A tandem mass spectrometric approach to determination of chondroitin/dermatan sulfate oligosaccharide glycoforms

May Joy C. Miller², Catherine E. Costello², Anders Malmström³, and Joseph Zaia¹,²

¹Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, R-806, Boston, MA 02118; and ²Department of Cell and Molecular Biology, Lund University, BMC B11, S-221 84, Lund, Sweden

Received on October 27, 2005; revised on January 26, 2006; accepted on February 15, 2006

Dermatan sulfate (DS) chains are variants of chondroitin sulfate (CS) that are expressed in mammalian extracellular matrices and are particularly prevalent in skin. DS has been implicated in varied biological processes including wound repair, infection, cardiovascular disease, tumorigenesis, and fibrosis. The biological activities of DS have been attributed to its high content of IdoA(α1–3)GalNAc4S(β1–4) disaccharide units. Mature CS/DS chains consist of blocks with high and low GlcA/IdoA ratios, and sulfation may occur at the 4- and/or 6-position of GalNAc and 2-position of IdoA. Traditional methods for the analysis of CS/DS chains involve differential digestion with specific chondroitinases followed by steps of chromatographic isolation of the products and disaccharide analysis on the individual fraction. This work reports the use of tandem mass spectrometry to determine the patterns of sulfation and epimerization of CS/DS oligosaccharides in a single step. The approach is first validated and then applied to a series of skin DS samples and to decorins from three different tissues. DS samples ranged from 74 to 83% CSB-like repeats for those samples from three different tissues. DS samples ranged from 74 to 83% CSB-like repeats for those samples from articular cartilage to 75% for those from sclera. These values agree with known levels of glucuronyl C5-epimerase in these tissues.

Key words: chondroitin sulfate/decorin/dermatan sulfate/glycosaminoglycan/mass spectrometry

Introduction

Glycosaminoglycan (GAG) glycoforms are of particular interest because of their varied biological properties. Proteoglycans (PGs)/GAGs are predominately found on cellular membranes and in the extracellular matrix of mammalian tissues. They interact with numerous proteins, growth factors, and cell surface receptors and thus serve a variety of important roles in cell proliferation, differentiation, adhesion, migration, and recruitment of neutrophils (Hocking et al., 1998; Bernfield et al., 1999; Trowbridge and Gallo, 2002). GAGs are linear, sulfated polysaccharides that are attached by a linker to a serine residue of the core protein of a proteoglycan; the structures of GAGs bound to a given core protein are known to vary depending on the environment (tissue, developmental disease, or cytokine state) (Faissner et al., 1994; Kitagawa et al., 1997; Clement et al., 1998; Tiedemann et al., 2005). The dynamic nature and biological importance of GAG glycoforms drive the need for a rapid and sensitive means of determination of their sulfation and epimerization patterns. Dermatan sulfate (DS) is a chondroitin sulfate (CS) variant that exhibits anticoagulant activity in both animals and humans and has been incorporated into antithrombic therapies (Maimone and Tolleson, 1990; Cofrancesco et al., 1994; Di Carlo et al., 1999). In contrast to other antithrombic GAGs, DS is effective on both free and fibrin-bound thrombin (Bendayan et al., 1994; Liaw et al., 2001) and has low hemorrhagic complications (Cofrancesco et al., 1994; Di Carlo et al., 1999). Scleral proteoglycans interact via their DS chains, demonstrating the multifaceted potentials of GAGs in promoting multifaceted binding with other macromolecules (Fransson et al., 1982).

DS interactions with fibroblast growth factors FGF-2 (Penc et al., 1998) and FGF-7 (Trowbridge et al., 2002) have been shown with respect to cellular proliferation and wound repair, respectively. Interactions with hepatic growth factor/scatter factor establish that DS plays roles in tumorigenesis and metastasis (Lyon et al., 2002). Oversulfated DS chains play roles in promoting neurite outgrowth in embryonic brain (Hikino et al., 2003). Decorin, a DS-containing proteoglycan predominant in skin, acts as an inflammation regulator through interaction with C1q (Krumdieck et al., 1992) and transforming growth factor-beta (Yamaguchi et al., 1990) and may play an important role in the inflammatory process of Lyme disease (Guo et al., 1995).

Although the IdoA(α1–3)GalNAc4S(β1–4) disaccharide unit is the most highly expressed in mammalian DS, analyses of several preparations reveal heterogeneity within the polysaccharide chain (Poblacion and Michelacci, 1986). This is due to the biosynthetic modifying reactions that generate DS from nascent chondroitin chains of composition [GlcA(β1–3)GalNAc(β1–4)]ₙ after the initial polymerization; epimerization of GlcA to IdoA occurs before sulfation (Malmstrom and Aberg, 1982; Malmstrom, 1984). Mammalian DS consists of domains of high percentage of IdoA(α1–3)GalNAc4S(β1–4) and those with high GlcA(β1–3)GalNAc4S(β1–4). Variable quantities of GlcA(β1–3)GalNAc6S(β1–4) are also present (Poblacion and Michelacci, 1986). DS chains from the body of an ascidian Ascidia nigra have a repeat unit of...
IdoA2S(α1–3)GalNAc6S(β1–4) (Pavao et al., 1995) and lack the anticoagulant activity of 4-sulfated DS (Vicente et al., 2001). Monoclonal antibodies generated against Ascidia DS have demonstrated the presence of IdoA2S(α1–3)GalNAc6S(β1–4) repeats in mouse brain (Bao et al., 2005).

