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# Multistage tandem mass spectrometry of chondroitin sulfate and dermatan sulfate

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ABSTRACT

Chondroitin/dermatan sulfate (CS/DS) is a glycosaminoglycan (GAG) found in abundance in extracellular matrices. In connective tissue, CS/DS proteoglycans play structural roles in maintaining viscoelasticity through the large number of immobilized sulfate groups on CS/DS chains. CS/DS chains also bind protein families including growth factors and growth factor receptors. Through such interactions, CS/DS chains play important roles in neurobiochemical processes, connective tissue homeostasis, coagulation, and cell growth regulation. Expression of DS has been observed to increase in cancerous tissue relative to controls. In earlier studies, $MS^2$ was used to compare the types of CS/DS isomers present in biological samples. The results demonstrated that product ion abundances reflect the types of CS/DS repeats present and can be used quantitatively. It was not clear, however, to which of the CS/DS repeats the product ions abundances were sensitive. The present work explores the utility of $MS^3$ for structural characterization of CS/DS oligosaccharides. The data show that $MS^3$ product ion abundances correlate with the presence of DS-like repeats in specific positions on the oligosaccharide chains.



## 1. Introduction

Chondroitin sulfate (CS) and dermatan sulfate (DS) proteoglycans are abundant extracellular matrix molecules the glycosaminoglycan (GAG) chains of which mediate both their physicochemical and adhesive properties and thus their biological activities. Protein-CS/DS binding is a hallmark for CS/DS proteoglycans and serves to modulate the activities of growth factors including the fibroblast growth factor family and hepatocyte growth factor [1–3]. CS chains bound to the aggrecan and related proteoglycans are present in high concentrations in cartilage and other connective tissues and function in the maintenance of tissue viscoelasticity [4]. The structure of the CS chains in cartilage changes during development and with the progression of osteoarthritis.

The structure of nascent CS chains is elaborated in the Golgi apparatus through the action of a series of biosynthetic enzymes [5]. Because these reactions do not go to completion, mature CS chains are heterogeneous mixtures of variants on a conserved domain structure [6]. The nascent chains consist of repeating units of $(4GlcA\beta1–3GalNAc\beta1–)_n$ attached to Ser residues via a tetrasaccharide core of structure 4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–. During biosynthesis, GlcA residues may undergo sulfation at the 4*O*- and/or 6*O*-positions of GalNAc and at the 2*O*-position of GlcA. Depending on the tissue location, CS may be modified by epimerization of the C5 position of GlcA to form IdoA by DS epimerases [7]. The mature chains typically reflect domains of high and low IdoA content, and such residues are often found adjacent to GalNAc-4*O*-sulfate residues. CS/DS chains are often classified based on their compositions. Those with a high content of GlcA–GalNAc4S are known as CS type A (CSA), those with a high content of IdoA–GalNAc4S as CSB (also known as DS) and those with GlcA–GalNAc6S as CSC.

The patterns of CSB-like repeats (4IdoAα1–3GalNAc4Sβ1-) in CS/DS chains arises from DS epimerases 1 and 2 [7,8] in conjunction with dermatan 4-*O*-sulfotransferase 1 [9]. The epimerase reaction occurs to the nascent chondroitin chain and is reversible. Subsequent 4-*O*-sulfation of the adjacent GalNAc residue is believed to be required to lock the residue as IdoA. As a result, low levels of DS 4-*O*-sulfotransferase are correlated with the presence interspersed patterns of repeats containing IdoA and GlcA. Thus the interplay of expression levels of epimerases and sulfotransferases, among other factors, gives rise to CS/DS structures specific to individual cellular phenotypes. DS epimerase 2 is expressed at high levels in brain, and has been linked genetically to bipolar disorder [8].

*Abbreviations:* CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; DS, dermatan sulfate; ESI, electrospray ionization; GAG, glycosaminoglycan; HILIC, hydrophilic interaction chromatography; MS, mass spectrometry.

