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Electron capture dissociation of divalent metal-adducted sulfated oligosaccharides

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ABSTRACT

κ-Carrageenan sulfated oligosaccharides containing one, two, and four sulfate groups were complexed with divalent metal cations and electrosprayed in positive ion mode. The resulting metal complexes were subjected to sustained off-resonance irradiation collision-activated dissociation (SORI-CAD) and electron capture dissociation (ECD). The presence of divalent metal cations in the electrospray solution facilitates formation of doubly positively charged precursor ions in the gas phase. Unsurprisingly, abundant sulfate group (SO_3) loss is observed in SORI CAD of sulfated oligosaccharides complexed with divalent metal cations. By contrast, sulfation is retained in ECD of metal-sulfated oligosaccharide complexes, providing information on the site of sulfation. Most product ions resulting from ECD are C/Z- or B/Y-type ions, corresponding to cleavage of glycosidic bonds linking two sugar units. However, several A/X-type product ions corresponding to sugar cross-ring cleavage were also observed in ECD. Such cross-ring fragments are important in carbohydrate analysis because they provide linkage information.

1. Introduction

Sulfated oligossacharides, extensively found in proteoglycans, glycoproteins, and glycolipids, play important roles in many biological processes [1–4]. For example, glycosaminoglycans (GAGs), one of the most important types of sulfated polysaccharides, are involved in processes such as regulation of cellular growth, anticoagulation of blood, and inflammation reactions [1]. The GAG family includes heparin/heparan sulfate, chondroitin sulfate, hyaluronan, and keratin sulfate [1]. Carrageenans, which are found in the cell wall of red seaweeds, constitute another type of sulfated polysaccharides [5–9]. Carrageenan structures involve a repeating unit of 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranos, the latter being replaced with 4-linked 3,6-anhydro-α-D-galactopyranose in commercial samples. Depending on the number of sulfate groups per monomer, carrageenans can be categorized into three branches, i.e., kappa (κ), iota (ι), and lambda (λ) carrageenans. Carrageenans mediate cell–cell recognition processes in host-pathogen interactions [5].

Analysis of sulfated oligosaccharides is a challenging task due to the extreme lability of sulfate groups and the structural diversity of oligosaccharides. Mass spectrometry (MS) has been extensively applied to sulfated oligosaccharides [4–47], including extensive and pioneering work by Costello and co-workers. MS-based ionization techniques used for sulfated oligosaccharides include fast atom bombardment (FAB) [10–13] matrix-assisted laser desorption/ionization (MALDI) [14–19] and electrospray ionization (ESI) [20–47]. ESI is the preferred ionization technique because sulfate groups of sulfated oligosaccharides can be retained under certain conditions. Tandem mass spectrometry (MS/MS) is essential for structural characterization of sulfated oligosaccharides. In previous studies, collision activated dissociation (CAD) was almost exclusively used as the MS/MS activation technique to dissociate sulfated oligosaccharides [4,7,10–13,23–41]. However, more recently, Amster and co-workers have applied alternative MS/MS fragmentation techniques, including electron detachment dissociation (EDD) [42–45], electron induced dissociation (EID) [46], and negative ion electron transfer dissociation (NETD) [47] to sulfate-containing GAG and dermatan oligosaccharides in negative ion mode. These techniques provide complementary and often more extensive structural information compared to CAD, due to unique

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activation mechanisms, i.e., radical-driven fragmentation in EDD and NETD and electronic excitation in EID.

Due to the acidity of sulfated oligosaccharides, negative ion mode is preferred [4–47]. In negative ion CAD, it has been found that fragmentation patterns of sulfated oligosaccharides are strongly dependent on the charge state of the precursor ion [29,30,32,34]. If the charge state of a sulfated oligosaccharide is smaller than the total number of sulfate groups, i.e., the sulfate groups are not completely deprotonated, negative ion CAD results in abundant loss of sulfate groups. For sulfated oligosaccharides with all sulfate groups deprotonated, CAD can produce product ions containing the sulfate groups [30]. However, even in this case, sulfate loss is a prevalent competing process. In CAD of sulfated oligosaccharides, the most abundant product ions are typically B and Y-type ions [10] from glycosidic bond cleavage. Cross-ring product ions (A/X-type ions [10]) are rarely observed in negative ion CAD of sulfated oligosaccharides. However, observation of cross-ring fragments is essential for unambiguously determining the position of sulfate groups. In its first application towards sulfated oligosaccharides, EDD was found to yield abundant A-type cross-ring product ions with retention of sulfate groups for a singly sulfated doubly deprotonated tetrasaccharide [42]. However, EDD of sulfated oligosaccharides carrying the same number of negative charges and sulfate groups resulted in extensive loss of the sulfate groups [42]. By contrast, sulfate groups can be retained in EDD product ions if the charge state of a multiply sulfated oligosaccharide is equal to one more than the number of sulfate groups [45]. Based on the exciting capabilities of EDD for characterization of sulfated oligosaccharides, we were interested in exploring the utility of electron capture dissociation (ECD), which also proceeds via radical-driven fragmentation, typically with higher fragmentation efficiency than EDD, but cationic precursor ions are required.