Because the ability of GAGs to bind protein receptors largely depends on their structure, the number of disaccharide repeats (size), and sulfation pattern, analytical techniques to determine GAG fine structure are highly valuable. Total uronic acid content for GAG preparations may be determined using the carbazole reaction (Meyer and Rapport, 1950; Rapport et al., 1951; Bitter and Muir, 1962). Chemical measurement of IdoA and GlcA content entails reaction of uronate carboxyl groups with a carbodi-imide, followed by reduction and acid hydrolysis. The monosaccharide products, differing for GlcA and IdoA, are then per-O-benzoylated, separated, and quantified using chromatography (Karamanos et al., 1988). The IdoA content of CS/DS chains may be determined by differential chondroitinase digestion followed by chromatographic separation (Coster et al., 1975). The differential chondroitinase digestion approach has been used in conjunction with subsequent size-exclusion chromatography (Malmstrom and Fransson, 1975), reversed-phase ion pairing high-performance liquid chromatography (RPIP-HPLC) (Karamanos et al., 1988), or slab gel electrophoresis (Fransson et al., 1990; Cheng et al., 1994). Oligosaccharides products of chondroitinase reactions isolated by RPIP-HPLC or other techniques may be subjected to additional chemical or enzymatic degradative steps (Theocharis et al., 2001) followed by monosaccharide or disaccharide analysis. Highly sensitive chromatographic (Toyoda et al., 1999, 2000), capillary electrophoretic (Karamanos et al., 1995; Mitropoulou et al., 2001; Lamari et al., 2002), fluorescence-assisted carbohydrate electrophoresis (Calabro, Benavides, et al., 2000; Calabro, Hascall, et al., 2000), and mass spectrometric (Desaire and Leary, 2000) methods are available for CS/DS disaccharide analysis. To determine uronic acid epimers from disaccharide analysis, one may use a chemical degradation method. The intact GAG chains are deacetylated by hydrazinolysis and subjected to deaminative cleavage using nitrous acid at pH 4. The resulting disaccharides, each containing dehydromannose at the reducing end and an unmodified uronic acid residue, may be reduced with sodium borotritide or tagged with a chromophore or fluorophore before chromatographic or electrophoretic separation (Guo and Conrad, 1989).

Measurement of the mass of a CS/DS oligosaccharide determines the composition with respect to the number of uronic acid, N-acetylatedhexosamine, and sulfate units (Carr and Reinhold, 1984; Takagaki et al., 1992; Sugahara et al., 1994). Tandem mass spectrometry (MS) determines the number of sulfate groups per disaccharide unit and provides a measure of IdoA(α1–3)GalNAc4S(β1–4) content in the target oligosaccharide (Zaia et al., 2001, 2003) based on product ion abundances.

We have used a liquid chromatography (LC)–MS platform for determining CS/DS glycoform distribution (Hitchcock et al., 2006). The present work describes the principles behind this approach and extends the analysis to longer oligosaccharides. A series of dermal DS samples isolated under different conditions are analyzed, in addition to a series of decorins from different tissues. The CS/DS chains are partially depolymerized using chondroitinase ABC and the resultant oligosaccharides analyzed using tandem MS. This approach is used to compare the expression of oligosaccharide glycoforms among the different samples. Such comparisons would be significantly more laborious using traditional techniques. These experiments demonstrate the principle and validity of a new method for glycoform analysis of CS/DS chains, one that will be useful in the emerging field of glycomics.

**Results**

**Partial depolymerization of CS/DS samples**

Oligosaccharides were generated from CS/DS samples by stopping chondroitinase ABC digestions at a time point found to produce 232 nm absorbance of 30% of its maximum value (see Materials and methods for details). The oligosaccharides were fractionated using high-performance size-exclusion chromatography with 232 nm detection. Oligosaccharide length will be given as degree of polymerization (dp) and the number of monosaccharide units. Peak area percentages for dp2, dp4, and dp6 for each of the samples are summarized in Table I. The size-exclusion chromatograms displayed no defined peaks greater than dp6, and the 232-nm baseline was elevated before the elution of this oligomer. Thus, the reported values include the area of this baseline elevation, listed as “>dp6.”