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Expression of CS/DS proteoglycans inhibits neuronal outgrowth and prevents repair of spinal cord injury [10]. This activity has been correlated with expression of specific CS/DS sulfotransferases [11]. The activity of CS/DS in brain has been correlated with spatio-temporal regulation of the expression of key sulfated disaccharide units, resulting from the activities of specific sulfotransferases and epimerases [12,13]. In order to expand the understanding of the functional roles of expression CS/DS disaccharides in the context of flanking oligosaccharide structures, there is clear potential for tandem MS. Such an approach has the potential to be used as a means of gas-phase decomposition of CS/DS oligosaccharides, enabling determination of key isomeric structural elements.

Given the presence of significant heterogeneity of CS/DS chains, methods are needed to assess both the chain compositions and the abundances of isomers present. Electrospray ionization mass spectrometry (ESI MS) has been shown to be effective for determination of the masses of CS oligosaccharides [14–17]. Mass determination serves to define the oligosaccharide chain length and number of sulfate groups. On-line liquid chromatography (LC) MS systems have been developed using size exclusion chromatography [14,18–20], graphitized carbon chromatography [21–23], reversed phase ion pairing [24–27] and hydrophilic interaction chromatography [28–32]. Approaches for analysis of CS/DS oligosaccharides using capillary electrophoresis/MS have also been described [33–35].

When exhaustively depolymerized using polysaccharide lyase enzymes, a mixture of disaccharides is produced. Determination of the abundances of the isomeric *2O*-, *4O*- and *6O*-sulfated disaccharides in such mixtures is an important part of characterization of a CS proteoglycan sample. A tandem mass spectrometric method for disaccharide analysis has been developed that compares product ion abundances of unknown disaccharide mixtures to those of purified standards to calculate mixture percentages [36–39]. For analysis of oligosaccharides, tandem MS is useful for assigning positions of over-sulfated GalNAc residues [40]. It is also important to be able to assign the abundances of 4*O*- and 6*O*-sulfation at each GalNAc residue [33,35] in the chain under conditions in which isomeric mixtures exist. Toward these ends, it has been demonstrated that the abundances of key product ions are diagnostic for the overall content of CSA-like, CSB-like and CSC-like repeats [41,42]. This approach has been used in an on-line LC/MS format to compare the CS/DS glycoforms present among isolated proteoglycans and among different tissues [29–32]. These results demonstrated the principle that product ion abundances reflect isomeric GalNAc sulfation positions in CS oligosaccharides. They do not produce information on sulfation site occupancy on individual GalNAc residues.

The focus of the present work is to develop methods to produce detailed information on the sulfation of individual residues in CS/DS chains. Towards these ends, a series of oligomers were prepared from CSA, CSB, CSC ranging from dp 10-dp 14 and analyzed $MS^3$. The results demonstrate the increased level of structural detail obtained through such a tandem MS approach for CS/DS analysis.

## 2. Experimental

### 2.1. Materials

CS type A (GlcA, GalNAc-4-sulfate), CSB (IdoA, GalNAc-4-sulfate) CSC (GlcA, GalNAc-6-sulfate), and chondroitinase ABC were obtained from Seikagaku America/Associates of Cape Cod (Falmouth, MA). 2-Anthranilic acid (2AA) was purchased from Fluka Chemika (Buchs, Switzerland).

### 2.2. Preparation of CS oligosaccharides

Fresh CSA, CSB, or CSC standards (5 μL, 2 mg/mL) were mixed with water (87 μL), Tris–HCl buffer (5 μL, 1 M, pH 7.4), ammonium acetate (0.5 μL, 1 M) and chondroitinase ABC (2.5 μL, 4 mU/μL), and digested at 37 °C to an absorbance value (232 nm) corresponding to liberation of 10% of the disaccharide units in the intact chains, and boiled for 1 min. Partial depolymerization products were lyophilized for subsequent reductive amination. Oligosaccharides were reductively aminated using 2AA and purified according to published procedures [29,31]. The reductively aminated oligosaccharides were purified using amide-silica hydrophilic interaction chromatography (HILIC) as follows: 2.1 mm × 5 cm Amide-80 (Tosoh Bioscience, Montgomery, PA, USA), mobile phase A = 10% acetonitrile, 0.05 M formic acid solution, pH 4.4 adjusted using ammonium hydroxide, B = 20% A in acetonitrile, flow rate = 250 μL/min, gradient from 80 to 20% B in 60 min.