Strategies for overcoming the challenges of performing positive ion mode analysis of sulfated oligosaccarides [5,7,40] have been proposed: in one approach, sulfated oligosaccharides were complexed with a basic peptide carrying positive charge [14,40]. Desaire and co-workers analyzed sulfated glycoproteins complexed with trilysine with both negative and positive ion mode CAD [40]. B/Y-type product ions were observed, however, abundant sulfate loss was seen from CAD in both detection modes although these authors concluded that negative ion CAD yielded more information than positive ion CAD for characterizing unknown glycopeptides. An alternative approach involves the use of alkali metal cation adducts. Dayrit and co-workers [7] utilized sustained off-resonance irradiation (SORI) CAD to characterize a series of κ-carrageenan sulfated oligosaccharides complexed with Na^+. In those experiments, all sulfate groups are sodiated, with additional Na^+ as charge carrier(s). Interestingly, sulfate group(s) can be retained in B/Y-type ions produced from SORI-CAD of sulfated oligosaccharides that are fully sodiated, thereby providing information on the position of sulfate group(s). However, A/X-type ions were completely absent in those SORI-CAD spectra.

Electron capture dissociation (ECD) [48–50] is a powerful tool for structural characterization of a variety of biomolecules. However, one limitation of ECD is that it is only applicable to positively charged ions with charge state $\geq$2, which is challenging to achieve for acidic, neutral, and low molecular weight analytes such as oligosaccharides with or without sulfation. We have previously shown that doubly positively charged ions, amenable to ECD, can be readily generated for sulfated peptides [51], non-sulfated oligosaccharides [52], and metabolites [53] upon complexation with divalent metal cations. O'Connor and co-workers utilized sodium adducts in a similar manner for permethylated oligosaccharides [54]. In all cases, structural information complementary to that from CAD is achieved. Specifically, ECD of divalent metal-adducted sulfopeptides provided sulfation site information [51]. By contrast, ECD of the corresponding protonated species resulted in sulfate loss. Burlingame and co-workers reached similar conclusions in ETD of alkali metal-adducted sulfopeptides [55]. Here, we apply divalent metal adduction and ECD towards three κ-carrageenan sulfated oligosaccharides containing up to four sulfate groups.

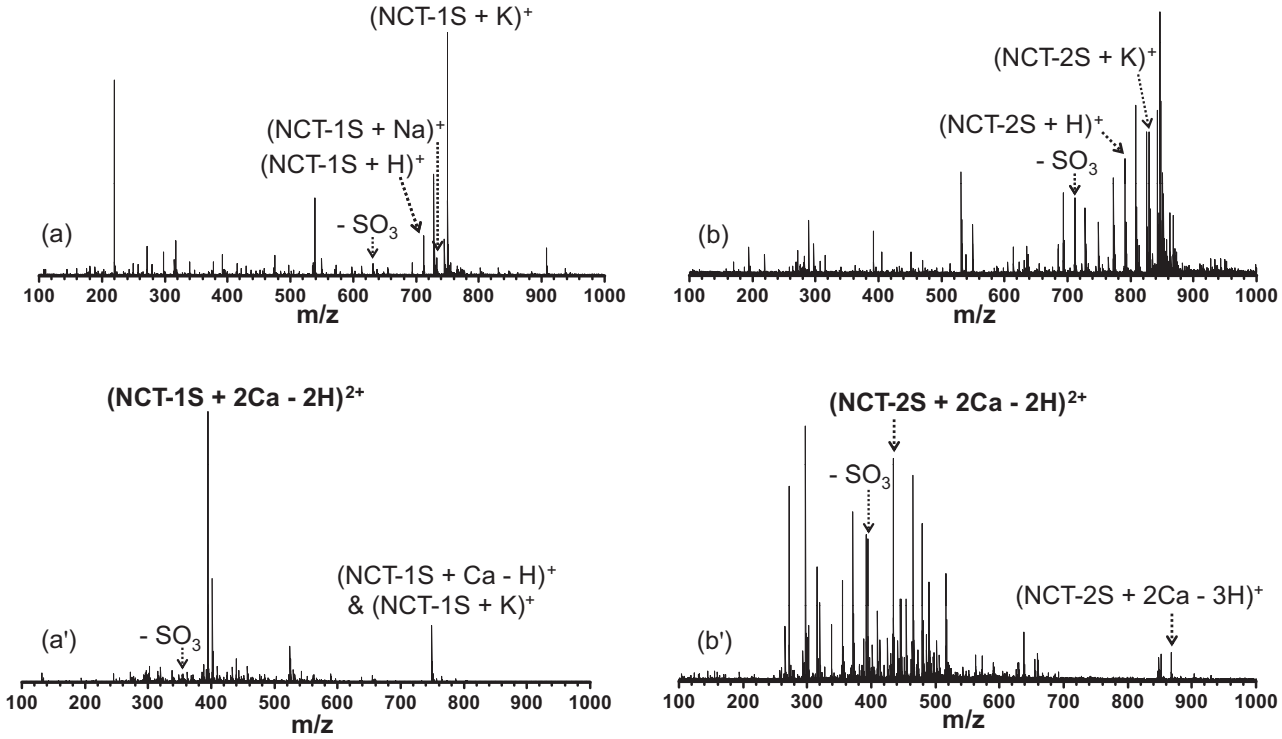

Fig. 1. Positive ion mode ESI FT-ICR mass spectra (single scan) from 5 μM NCT-1S and 10 μM NCT-2S in the presence of 2% acetic acid (a and b) and 20 μM Ca^{2+} (a' and b').