**MS of CS/DS oligosaccharides**

The compositions of oligosaccharides with a 4,5-unsaturated uronic acid residue at the nonreducing end will be given preceded with the Δ symbol. Figure 1 is a representative electrospray (ESI) mass spectrum of size-exclusion chromatography fractions corresponding to dp4 (A) and dp6 (B). The ion at m/z 458 corresponds to [M-2H]− for Δdp4 (A).

| Table I. Relative chromatographic peak areas for CS/DS samples partially digested with chondroitinase ABC |
|-------------------|-------------|-------------|-------------|-------------|
|                  | dp2         | dp4         | dp6         | >dp6        |
| CSA               | 48.35       | 9.16        | 9.98        | 32.5        |
| CSB               | 38.92       | 18.27       | 18.53       | 24.3        |
| CSC               | 55.30       | 19.19       | 9.48        | 16.0        |
| CS6               | 58.02       | 21.38       | 9.13        | 11.5        |
| DS18              | 35.80       | 14.48       | 16.59       | 33.1        |
| DS36              | 37.93       | 10.91       | 11.53       | 39.6        |
| DS50              | 43.13       | 15.24       | 12.71       | 28.9        |
| ACD               | 39.80       | 15.72       | —           | 44.5        |
| CD                | 55.16       | 29.19       | —           | 15.7        |
| SD                | 42.69       | 23.03       | —           | 34.3        |

Chondroitinase ABC digestions were stopped at 30% completion, and separated using high-performance size-exclusion chromatography (SEC), as described in the Materials and methods section. The relative peak areas for Δ-unsaturated dp2, dp4, and dp6 oligosaccharides are given.
Because this ion is isobaric for \( \Delta \)-unsaturated CS/DS oligosaccharides of composition \( \Delta \)(HexA)\(_n\)(GalNAc)\(_n\)(SO\(_3\))\(_n\), careful isolation of the individual oligomers was performed using high-performance size-exclusion chromatography. The isotope pattern of the ion at \( m/z \) 458 (Figure 1A, inset) shows peaks separated by 0.50 \( \mu \)m that signify a charge state of 2\( ^- \), the neutral mass of which (918.10 Da; Table II) matches a composition of \( \Delta \)(HexA)\(_2\)(GalNAc)\(_2\)(SO\(_3\))\(_2\). The inset in Figure 1B shows peaks separated by 0.33 \( \mu \)m, signifying a charge state of 3\( ^- \), the neutral mass of which (1377.24 Da; Table II) is consistent with a composition of \( \Delta \)(HexA)\(_3\)(GalNAc)\(_3\)(SO\(_3\))\(_3\). The absence of an ion at \( m/z \) 458.38 from Figure 1A shows that the dp4 fraction is not contaminated with dp6. The absence of an ion at \( m/z \) 458.55 from Figure 1B shows that there is no dp4 in the dp6 fraction. These negative electrospray ionization patterns produced from CS/DS oligosaccharides are consistent with the previous reports (Desaire and Leary, 2000; Zaia and Costello, 2001; Zaia et al., 2001, 2003; Chai et al., 2002; Zamfir et al., 2003).

Assignments for all ions observed in the mass spectra of CS/DS dp4 and dp6 fractions are listed in Table II. The ions at \( m/z \) 498 in Figure 1A correspond to \( \Delta \)(HexA)\(_2\)(HexNAc)\(_2\)(SO\(_3\))\(_3\) and \( m/z \) 484 in Figure 1B to \( \Delta \)(HexA)\(_3\)(HexNAc)\(_3\)(SO\(_3\))\(_4\) (Table II). These CS/DS oligosaccharides contain more than one sulfate group per disaccharide repeat and are termed oversulfated. In addition, an \([\text{M}-2\text{H}]^2^-\) ion corresponding to a saturated oligosaccharide of composition of \( \Delta \)(HexA)\(_3\)(GalNAc)\(_3\)(SO\(_3\))\(_3\) was detected at \( m/z \) 696.5 in Figure 1B. This composition matches that expected for an oligosaccharide derived from the nonreducing terminus of the intact CS/DS chain, by virtue of the fact that the neutral mass is consistent with a saturated structure. The ion at \( m/z \) 464 in Figure 1B corresponds to \([\text{M}-3\text{H}]^3^-\) for the same composition. The ion at \( m/z \) 899 in Figure 1B corresponds to the composition \( \Delta \)(HexA)\(_2\)(HexNAc)\(_2\)(SO\(_3\))\(_2\)-H\(_2\)O (Table II). Gentle ionization conditions were used, and no losses of SO\(_3\) or other signs of in-source fragmentation were observed in Figure 1. It is therefore likely that the species giving rise to \( m/z \) 899 exists in solution and is not an artifact of the MS measurement. Although the oligosaccharide composition may be determined from the molecular weight information obtained from the ESI mass spectra,
detailed structural information such as sulfation positions and epimerization state is not produced.