### 2.3. Tandem MS of CS oligosaccharides

CS oligosaccharides were analyzed using a ThermoFisher Scientific LTQ-Orbitrap mass spectrometer. Samples were dissolved at a concentration of 10 μM in a solution of methanol:water:ammonium hydroxide (30:70:0.2%) and analyzed using a pulled silica capillary nano-scale electrospray interface using negative polarity. The following negative polarity source conditions were used: spray voltage 1500, capillary temperature 240 °C, capillary voltage −15, tube lens voltage −34. The masses of all oligosaccharides were determined using high resolution detection in the Orbitrap analyzer. All tandem mass spectra were acquired in the LTQ analyzer using an isolation width of 3 u. For $MS^2$ experiments, collision voltage was set to 20. For $MS^3$ experiments, collision voltage was set to 20 for both stages of dissociation. Tandem mass spectra were summed from approximately 1 min of accumulated data. The following product ions observed in $MS^2$ were selected for $MS^3$: $Y_3^{2-}$, $Y_5^{3-}$, $Y_7^{4-}$, $Y_9^{5-}$ and $B_5^{2-}$, $B_7^{3-}$, $B_9^{4-}$. Data were acquired in triplicate technical replicates. Relative product ion abundances were calculated from the peak intensities determined using the Xcalibur data system.

## 3. Results

CS chains were subjected to limited digestion using chondroitinase ABC, after which the oligosaccharide products were reductively aminated using 2AA [31,32]. The resultant 2AA labeled oligosaccharides were fractionated using HILIC and used for tandem mass spectrometric studies. Fig. 1A shows the negative ion ESI tandem mass spectrum of CSC Δdp12-2AA acquired by selecting the $[M-6H]^{6-}$ ion. Typical of CS oligosaccharides, a series of B- and Y-type ions was observed, corresponding to cleavage of every HexA–GalNAc bond. An ion corresponding to $[M-SO_3-6H]^{6-}$ was observed, the abundance of which is highest for the CSC isomer relative to CSA and CSB, respectively, as has been observed using smaller oligosaccharides [32,43]. Ions produced from loss of sulfate from some B- and Y-type ions were observed in relatively low abundances.

Oligosaccharides derived from CSA, CSB, and CSC produced the same product ions but in distinct abundance patterns. Fig. 1B compares the product ion abundances for dp12 for CSA, CSB and CSC. The results showed the $Y_3$ ion was most abundant for CSA dp12, and therefore diagnostic for this CS isomer. The abundances of $Y_9$ and $Y_{11}$ were diagnostic of CSB. Those of the $[M-SO_3-6H]^{6-}$ and $B_{11}$ ions were diagnostic of CSC. These results are consistent with studies of dp4-dp8 CS oligosaccharides [30,41,42]. The abundances