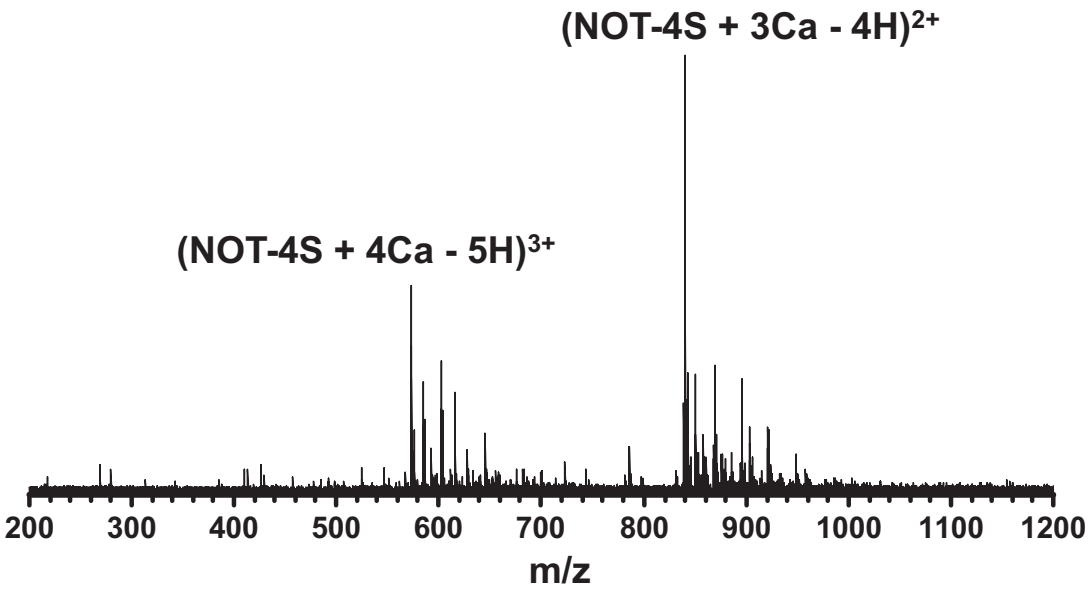

Fig. 2. Positive ion mode ESI FT-ICR mass spectrum (single scan) from 5 μM NOT-4S and 20 μM Ca^{2+}.

2. Experimental

2.1. Reagents and sample preparation

κ-Carrageenan sulfated oligosaccharides, neocarratetraose 4^1-sulfate (NCT-1S) sodium salt (purity ≥98%), neocarratetraose 4^1,4^3-disulfate (NCT-2S) disodium salt (purity unknown), neocarraoctaose 4^1,4^3,4^5,4^7-tetrasulfate (NOT-4S) tetrasodium salt (purity 95%) were purchased from Aldrich (St. Louis, MO) and used without further purification. Protonated sulfated oligosaccharides were generated by external ESI at 70 μL/h (Apollo or Apollo II ion source (as specified below), Bruker Daltonics, Billerica, MA) of solutions containing 5 μM sulfated oligosaccharides and 2% acetic acid (1:1 MeOH:H_2O), while metal-adducted sulfated oligosaccharide ions were produced from ESI of solutions containing 5 μM sulfated oligosaccharides and 20 μM metal salt ($MnCl_2$ from Aldrich (St. Louis, MO) and $CaCl_2{\cdot}2H_2O$ from Fisher (Fair Lawn, NJ)). No acid was added to the metal-containing ESI solutions.

2.2. Mass spectrometry and data analysis

Data for NCT-1S and NCT-2S were collected with an actively shielded 7 Tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer with a quadrupole front-end (ApexQ, Bruker Daltonics, MA) and an Apollo ESI source as previously described [56]. However, we failed to observe metal-adducted NOT-4S with this ion source. Following upgrade of the original Apollo ESI source to the Apollo II source (Bruker Daltonics, MA), containing a dual stage ion funnel and operating at elevated pressure to assist ion collection and transfer, we were able to detect multiply charged metal-adducted NOT-4S. Ions produced by ESI were mass-selectively externally accumulated (10–40 m/z isolation window) in a hexapole for 2 s, transferred via high voltage ion optics, and captured in the ICR cell by dynamic trapping. This accumulation sequence was looped six times to optimize precursor ion signal-to-noise (S/N) ratio prior to ECD. Further precursor ion isolation was accomplished by correlated harmonic excitation fields (CHEF) [57] inside the infinity ICR cell [58]. An indirectly heated hollow dispenser cathode [59] with inner and outer diameters of 3.5 and 7.6 mm, respectively, provided the electrons. ECD was performed at a −1 V cathode bias voltage with 100–300 ms irradiation time. For SORI CAD, an RF frequency offset by −1000 Hz from the precursor ions' cyclotron frequency was applied for 20–40 ms while argon was pulsed into the ICR cell. All data were acquired with XMASS (version 6.1, Bruker Daltonics) in broadband mode from m/z = 200–1600 with 256–512 k data points and summed over 30–50 scans. Mass spectra were analyzed with the MIDAS analysis software [60].