**Tandem MS of CS/DS Δdp4 glycoforms**

To determine positions of sulfation and epimerization, we compared abundances of the fragment ions formed from DS oligosaccharides with those produced from commercial CS/DS standards with known epimerization and GalNAc sulfate positions. It was established previously that tandem MS product ion abundances of CS oligosaccharides reflect sulfation position at GalNAc residues (Zaia *et al.*, 2001; McClellan *et al.*, 2002) and epimerization of HexA residues (Zaia *et al.*, 2003). Thus, three commercial standards were used, each of which is ~90% pure. These were CSA (GlcA(β1–3)GalNAc4S(β1–4))ₙ, CSB (IdoA(α1–3)GalNAc4S(β1–4))ₙ, and CSC (GlcA(β1–3)GalNAc6S(β1–4))ₙ. Furthermore, to focus the analysis on a single uronic acid that is unaltered by lyase digestion, we first investigated Δdp4 series.

Figure 2 shows the tandem mass spectra of Δdp4 oligosaccharides generated from CS standard preparations, CSA (Figure 2A), CSB (Figure 2B), and CSC (Figure 2C). The doubly charged precursor ion is typically present as the most abundant charge state, and product ions resulting from glycosidic bond cleavages are abundant, whereas those from losses of SO₃ are in low abundance. Although the three CS/DS Δdp4 isomers dissociate to form ions with identical m/z values, differences in abundances of the fragment ions characterize each other. Specifically, there are six signature ions, the abundances of which differentiate the three isomers. The Δdp4 oligosaccharides derived from CSA are characterized by high abundance of Y₁¹⁻ (m/z 300) and B₃⁻¹ (m/z 616) ions relative to those observed for the other two isomers (Figure 2A). CSB Δdp4 oligosaccharides are characterized by the high abundance of 0₂X₂⁻² (m/z 400) and Y₃⁻² (m/z 379) ions relative to the other two isomers. Abundant [M-SO₃]⁻² (m/z 418) and C₃⁻² (m/z 316) ions characterize CSC Δdp4 oligosaccharides. These fragment ions are summarized in Table III. In low-energy collision-induced dissociation (CID) experiments, product ion abundances reflect the lability of the covalent bonds that are cleaved during the tandem MS dissociation. Thus, the sulfation and epimerization positions influence the lability of certain bonds in the oligosaccharide ions that are reflected by the observed ion abundances.

The Y₁¹⁻ ion contains the sulfate group on the reducing terminal GalNAc residue of the precursor oligosaccharide ion. Abundance of this ion correlates directly with the presence of an oligosaccharide with a CSA-like GlcA(β1–3)GalNAc4S(β1–4) repeat. Similarly, the B₃⁻¹ ion, which contains the internal sulfated GalNAc residue, is produced by cleavage of the same glycosidic bond, and its abundance correlates with the presence of the same repeat. It is likely that the abundance of both the Y₁¹⁻ and the B₃⁻¹ ions reflect the position of sulfation on the reducing end GlcA(β1–3)GalNAc4S disaccharide. The [M-2H]⁻² ion of Δdp4 derived from CSC dissociates to produce abundant [M-SO₃]⁻² and C₃⁻² ions. The C₃⁻² ion contains the internal sulfated GalNAc residue, but the ion abundance of the glycosidic bond cleavage could be influenced by the sulfate...
position of the reducing end GlcA(β1–3)GalNAc6S disaccharide during the CID process. Loss of sulfate, [M-SO3]2–, is nonspecific as to the originating GlcA(β1–3)GalNAc6S disaccharide but is indicative of CSC-like structures. The 0,2X32– and Y32– ion abundances are enhanced when CSB-like IdoA(α1–3)GalNAc4S(β1–4) repeats are present.

Standards for Δdp4 and Δdp6 oligosaccharides containing mixed GalNAc 4- and 6-sulfation patterns are not available. However, for Δdp4, the abundances of the CSA signature ions, Y11– and B31–, reflect the position of sulfation of the reducing end GalNAc residue. It is therefore likely that all tetrasaccharides with GalNAc-4-sulfate on the reducing end will produce abundant CSA-like signature ions. CSC-like signature ions correspond to C32– and [M-SO3]2–. The abundance of the C32– ion varies according to the position of sulfation of the reducing terminal GalNAc residue and that of the [M-SO3]2– ion varies according to the presence of GalNAc-6-sulfate at either position in the tetramer. Thus, it would be expected that a mixed tetramer with GalNAc-6-sulfate on the reducing end would produce an abundant C32– ion, but that of the [M-SO3]2– ion would be intermediate.