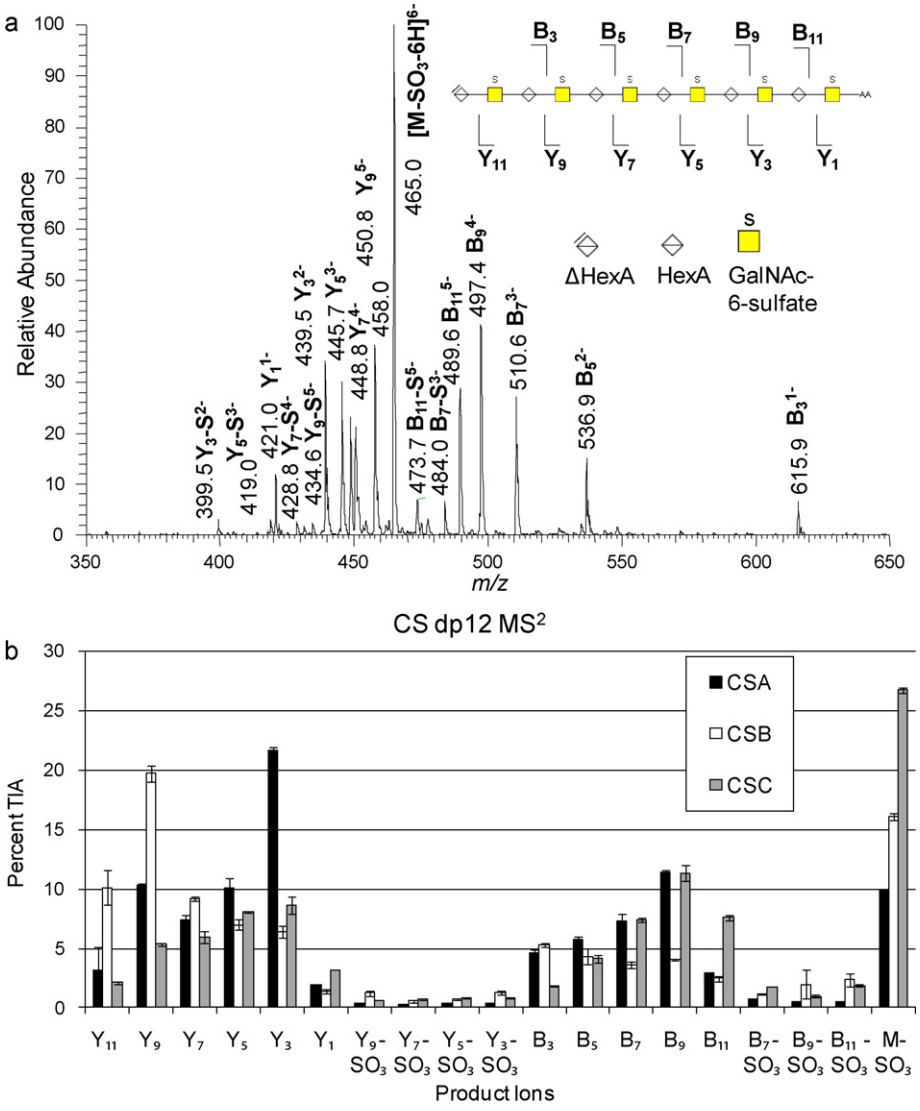


**Fig. 1.** (A) Representative tandem mass spectrum of 2AA labeled CS: CSC dp12-2AA. The ion at m/z 458 is an internal fragment ion commonly observed for CS oligosaccharides. The inset diagrams the product ions observed. The $Y_{11}$ is observed as a low abundance ion at *m/z* 451.8 (not labeled). Loss of $SO_3$ is abbreviated as "S". (B) Comparison of product ion abundances from CAD tandem MS of Δdp 12-2AA from CSA, CSB, and CSC, respectively. TIA = total ion abundance.

of individual product ions are likely to reflect the CS oligosaccharide structure in the vicinity of the cleaved bond. Thus, in order to increase the amount of structural information produced, multistage MS was used as a means of degrading the CS oligosaccharides in the gas phase.

A series of $MS^3$ dissociation experiments were conducted on the $Y_3^{2-}$, $Y_5^{3-}$, $Y_7^{4-}$, and $Y_9^{5-}$ ions observed in the $MS^2$ stage for dp 12 $[M-6H]^{6-}$ precursor ions for CSA, CSB and CSC. All measurements were performed in triplicate. The product ion abundances for each Y ion are shown in Fig. 2. The $MS^3$ product ion patterns of $Y_9$ (2A), $Y_7$ (2B), and $Y_5$ (2C) all show that the abundance of $Y_3$ is diagnostic for the CSA isomer. Thus, the information on the CSA isomers produced from $MS^3$ of $Y_n$ ions was the same as that observed in the $MS^2$ product ion pattern. When $MS^3$ was acquired on the $Y_3$ ion (2D), the resultant $Y_1$ product ion was diagnostic for CSA. This observation is consistent with the abundance of this ion correlating with the sulfation position at the reductively aminated GalNAc residue.