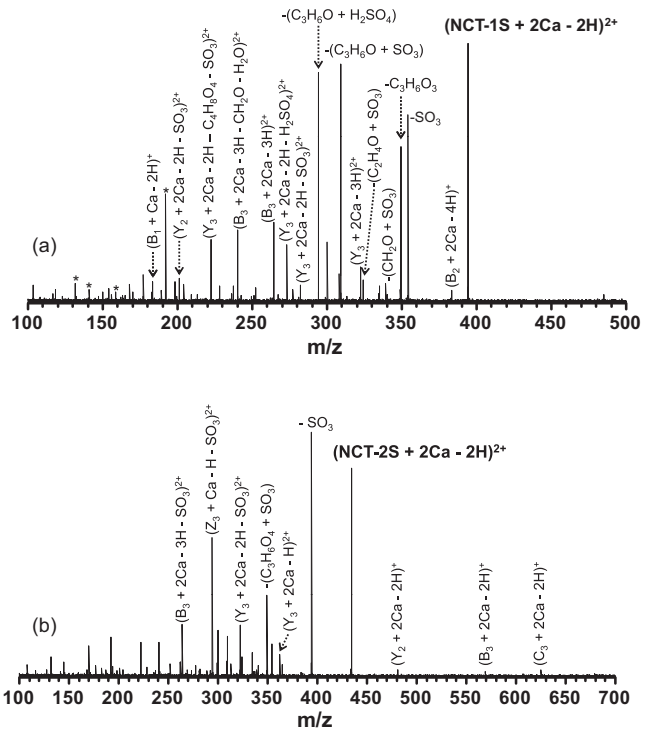

Fig. 3. Positive ion mode SORI-CAD spectra of NCT-1S (a) and NCT-2S (b) complexed with Ca^{2+} (8 scans). Electronic noise peaks are labeled with asterisks.