**Signature ion patterns**

The use of signature ions to distinguish the three CS isomeric oligosaccharides can best be seen when their percent total ion abundances (Table IV) are plotted. The Y11– and B31– ions are most abundant, expressed as a percent of the sum of all product ion abundances (Figure 3A), for CSA Δdp4. The Y32– and 0,2X32– ions are most abundant for Δdp4 derived from CSB. The C32– and [M-SO3]2– ions are most abundant for Δdp4 derived from CSC. The data are replotted in Figure 3B to show how the ion abundances vary according to the type of CS from which the Δ-unsaturated dp4 was derived.

These ions will be used to characterize oligosaccharides derived from CS/DS samples of biological interest, as discussed in the Analysis of varied DS samples and decorin section.
Tandem MS of CS/DS Δdp6 glycoforms

As with the CS/DS Δdp4, signature ions characterize the Δdp6 oligosaccharides derived from standard CSA, CSB, and CSC. A summary of these ions is provided in Table III. The percent total ion abundances for the $Y_{1}^{+}$ ($m/z$ 300) and $B_{5}^{2-}$ ($m/z$ 537) ions are highest for CSA-derived Δdp6 oligosaccharides (Figure 4A). Although these ions are abundant for Δdp6 oligosaccharides derived from CSC, the abundance as a percent of the total is higher for Δdp6 derived from CSA because of the lower abundances of other product ions. The $0.2X_{5}^{3-}$ ($m/z$ 419) and $Y_{5}^{3-}$ ($m/z$ 405) ions have the highest percent total ion abundances for Δdp6 derived from CSB (Figure 4B), whereas [M-SO$_3$]$^{3-}$ ($m/z$ 431) and $C_{5}^{2-}$ ($m/z$ 546) ions are observed in greatest abundance for those from CSC (Figure 4C). Figure 5A shows how the abundances of the signature ions vary for different types of CS. The data are replotted in Figure 5B to show how the different forms of CS Δdp6 produce characteristic ion patterns.

Analysis of varied DS samples and decorin

To demonstrate the utility of this method, we analyzed several CS/DS samples, the Δdp4 signature ion profiles of which are shown in Figure 6. The CS6 sample was purified
From horse nasal septum and several DS preparations, namely DS18, DS36, and DS50 were purified from pigskin. From the signature ion patterns, it can easily be confirmed that Δdp4 oligosaccharides derived from DS18, DS36, and DS50 display signature ion patterns similar to those of standard CSB. This indicates that a high percentage of the Δdp4 oligosaccharides in each of these samples contain the IdoA(α1–3)GalNAc4S(β1–4) sequence. Those from CS6, by contrast, display signature ion patterns consistent with a high percentage of GlcA(β1–3)GalNAc6S(β1–4) repeats. Although the signature ion patterns of these samples qualitatively resemble those of the standards, the heights of the bars differ. For instance, samples DS18, DS36, and DS50 show an apparent increase in the height of CSA-like ions, suggesting varying GlcA(β1–3)GalNAc6S(β1–4) content in the DS samples. However, the CSC-like signature ions are observed consistently in low abundances among the Δdp4 oligosaccharides generated from the three decorins. Moreover, the CSB-like ions have the greatest contribution for ACD compared with almost insignificant contribution for CD and SD, suggesting that ACD contains the greatest GlcA(β1–3)GalNAc6S(β1–4) percentage among the three decorins. For the Δdp6 series, the signature ion profile of DS samples and decorin is similar to that of the Δdp4 series.

Quantitative analysis of each isomeric contribution

As was pointed out earlier, the signature ion abundances vary directly with the abundances of CSA-, CSB-, and CSC-like disaccharide repeats in each sample. Because the signature ion abundances for a given sample are a linear combination of the three repeat types, the percentages of CSA-, CSB-, and CSC-like ions required to produce the observed patterns can be calculated. Tandem MS ion abundances have been used to quanitate CS and heparin disaccharides (Desaire and Leary, 2000; Saad and Leary, 2003). Using similar principles applied to the Δdp4 oligosaccharide signature ion abundances, the following equations were applied to calculate the percent contribution of CSA, CSB, and CSC:

$$AY_1 + BY_1 + CY_1 = DY_1,$$

$$AY_3 + BY_1 + CY_3 = DY_3,$$

and

$$A(M-SO_3) + B(M-SO_3) + C(M-SO_3) = D(M-SO_3)$$

where $Y_1$, $Y_3$, and $M-SO_3$ are the signature ions for CSA-, CSB-, and CSC-like, respectively; $A$, $B$, and $C$ are the unknown contributions of CSA, CSB, and CSC isomers; and $D$ is the contribution from the unknown sample. The percent total ion abundance of each signature ion from the standards is substituted on the left side of the equations and

![Fig. 6. Signature ion profile for Δ-unsaturated dp4 oligosaccharides derived from CS and DS samples. CS6 is from horse nasal septum. DS18, DS36, and DS50 are from pigskin.](https://academic.oup.com/glycob/article-abstract/16/6/502/754628)