For $MS^3$ dissociation of $Y_9$, the $Y_7$ product ion was most abundant for the CSB isomer (2A). Similarly, $MS^3$ of $Y_7$ (2B) showed $Y_5$ to be diagnostic for CSB. For $MS^3$ of $Y_5$ (2C), however, the analogous pattern was not observed, possibly due to the high abundance of $Y_3$ for the CSA isomer. Thus, it appears that additional information on the presence of CSB-like repeats was generated from $MS^3$ of the $Y_9$ and $Y_7$ ions. In addition, $MS^3$ of all $Y_n$ ions showed a $Y_3$–$SO_3$ ion diagnostic for CSB. The results are consistent with improved coverage of CSB-like disaccharide repeats, reflecting the epimeric state of the HexA residue adjacent to the cleaved glycosidic bond.

For $MS^3$ of all $Y_n$ ions, the abundances of ion produced by loss of $SO_3$ from the $MS^3$ precursor were diagnostic for CSC. A similar series of data were acquired for CSA, CSB, and CSC dp 10 as shown in Supplementary Figs. 1 and 2 and dp14 in Supplementary Figs. 3 and 4. The ion abundance trends are analogous to those observed for dp12, and support the same conclusions.

The product ion abundances resulting from $MS^3$ for $B_5^{2-}$, $B_7^{3-}$ and $B_9^{4-}$ ions for CS dp 12 are shown in Fig. 3. The data showed that the $MS^3$ $B_3$, and $B_5$ product ion abundances to be diagnostic for CSA, although the abundance differences were moderate. Ions produced from loss of $SO_3$ from the $MS^3$ precursor ion were observed to be diagnostic for CSC. The only ion in the $MS^3$ profiles of $B_n\rightarrow$ ions diagnostic for CSB was $B_4$, although the low abundance made this ion of limited practical value.