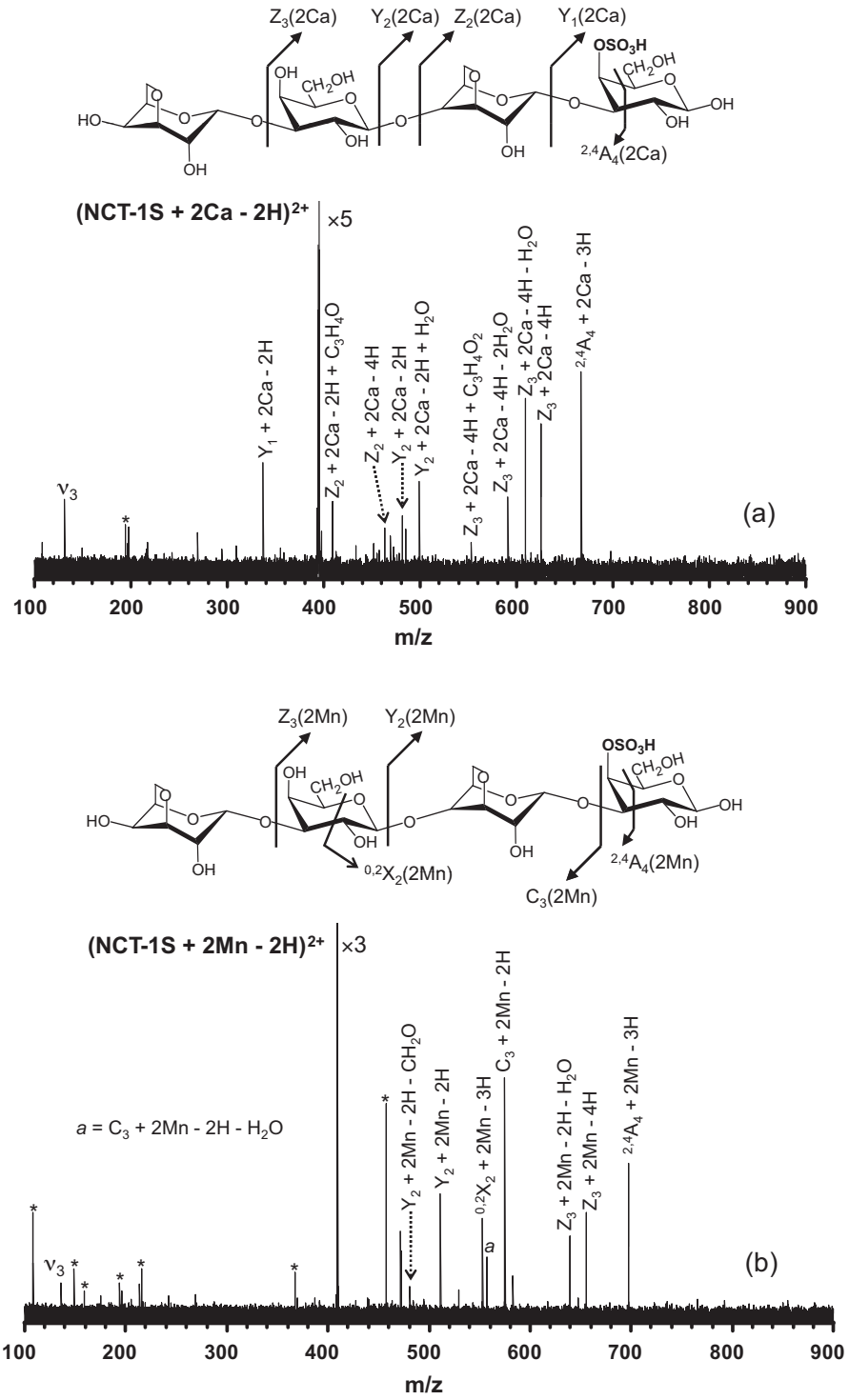

Fig. 4. ECD spectra of NCT-1S complexed with Ca^{2+} (a) and Mn^{2+} (b) (300 ms electron irradiation time, −1 V bias voltage, 50 and 32 scans for Ca^{2+} and Mn^{2+}, respectively). Electronic noise peaks are labeled with asterisks, ν_3 = 3rd harmonic.

3. Results and discussion

3.1. ESI of divalent metal-adducted NCT-1S, NCT-2S, and NOT-4S

Figs. 1 and 2 show positive mode ESI mass spectra of NCT-1S, NCT-2S, and NOT-4S from solutions containing acid, or divalent metal cations. ESI of NCT-1S and NCT-2S in the presence of acid produced only singly positively charged NCT-1S and NCT-2S, respectively (Figs. 1(a) and 2(a)), resulting from the addition of a proton or an alkali metal cation (Na^+ or K^+). However, we failed to detect singly positively charged precursor ions for NOT-4S. Unsurprisingly, multiply positively charged NCT-1S and NCT-2S were not observed due to their high acidity. By contrast, in the presence of excess Ca^{2+}, multiply positively charged NCT-1S, NCT-2S, and NOT-4S were efficiently generated (see Figs. 1(a', b') and 2) in the form of Ca-adducted species. For NCT-1S and NCT-2S, abundant doubly Ca-adducted species, $[NCT\text{-}1S/NCT\text{-}2S + 2Ca - 2H]^{2+}$ were observed. These doubly charged ions are particularly abundant for NCT-1S

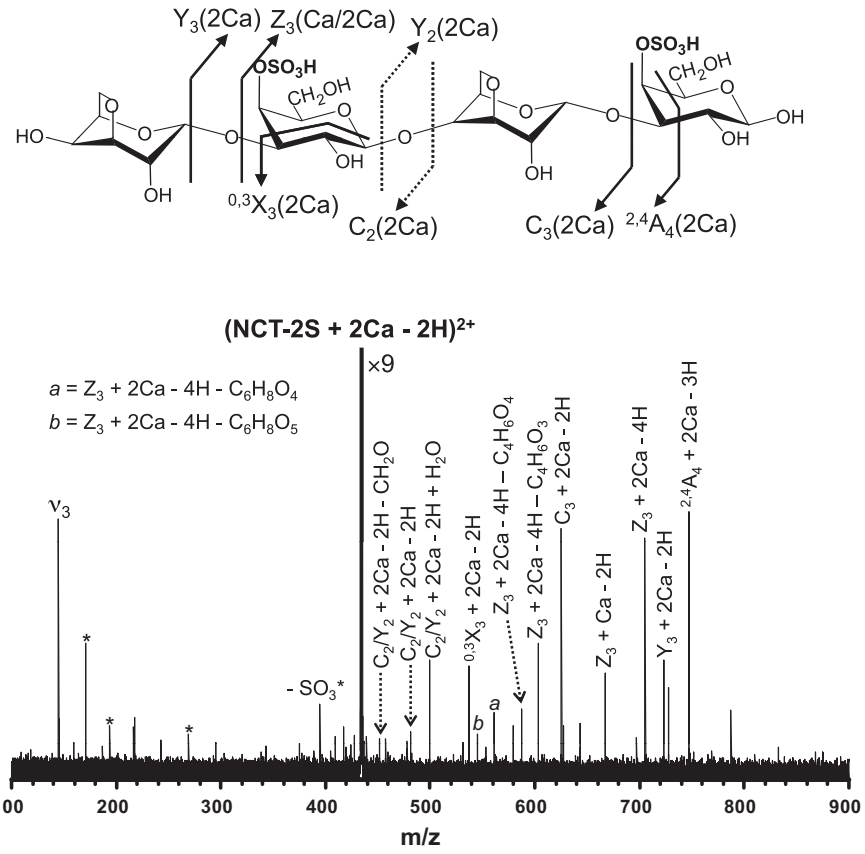

Fig. 5. ECD spectrum from NCT-2S complexed with Ca^{2+} (100 ms electron irradiation time, −1 V bias voltage, 50 scans). Electronic noise peaks are labeled with asterisks, ν_3 = 3rd harmonic. The peak corresponding to loss of SO_3 from the precursor ion was present in the mass spectrum prior to ECD. Due to identical m/z of C_2- and Y_2-type ions, glycosidic cleavage corresponding to these two ions is denoted by dashed lines in the inset.