![Fig. 7. Signature ion profile for Δ-unsaturated dp4 oligosaccharides derived from decorin samples: articular cartilage decorin (ACD), human cervix decorin (CD), and bovine sclera decorin (SD).](https://academic.oup.com/glycob/article-abstract/16/6/502/754628)
those from the sample on the right side, producing three
equations with three unknowns. A summary of the calcu-
lated percent CSA, CSB, and CSC for DS18, DS36, and
DS50 is provided in Table V. The percent of CSA-like
sequences (GlcA(β1–3)GalNAc4S(1–4)) is observed
to increase, from DS18 to DS36 to DS50, and that for CSB
(1doA(α1–3)GalNAc4S(1–4)) to decrease. The absence
of CSC-like sequences (GlcA(β1–3)GalNAc6S(1–4)) is
observed.

Although the level of CSC-like sequences for the CSC
Δdp4 oligosaccharides in Table V is 58.18%, the level of
ΔHexAGalNAc6S disaccharides in Table VI is 78.88%. The
mass spectral method compares ion signatures for a given sample against those of the standard CS preparations. The method thus serves as a means of comparing a series of samples but does not provide absolute quantification of the levels CS repeats. The CSC sample contains significantly lower content of ΔHexAGalNAc6S (78.88%) than CS6 (93.97%). CS6 is therefore used as a mass spectral standard against which CSC is compared. It is important to emphasize that the trend is the same for the two sets of data. Measurement of relative changes of glycoform expression

Table V. Percent compositions for glycoforms of CS/DS Δdp4, calculated from tandem mass spectrometric signature ion abundances

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSA-like</td>
<td>CSB-like</td>
<td>CSC-like</td>
</tr>
<tr>
<td></td>
<td>GlcAGalNAc4S</td>
<td>IdoAGalNAc4S</td>
<td>GlcAGalNAc6S</td>
</tr>
<tr>
<td>CSC</td>
<td>41.81 ± 1.72</td>
<td>0 ± 0.33</td>
<td>58.18 ± 0.22</td>
</tr>
<tr>
<td>DS18</td>
<td>0.85 ± 1.42</td>
<td>99.15 ± 0.35</td>
<td>0 ± 0.14</td>
</tr>
<tr>
<td>DS36</td>
<td>11.21 ± 1.31</td>
<td>88.79 ± 0.41</td>
<td>0 ± 0.13</td>
</tr>
<tr>
<td>DS50</td>
<td>25.66 ± 1.22</td>
<td>74.35 ± 0.35</td>
<td>0 ± 0.15</td>
</tr>
<tr>
<td>ACD</td>
<td>50.94 ± 1.35</td>
<td>31.13 ± 0.23</td>
<td>17.92 ± 0.41</td>
</tr>
<tr>
<td>CD</td>
<td>35.92 ± 1.22</td>
<td>64.08 ± 0.28</td>
<td>0 ± 0.47</td>
</tr>
<tr>
<td>SD</td>
<td>21.56 ± 1.73</td>
<td>74.51 ± 0.47</td>
<td>3.92 ± 0.36</td>
</tr>
</tbody>
</table>

CS6 was used as the CSC-like standard.

Table VI. CE-LIF percentage compositions of ΔHexAGalNAc4S, ΔHexAGalNAc6S, ΔHexA2SGalNAc4S, and ΔHexA2SGalNAc6S after an exhaustive
digestion of the whole DS polymer chain

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔHexAGalNAc4S</td>
<td>94.65 ± 0.29</td>
<td>5.34 ± 0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexAGalNAc6S</td>
<td>91.10 ± 0.39</td>
<td>7.10 ± 0.03</td>
<td>1.79 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc4S</td>
<td>17.45 ± 0.17</td>
<td>78.88 ± 0.66</td>
<td>0</td>
<td>3.66 ± 0.02</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>6.03 ± 0.07</td>
<td>93.97 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>95.51 ± 1.00</td>
<td>2.65 ± 0.13</td>
<td>1.84 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>94.39 ± 1.21</td>
<td>1.98 ± 0.09</td>
<td>3.69 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>84.16 ± 2.03</td>
<td>13.69 ± 0.27</td>
<td>2.14 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>60.77 ± 0.25</td>
<td>39.23 ± 0.16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>86.75 ± 0.08</td>
<td>9.64 ± 0.01</td>
<td>3.61 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>ΔHexA2SGalNAc6S</td>
<td>79.39 ± 0.01</td>
<td>15.59 ± 0.01</td>
<td>5.01 ± 0.06</td>
<td>0</td>
</tr>
</tbody>
</table>

Percentage Δ-disaccharide compositions were calculated using CE fluorescence peak areas normalized to the area of the internal standard.