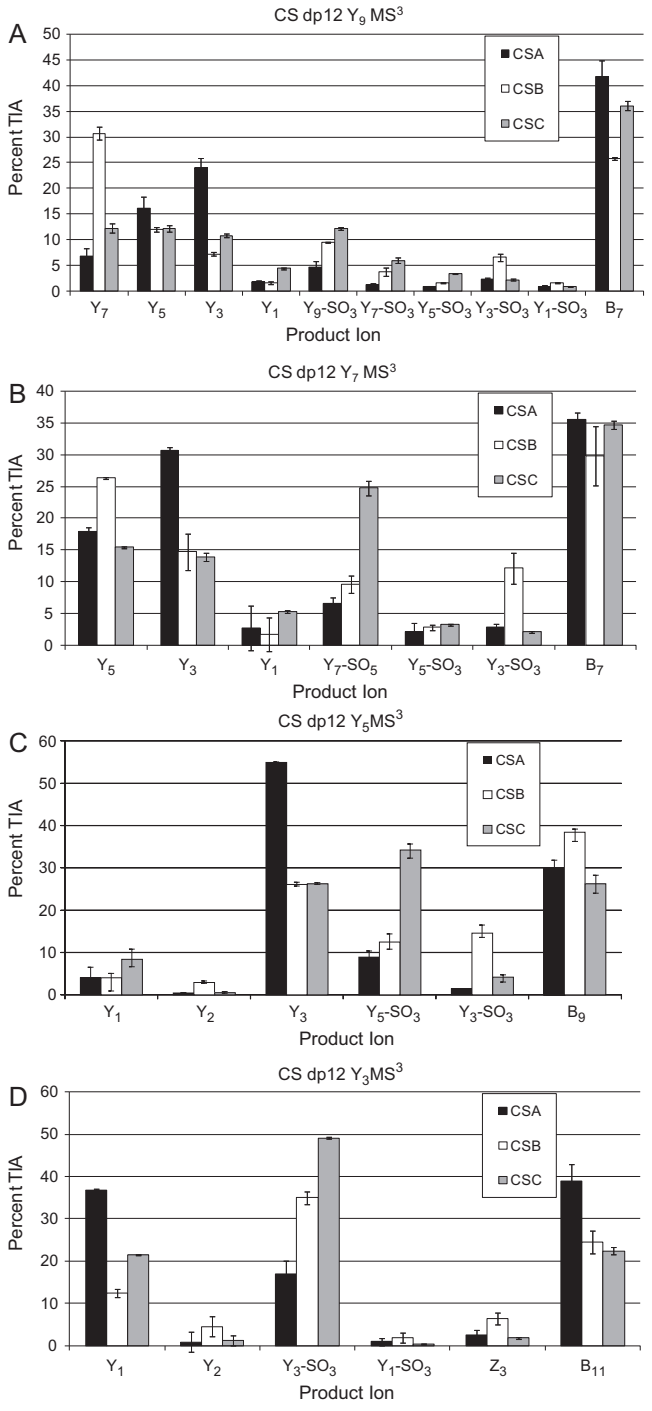


**Fig. 2.** (A) Comparison of Y-type $MS^3$ product ion abundances for Δdp12 $[M-6H]^{6-}$ (A) $Y_9^{5-}\rightarrow$, (B) $Y_7^{4-}\rightarrow$, (C) $Y_5^{3-}\rightarrow$, and (D) $Y_3^{2-}\rightarrow$.

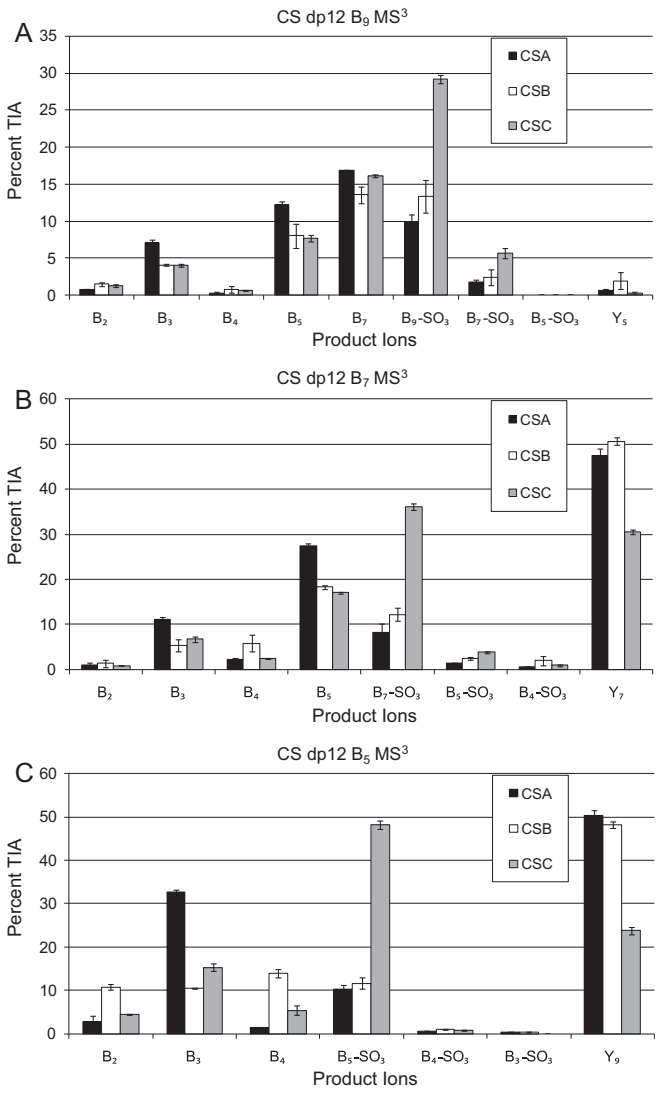


**Fig. 3.** Comparison of B-type $MS^3$ product ion abundances for Δdp12 $[M-6H]^{6-}$ (A) $B_9^{4-}\rightarrow$, (B) $B_7^{3-}\rightarrow$, and (C) $B_5^{2-}\rightarrow$.