(Fig. 1(a')) where they dominate the ESI spectrum. ESI of NOT-4S produced abundant doubly and triply charged cations (Fig. 2), $(NOT\text{-}4S + 3Ca - 4H)^{2+}$ and $(NOT\text{-}4S + 4Ca - 5H)^{3+}$, respectively. The addition of more metal cations for NOT-4S than for NCT-1S and NCT-2S is presumably due to the presence of more sulfate groups, which likely constitute the metal binding site [7,51]. We have previously shown that metal complexation facilitates formation of multiply charged cations for sulfated peptides [51], non-sulfated oligosaccharides [52], and for metabolites [53]. Here, we show that ionization of sulfated oligosaccharides in positive ion mode can also be enhanced via divalent metal complexation. Abundant peaks corresponding to divalent metal-adducted sulfated oligosaccharides are observed without any further purification and desalting of the samples. Signal from singly sodiated or potassiated NCT-1S is either absent or significantly suppressed in the presence of Ca^{2+} (Figs. 1(a and a')). It should be noted that sulfate loss is not prevalent in ESI under these conditions, except for NCT-2S where moderate loss of one sulfate group is observed. Also, ESI mass spectra of NCT-2S contain many unknown peaks, which are likely due to impurities (the purity of this sample is unknown, see Section 2). Thus, the observed sulfate loss product ions may already be present in the ESI solution rather than being formed in the gas phase. Nevertheless, abundant divalent metal-adducted NCT-2S containing the sulfate groups could still be generated.

The successful generation of multiply positively charged ions for these sulfated oligosaccharides allows application of ECD, which is known to be a superior tool for analyzing labile modifications [48–50]. Here, we are focusing on calcium adducts because our previous work [51–53] showed that Ca^{2+} is superior to other divalent metals due to its propensity to form abundant complexes with molecules of interest and the analytical utility of the corresponding ECD spectra. However, abundant complexes were also obtained between NCT-1S (but neither NCT-2S nor NOT-4S) and manganese. ECD data of Mn^{2+}-adducted NCT-1S are shown for comparison.

3.2. SORI-CAD of divalent metal-adducted NCT-1S and NCT-2S

Sulfate groups are typically preferentially lost in positive ion mode CAD of sulfated species due to their extreme lability [4]. Dayrit and co-workers found that positive ion mode SORI-CAD of sodiated κ-carrageenan sulfated oligosaccharides, in which all H of SO_3H are replaced with Na, produced B and Y-type product ions containing the sulfate groups. However, positive ion mode CAD of divalent metal-adducted sulfated oligosaccharides has not, to our knowledge, been reported. SORI-CAD spectra of doubly calcium-adducted NCT-1S and NCT-2S are shown in Fig. 3(a and b), respectively. Apparently, sulfate loss is the dominant process in SORI-CAD of these two metal complexes. For example, the three most abundant product ions in SORI-CAD of $[NCT\text{-}1S + 2Ca - 2H]^{2+}$ (Fig. 3(a)) are due to loss of SO_3 or combined loss of SO_3/H_2SO_4 and C_3H_6O from the precursor ions. In addition, all but one B/Y-type ions observed in Fig. 3(a) do not contain the sulfate group. The only ion containing the sulfate group is $(Y_3 + 2Ca - 3H)^{2+}$, which is of low abundance. Similarly, sulfate loss dominates in SORI-CAD of $[NCT\text{-}2S + 2Ca - 2H]^{2+}$ (Fig. 2(b)). Although there are several B/Y-type ions containing one sulfate group, i.e., $(Y_3 + 2Ca - H)^{2+}$, $(Y_2 + 2Ca - H)^{+}$, and $(B_3/C_3 + 2Ca - H)^{2+}$, these ions are of much lower abundance than the corresponding ions without the sulfate group. Therefore, determination of the position of sulfate groups may be challenging with CAD alone.