Disaccharide analysis

Disaccharide analysis of CS/DS standards and unknown samples was achieved by exhaustive digestion using chondroitinase ABC, subsequent fluorescent derivatization using 2-aminoacridone (AMAC) and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection to analyze the disaccharides (Militospoulou et al., 2002). Migration times of Δ-disaccharides generated from standard CS preparations CSA, CSB, CSC, and those from DS samples and decorin were verified against those of AMAC-derivatized commercial disaccharide standards. An AMAC-derivatized trisulfated disaccharide derived from heparin (HSIS) was used as an internal standard for each run, as it has a unique migration time compared with the CS/DS samples analyzed. Tables VI and VII list the Δ-disaccharide composition of the whole polymer chain and the dp6 fraction, respectively. Percentage Δ-disaccharide compositions were calculated using the CE fluorescence peak areas, normalized to the area of the internal standard.

Partial chondroitinase ABC digestion was used to produce oligosaccharide mixtures for subsequent mass spectral analysis. Although the distribution of oligosaccharides was dependent to some extent on the sample, the dp4 and dp6 fractions are representative of the glycoform distribution of the entire CS/DS chain. This can be seen from the good agreement in disaccharide analysis values obtained from isolated dp6 fractions versus those of the entire CS/DS chain from which they were derived (Tables VI and VII).

For the GlcA-containing standards, CSA is shown to contain 94.65% ΔHexA-GalNAc4S and 5.34% ΔHexA-GalNAc6S, whereas CSC is a mixture of 17.45% ΔHexA-GalNAc4S, 78.88% ΔHexA-GalNAc6S, and 3.66% of the 2-sulfated disaccharide, ΔHexA2SGalNAc6S (Table VI). The CS6 sample contains 93.97% ΔHexA-GalNAc6S, significantly greater than the value observed for the commercial CSC, a trend that was observed in the signature ion plots for the Δdp4 and Δdp6 oligosaccharides (Figure 6). Thus, between a given sample and the CS standards is the most appropriate use of the mass spectral method.
the higher abundance of GlcA(β1–3)GalNAc6S(β1–4) in CS6 compared with that in the CSC standard from the MS ion signature data is supported by the CE-based Δ-disaccharide analyses of the abundances of the ΔHexAGalNAc6S disaccharides. Likewise, lower abundances of GlcA(β1–3)GalNAc4S(β1–4) signature ions in CS6 relative to commercial CSC is reflected by the lower percentage of ΔHexAGalNAc4S in the CE-LIF data.

### Discussion

The use of MS ion signatures to quantify CSA-, CSB-, and CSC-like repeats in CS/DS oligosaccharides allows the comparison of sulfation pattern and epimerization state among samples derived from different tissue contexts or purification schemes. Because the mass spectral ion signatures of each are measured against the same standard CS/DS preparations, it is possible to compare glycoform distributions among a series of GAG or PG samples derived from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).

Dermal DS samples show a consistent decrease in total CSA, CSB, and CSC from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).

Dermal DS samples show a consistent decrease in total CSA, CSB, and CSC from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).

Dermal DS samples show a consistent decrease in total CSA, CSB, and CSC from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).

Dermal DS samples show a consistent decrease in total CSA, CSB, and CSC from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).

Dermal DS samples show a consistent decrease in total CSA, CSB, and CSC from different environments. The method entails digestion with a single nonspecific enzyme, a single stage of chromatography followed by mass spectral analysis. We have also used this approach in an LC–tandem MS platform (Hitchcock et al., in press). This platform is limited to the analysis of tetrasaccharides, and the present work extends the analysis to hexasaccharides.

Decorins with GAG chains containing high DS content have been shown to be expressed in articular cartilage (Rosenberg et al., 1985; Sampaio et al., 1988; Cheng et al., 1994). The activity of β-glucuronol C5-epimerase is high in fibroblasts, intermediate in articular cartilage, and low in nasal cartilage (Tiedemann et al., 2001); the results of this work (Table V), showing that levels of CSB-like repeats are generally higher for the dermal DS than for articular cartilage decorins, are consistent with this trend in enzyme activities. The results for all decorin samples tested indicate significant abundances of CSB-like (IdoA(α1–3)GalNAc4S(β1–4)) repeats from 31.1 to 74.5% (Table V).
LC/MS platform for glycomics profiling so as to streamline the workflow. Thus, partially depolymerized CS/DS oligosaccharides will be separated using high-performance size-exclusion chromatography with online tandem MS detection. In principle, the concept demonstrated for CS/DS oligosaccharides should be applicable to any class of glycans. The nature of the separation system will change depending on the chemistry of the glycan class in question, and the structural detail produced will depend on the nature of the standards against which unknown glycoconjugate samples are compared.