The product ion patterns diagrammed in Fig. 4 were observed for CS/DS Δ-oligosaccharides dp10 (Supplementary data Figs. 1 and 2), dp 12 (Figs. 1–3) and dp14 (Supplementary Figs. 3 and 4). A summary of the results for $MS^3$ of Y-type product ions is shown in Fig. 4A. The diagnostic ion for CSA is $Y_3$ for all CS oligomers tested, indicating that it is possible to produce information on the presence of CSA-like repeats corresponding to residues #3 and #4 in Fig. 4A. For CSB, information is produced at the non-reducing end of the precursor ion oligosaccharide. This pattern is consistent with the conclusion that the abundances of such ions are diagnostic for the epimerization of the adjacent HexA residue, #10 in the $MS^2$ stage, #8 in the $MS^3$ $Y_9\rightarrow$ stage and #6 in the $MS^3$ $Y_7$ stage. In addition, the $Y_3$–$SO_3$ ion was diagnostic for the epimerization state of #4 in the $MS^3$ $Y_7\rightarrow$ and $Y_5\rightarrow$ stages. The abundance of the $Y_2$ ion appeared to be diagnostic for the epimerization state of #2 in the $MS^3$ $Y_5\rightarrow$ and $Y_3\rightarrow$ stages. Thus, there is evidence that $MS^3$ dissociation of $Y_n$ ions produces information on the epimerization state of every uronic acid residue in the oligosaccharides. For CSC, diagnostic ions result from loss of $SO_3$ from the precursor selected for each $MS^3$ stage. It is not clear which sulfate group on the chains dissociates preferentially to produce this pattern. In summary, there is clear value in performing $MS^3$ stages on $Y_n$ ions, in particularly for determining patterns of CSB-like repeats.

As shown in Fig. 4B, $MS^3$ dissociation of $B_n$ ions produced $B_3$ and $B_5$ ions the abundances of which were diagnostic for CSA repeats. It is likely that these ions were diagnostic for the position of sulfation at positions #9 and #7, respectively. Losses of $SO_3$ from the $MS^3$ precursor ions were again diagnostic for CSC, but it was not possible to surmise the position of the sulfate group lost. In summary, the amount of structural information produced on the presence of CSA-like repeats in the oligosaccharides was increased by $MS^3$ of $B_n$ ions.