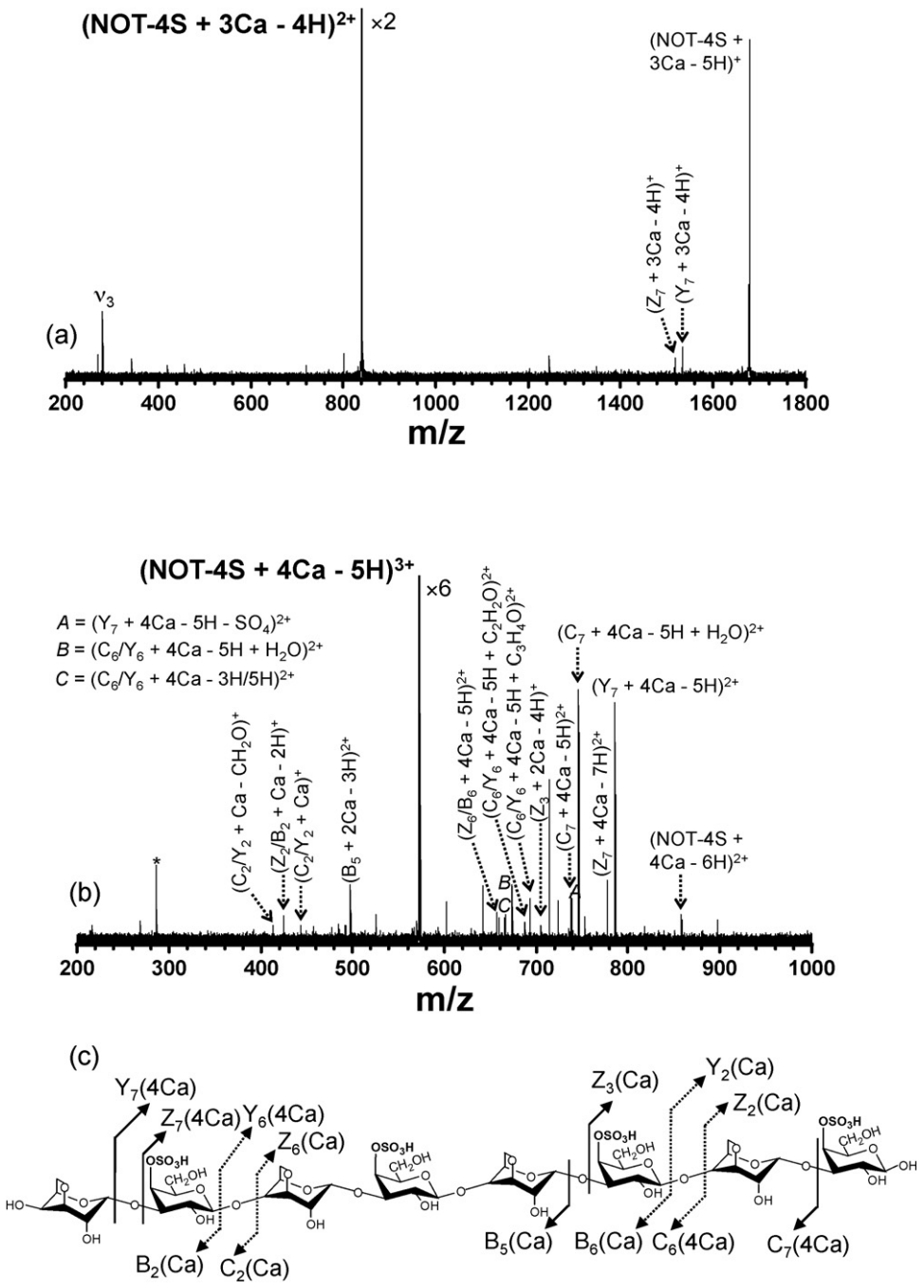

Fig. 6. ECD spectra from NOT-4S complexed with three and four Ca^{2+}, corresponding to charge states of 2+ (a) and 3+ (b) (300 ms electron irradiation time, −1 V bias voltage, 32 scans), respectively, and the ECD fragmentation pattern of NOT-4S (c). Electronic noise peaks are labeled with asterisks, ν_3 = 3rd harmonic. Due to identical m/z of C_2- and Y_2-, C_6- and Y_6-, Z_2- and B_2-, and Z_6- and B_6-type ions, the corresponding glycosidic cleavage is denoted by dashed lines in (c).

3.3. *ECD of divalent metal-adducted NCT-1S, NCT-2S, and NOT-4S*

We have previously shown that the combination of divalent metal complexation and ECD is highly useful for locating sulfate groups in sulfated peptides [51]. Divalent metal complexation enhanced the ionization of sulfated peptides in positive ion mode, while ECD produced extensive backbone fragmentation without loss of the sulfate groups. The generation of multiply positively charged sulfated oligosaccharides (Figs. 1 and 2) makes application of ECD to sulfated oligosaccharides possible. Figs. 4–6 show ECD spectra of NCT-1S, NCT-2S, and NOT-4S complexed with Ca^{2+} (and Mn^{2+} for NCT-1S). All product ions observed in ECD of NCT-1S and NCT-2S are singly charged. Thus, product ion charge state has been omitted from figure labels.

ECD of NCT-1S complexed with Ca^{2+} and Mn^{2+} (Fig. 4) yielded C-, Z-, and Y-type product ions from glycosidic bond cleavage and one A/X-type product ion from cross-ring cleavage. Specifically, two Z-type, two Y-type, and one A-type fragment (Z_2, Z_3, Y_1, Y_2, and $^{2,4}A_4$ with two Ca adducts) were produced in ECD of [NCT-1S + 2Ca − 2H]$^{2+}$ (Fig. 4a). No C- and X-type product ions were observed in ECD of NCT-1S complexed with Ca^{2+}. The most abundant product ion found from ECD of [NCT-1S + 2Ca − 2H]$^{2+}$ can be assigned as ($^{2,4}A_4$ + 2Ca − 3H), while two Z/Y-type ions, assigned as

