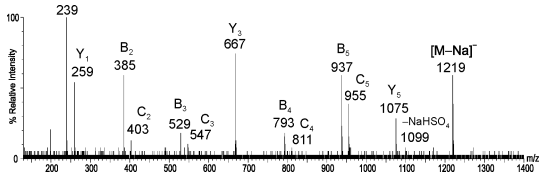

Figure 6. Negative-ion ES CID MS/MS product ion spectrum of 5 pmol of neocarra-hexa-3S using flow injection and conventional spray.

neocarra-hexa-3S (Figure 2a), the main difference is the absence of the intense Y_3 ion while the B- and C-ions are similar, from which the sequence can be deduced (Figure 5c and Table 2). The other difference is the occurrence of desulfated fragment ions, in the forms of $-NaSO_3 + H$ (-102 Da) or $-NaHSO_4$ (-120 Da), due to the presence of a labile 2-*O*-sulfate on the A residue. Again, similar to the A2S-containing carra-tri-3S, the C-ion at this residue (C_3) tends to lose sulfate more readily and desulfated C_3 at m/z 529 is more prominent. Again, the B-, C-, and Y-ions can be used to derive its hybrid sequence of κ'-ι'-κ'. The origin of the ion at m/z 791 is not known, presumably from either an impurity or a double cleavage, e.g., Y_5-C_4 cleavages.

Sensitivity. Using neocarra-hexa-3S as an example, the detection sensitivity was assessed for negative-ion ES CID MS/MS. With a sample concentration of 1 pmol/μL, 5 μL was used for conventional-scale flow injection and 1 μL for nanospray using a capillary needle. Similar spectra were obtained, and as representative, the conventional spray spectrum is shown in Figure 6. Clearly all the fragment ions are present (compare with Figure 1b for which 40 pmol was used) with good signal-to-noise ratio for sequence assignment.

CONCLUSIONS

Carrageenans are linear polysaccharides, and if desulfation can be prevented during CID, it is possible to derive the oligosaccharide sequence information directly from the glycosidic cleavage fragmentation in the product ion spectra.[35–37] Here, we have demonstrated that negative-ion ES CID MS/MS using the sodiated molecular species as precursors permit complete sequence assignment from the extensive B- and C-ion doublets and Y-ions at the sulfated residues. Isomeric neocarra- and carraoligosaccharides can also be readily differentiated as illustrated with neocarra- and carra-tetra-2S. However, no cross-ring fragmentation was observed for the sulfated carrageenan oligosaccharides investigated. Due to the anionic nature of sulfate, negative-ion detection of carrageenan oligosaccharides is the natural choice for high-sensitivity detection. Low picomole amount (1–5 pmol depending on the spraying method) is required for CID MS/MS whereas in the positive-ion mode a sample concentration of 0.5–2 nmol/μl is generally required.[24,30]

The biose units of the major classes of carrageenans have different and unique masses, e.g., β, 306 Da (G–A); κ, 386 Da (G4S–A); γ, 404 Da (G–D6S); ι, 466 Da (G4S–A2S); μ, 484 Da (G4S–D6S); and λ, 564 Da (G2S–D2S6S). Some other biose units

(35) Zaia, J.; Costello, C. E. *Anal. Chem.* **2003**, *75*, 2445–55.
(36) Leteux, C.; Chai, W.; Nagai, K.; Herbert, C. G.; Lawson, A. M.; Feizi, T. *J. Biol. Chem.* **2001**, *276*, 12539–45.
(37) Chai, W.; Leteux, C.; Westling, C.; Lindahl, U.; Feizi, T. *Biochemistry* **2004**, *43*, 8590–99.

may have identical masses, but their monosaccharide units may be different. For example, the mass of a δ-biose unit is 484 Da, identical to that of a μ-unit, but they differ in monosaccharide units: δ (G−D2S6S, 162 + 322 Da) and μ (G4S−D6S, 242 + 242 Da). The glycosidic cleavage in CID MS/MS is able to produce sequence ions with different masses to characterize these oligosaccharide sequences. However, a few biose units, e.g., ν- (G4S−D2S6S, 242 + 322 Da) and λ-units (G2S−D2S6S, 242 + 322 Da), have identical masses of di- and monosaccharide units and the masses of sequence ions alone are not possible to distinguish these two. Their difference in sulfate substitution, i.e., G4S in ν and G2S in λ, may provide clues to their identities. As illustrated in the spectra of A2S-containing neocarra-hexa-4S and carra-tri-3S, under identical conditions, the 2-*O*-sulfate is more labile and a desulfated C-ion at the 2-O-sulfated residue occurs whereas 4-*O*-sulfate is stable. Hence, following further detailed study of a more comprehensive series of carrageenan oligosaccharides, it may become possible to sequence all types of carrageenan oligosaccharide by ES CID MS/MS.

ACKNOWLEDGMENT

This study was supported in part by National Basic Research Program of China (2003CB716401), National Science Foundation of China (30370345), OUC Luka Program (1405-814147), and a U.K. Medical Research Council program grant (G9601454).

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 1, 2006. Accepted September 29, 2006.

AC061416J