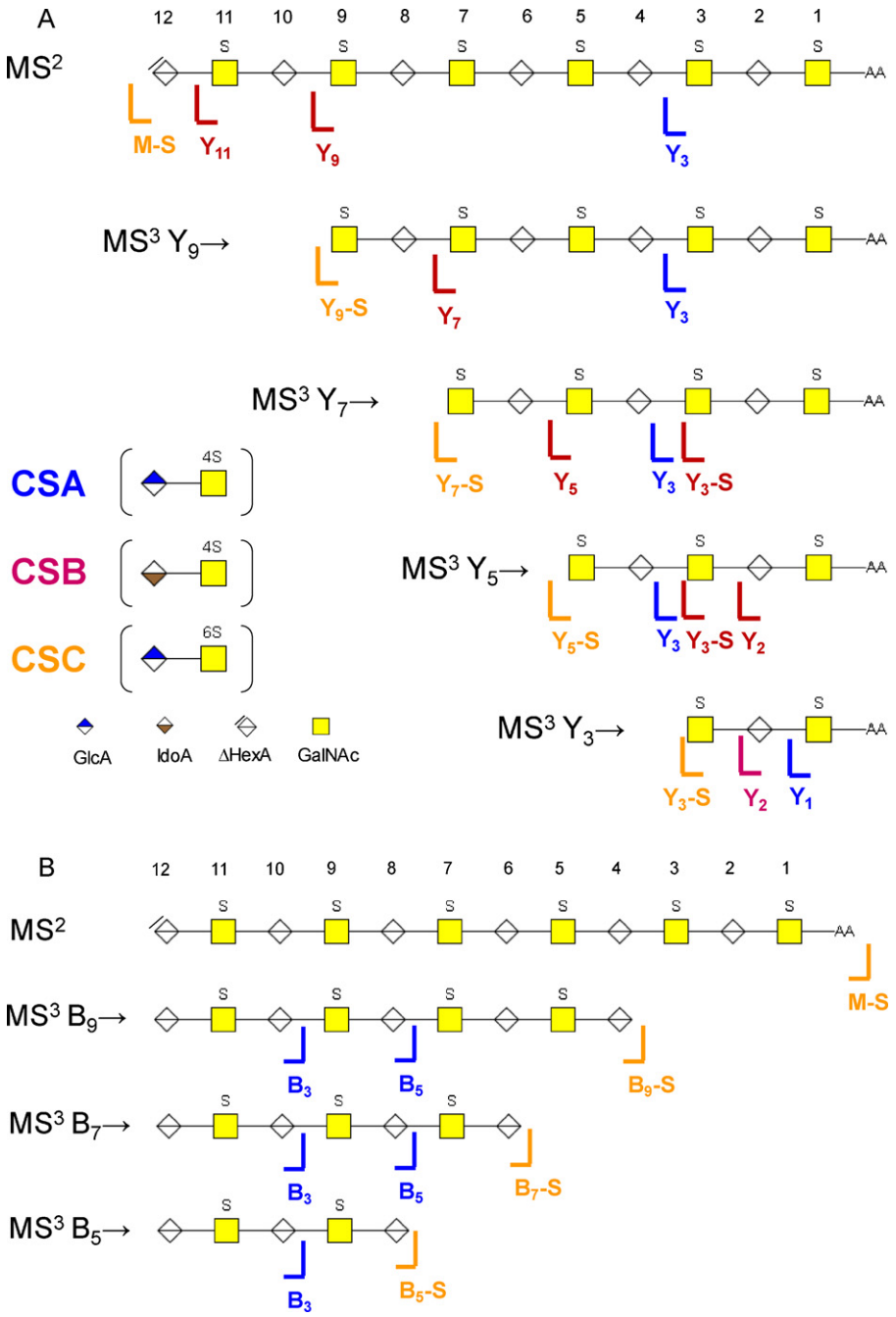


**Fig. 4.** Summary of diagnostic product ions for (A) Y-type $MS^3$ product ions and (B) B-type $MS^3$ product ions. For this figure, $SO_3$ loss is abbreviated as S.

## 4. Conclusions

The importance of determining patterns of CSB-like repeats in CS/DS is highlighted by the recent findings that DS epimerase enzyme activity levels are elevated in cancerous tissue [7,44,45]. In the presence of high levels of DS epimerase 1 and dermatan 4-*O*-sulfotransferase 1, extended repeats of IdoA-GalNAc4S result in the CS/DS chains [7–9]. In the presence of lower levels of these enzymes, interspersed repeats of GlcA–GalNAc and IdoA–GalNAc result. The presence of interspersed versus extended patterns of CSB-like repeats has considerable biological relevance, due to the strong potential influence on protein binding interactions. Thus, there is clear need for effective methods for analysis of such patterns.

In considering the tandem mass spectrometric options, CAD-based methods are compatible with tandem mass spectrometric glycomics analysis. The mass spectral acquisition may also be automated to facilitate generation of robust and reproducible data. The present work showed that the amount of information produced on CS/DS oligosaccharide fine structure was dramatically improved by the addition of an $MS^3$ stage. The results were consistent with $MS^3$ ion abundances indicative of the epimerization or sulfation states of individual residues.

In order to use tandem MS for CS/DS oligosaccharides, it is recommended that commercial CSA, CSB and CSC oligosaccharides be prepared and analyzed as standards on the same day as oligosaccharides from unknown samples. In addition, it is recommended that a mixture of all three standards be analyzed on each day of instrument operation as a test of instrument performance over time. It is recommended that all samples (standards and unknowns) be analyzed in random order. In this manner, the investigator will be assured

that all instrumental conditions are set to achieve reproducible results.

Activated electron dissociation, particularly electron detachment dissociation, results in the formation of cross-ring cleavage pathways that directly differentiate uronic acid epimers [46–50]. The challenge to this work is to increase the dissociation efficiency to enable glycomics studies of CS/DS expression patterns. It is envisioned that practical analysis of CS/DS fine structure will entail a combination of approaches. Multistage MS using CAD will be useful for comparative studies of fine structure of unknown CS/DS samples relative to those of CSA, CSB and CSC reference samples, as described in the present work. Activated electron dissociation will be useful for providing absolute structural information on selected samples.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.10.017.

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