(Z_3 + 2Ca − 4H) and (Y_1 + 2Ca − 2H) are slightly less abundant. The water-loss product of (Z_3 + 2Ca − 4H) is also abundant. Other minor Z/Y-type product ions include (Z_2 + 2Ca − 4H) and (Y_2 + 2Ca − 2H). It should be pointed out that all Z- and A-type product ions observed in ECD of $[NCT\text{-}1S + 2Ca - 2H]^{2+}$ were not observed in SORI-CAD of the same species (Fig. 4a). Metal-adducted Z_3, Y_2, and $^{2,4}A_4$ ions observed for Ca-adducted NCT-1S were also observed for Mn-adducted NCT-1S (Fig. 4b). In addition, one C-type and one X-type product ion [(C_3 + 2Mn − 2H) and ($^{0,2}X_2$ + 2Mn − 3H)] were seen in ECD of NCT-1S complexed with Mn^{2+} (Fig. 4b), the former being the most abundant peak in this ECD spectrum. Another major difference between ECD spectra of Ca- and Mn-adducted NCT-1S is that the Y_1 and Z_2 ions observed for the Ca-adducted species were not detected for the Mn-adducted species. Nevertheless, product ions observed for Ca- and Mn-adducted NCT-1S provide information on the oligosaccharide sequence. Interestingly, sulfate loss was completely absent in ECD of NCT-1S complexed with Ca^{2+} and Mn^{2+}, in stark contrast to the SORI-CAD results discussed above (Fig. 3). Thus, ECD should greatly facilitate localization of the sulfate group. Particularly, the position of the sulfate group in NCT-1S can be unambiguously determined from the metal adducted $^{2,4}A_4$ ion.

Fig. 5 displays the spectrum resulting from ECD of NCT-2S complexed with Ca^{2+}. Again, a variety of C/Z-, A/X-, and Y-type product ions were detected. Note that C_2 and Y_2 have the same molecular weight and thus cannot be distinguished. The C_3, Z_3, and A_4 ions with two Ca adducts are nearly equally abundant, while they are much more abundant than other product ions. As found for NCT-1S, sulfate loss was absent in ECD of $[NCT\text{-}2S + 2Ca - 2H]^{2+}$. Z_3, Y_3 and A_4 ions with one or two Ca adducts contain both sulfate groups, while the C_2/Y_2, C_3 and X_3 ions with two Ca contain one of two sulfate groups. The position of two sulfate groups can be determined from analysis of these product ions, particularly the calcium-adducted $^{0,3}X_3$ and $^{2,4}A_4$ ions. For NOT-4S, containing four sulfate groups, both the doubly and triply positively charged precursor ions observed in ESI (Fig. 2) were subjected to ECD.

Unlike ECD of doubly charged NCT-1S and NCT-2S (Figs. 4 and 5), ECD of doubly charged NOT-4S (Fig. 6a) yielded predominantly charge reduced product ions, $(NOT\text{-}4S + 3Ca - 5H)^{+}$. Other than that, only one Z- and one Y-type product ion (Z_7 and Y_7 with three Ca adducts) were observed at low abundance. Although these two product ions contain all four sulfate groups, they provide insufficient information about the position of these four sulfate groups. However, when triply charged NOT-4S, complexed with four Ca^{2+}, was subjected to ECD (Fig. 6b), significantly more product ions were generated. Note that each pair of C_2/Y_2, C_6/Y_6, Z_2/B_2, and Z_6/B_6 ions has identical mass. Most product ions observed in ECD of triply charged NOT-4S are doubly charged, but some singly charged fragments are also seen. There are product ions containing one sulfate group (C_2/B_2 and Z_2/Y_2 with one Ca), two sulfate groups (Z_3 and B_5 with one and two Ca, respectively), three sulfate groups (C_7, Z_6/Y_6, and C_6/B_6 with four Ca), and all four sulfate groups (Z_7/Y_7 with four Ca). The two most abundant product ions are Y_7 with four Ca, $(Y_7 + 4Ca - 5H)^{2+}$, and a hydrated C_7 ion with four Ca, $(C_7 + 4Ca - 5H + H_2O)^{2+}$. All product ions observed in ECD of NOT-4S are associated with glycosidic cleavage, and contain at least one sulfate group. Analysis of these product ions may aid determination of the position of each sulfate group, although no product ions from cross-ring cleavage were observed. From the results obtained for sulfated oligosaccharides studied here and those for sulfated peptides [51], we conclude that the combination of divalent metal complexation and ECD is a highly useful tool for analyzing sulfate-containing molecules, which are otherwise challenging to characterize in positive ion mode.

4. Conclusions

Three κ-carrageenan sulfated oligosaccharides containing up to four sulfate groups (NCT-1S, NCT-2S, and NOT-4S) have been analyzed with the combination of divalent metal complexation and ECD. Divalent metal complexation was found to enhance the ionization of highly acidic sulfated oligosaccharides in positive ion mode without significant loss of the labile sulfate group(s). Multiply charged precursor cations complexed with divalent metals were generated and subjected to SORI-CAD and ECD after quadrupole isolation. Unsurprisingly, SORI-CAD resulted predominantly in sulfate loss, eliminating the possibility of sulfate site determination. In contrast, sulfate loss was completely absent in ECD of all three sulfated oligosaccharides complexed with divalent metal cations. Analysis of sulfated product ions provides information on the position of the sulfate group(s) for these sulfated oligosaccharides.

Acknowledgments

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