Materials and methods

CS type A (GlcA, GalNAc4S), CSB (IdoA, GalNAc4S), CSC (GlcA, GalNAc6S), and chondroitinase ABC were obtained from Seikagaku America/Associates of Cape Cod (Falmouth, MA). Purified CS Δ-disaccharide standards were purchased from V-Labs (Covington, LA). Bovine articular cartilage decorin was purchased from Sigma Chemical (St. Louis, MO). DS samples were prepared by precipitation from calcium acetate/ acetic acid solution with ethanol. AMAC was purchased from Fluka Chemical (Milwaukee, WI), sodium cyanoborohydride was from Aldrich Chemical (Milwaukee, WI), and cellulose spin columns were purchased from Harvard Apparatus (Holliston, MA).

Partial digestion of chondroitin and dermatan sulfate

The method for digestion was described previously (Zaia et al., 2003). Briefly, 100 μg of CS/DS was mixed with 10 μL of water, 2 μL of 1 M Tris-HCl buffer (pH 7.4), 1 μL of 1 M NH₄OAc, and 4 μL of chondroitinase ABC and was digested at 37°C. After 8 min (30% digestion), the digestion was terminated by boiling for 2 min. Oligosaccharides were fractionated using a Superdex Peptide 3.2/30 column (Amersham Biosciences, Piscataway, NJ), which was equilibrated in 10% acetonitrile, 0.1 M ammonium acetate at 100 μL/min, with detection at 232 nm.

Electrospray MS

Mass spectra were acquired in the negative mode using an Applied Biosystems/MDS-Sciex (Toronto, Canada) API QSTAR Pulsar i quadrupole orthogonal time-of-flight mass spectrometer fitted with a nanospray ion source. Samples were dissolved in 10% isopropanol and diluted with 10% isopropanol and 0.1% NH₄OH to achieve a 1–5 pmol/μL solution. Aliquots of 5 μL were sprayed into the mass spectrometer using 1 μm orifice nanospray tips pulled in-house using a capillary puller (Sutter Instrument P 80/PC micropipette puller; San Rafael, CA). An ionization potential of ~1150 V produced a steady ion signal, and all spectra were calibrated externally. The collision energies were set so that the precursor ion remained the most abundant. For CID, these were best obtained at collision energy of ~18 and ~16 V for the Δdp4 and Δdp6 series, respectively.

Exhaustive digestion of CS/DS

To 100 μg of CS/DS was mixed 10 μL of water, 2 μL of 1 M Tris- HCl buffer (pH 7.4), 1 μL of 1 M NH₄OAc, and 20 mU of chondroitinase ABC, and digestion was allowed to proceed at 37°C. After 12 h, an additional 20 mU of chondroitinase ABC was added. The mixture was allowed to digest for 24 h after which the reaction was stopped by boiling the mixture for 2 min. The disaccharide collected after size-exclusion chromatography was derivatized with AMAC.

AMAC derivatization

Derivatization with AMAC was performed following the procedure described by Militsopoulou (Militsopoulou et al., 2002). To the lyophilized disaccharide CS/DS (at least 100 pmol) was added 5 μL of 0.1 M AMAC solution in acetic acid : dimethyl sulfoxide (DMSO) (3:17, v/v) and 5 μL of a freshly prepared 1 M NaBH₃CN solution in water. The mixture was vortexed for 3 min and was incubated at 45°C for 4 h after which 50 μL of DMSO was added. Excess reagent was removed using cellulose spin columns.

CE analysis

The AMAC-derivatized DS samples were analyzed using a Beckman Coulter (Fullerton, CA) P/ACE MDQ capillary electrophoresis instrument. The uncoated, fused silica capillary tube (75 μm ID, 60 cm total length) was regenerated with 0.1 M HCl, 0.1 M NaOH, and HPLC grade water before each run, and analyses were performed using 50 mM NaH₂PO₄ buffer, pH 3.5, at 30 kV at reverse polarity and detection using the AMAC chromophore at 254 nm. Distinct separation of the CS/DS isomers was obtained in the reverse polarity, and reproducibility was also obtained when fresh buffer solution was used for each run. An HSIS was used as an internal standard for each run, as it has a unique migration time compared with the CS/DS samples.

Acknowledgments

This work was supported by NIH grants P41RR10888 and R01HL74197.

Conflict of interest statement

None declared.

Abbreviations

ACD, bovine articular cartilage decorin; AMAC, 2-aminoacridone; CD, human cervix decorin; CE, capillary electrophoresis; CID, collision-induced dissociation; CS, chondroitin sulfate; CSA, chondroitin sulfate type A; CSB, chondroitin sulfate type B; CSC, chondroitin sulfate type C; dp, degree of polymerization; DS, dermatan sulfate; ESI, electrospray; FGF, fibroblastic growth factor; GAG, glycosaminoglycan; HSIS, AMAC-derivatized tri sulfated disaccharide derived from heparin; LC, liquid chromatography; LIF, laser-induced fluorescence; MS, mass spectrometry; PG, proteoglycan; RPIP-HPLC, reversed-phase ion pairing high-performance liquid chromatography; SD, bovine sclera decorin; Δ, 4,5-unsaturated uronic acid.


