Analysis of heparan sulfate oligosaccharides by nano-electrospray ionization mass spectrometry

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A highly sensitive method to identify and quantify heparan sulfate (HS) oligosaccharides by using nano-electrospray ionization mass spectrometry (nESI-MS) is described. The new approach allows us to detect approximately 50 nM of a chemically synthesized pentasaccharide with a structure of GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6SOMe (3-OH pentasaccharide). Typically, solutions were infused for a total of 5 min, at an average flow rate of 30 nl/min, and the remaining sample was recovered from the nanovial. The spectra shown were obtained by summing scans for 1–3 min. Hence, our data indicated that as little as 3 × 10⁻¹⁵ mole of the pentasaccharide was consumed to obtain a reasonable spectrum at the concentration as low as 50 nM. In addition, we found a linear relationship between the relative response of the molecular ion and the concentration of the analyzed 3-OH pentasaccharide, demonstrating that this approach can be used to determine the amount of HS oligosaccharides. To this end, a 3-O-sulfated pentasaccharide was prepared by incubating the 3-OH pentasaccharide with purified HS 3-O-sulfotransferase-1 and 3'-phosphoadenosine-5'-phosphosphate[S]sulfate. The resulting 3-O-sulfated pentasaccharide was purified and analyzed by nESI-MS. Based on the standard curve constructed with the 3-OH pentasaccharide, we calculated the concentration of the 3-O-sulfated pentasaccharide by the relative response. The result indicates that this value is very close to the value measured by [S]sulfate radioactivity. In conclusion, nESI-MS provides both high sensitivity and the capacity to quantify HSs. This approach is likely to become a very important tool for structural analysis and sequencing of HS and heparin oligosaccharides.

Key words: heparan sulfate/oligosaccharides/nano-electrospray/mass spectrometry/antithrombin

Introduction

Heparan sulfates (HSs) are highly negatively charged polysaccharides with 1→4-linked sulfated glucosamine and uronic acid repeating disaccharide units. HSs are present on the cell surface as well as in the extracellular matrix and bind to proteins involved in anticoagulation, angiogenesis (Rosenberg et al., 1997), viral infection (Wu et al. and Spear, 1989), and monocyte adhesion (Guiffre et al., 1997). HS is a structurally complex and heterogeneous macromolecule. It is initially biosynthesized as a copolymer of glucuronic acid and N-acetylated glucosamine by D-glucuronyl and N-acetyl-D-glucosaminyl transferase, followed by various modifications (Lindahl et al., 1998). These include C5-epimerization of glucuronic acid to form an iduronic acid residue, 2-O-sulfation of iduronic and glucuronic acid, N-deacetylation and N-sulfation of glucosamine, as well as 6-O-sulfation and 3-O-sulfation of glucosamine. Several enzymes that are responsible for the biosynthesis of HS have been cloned (Hashimoto et al., 1992; Eriksson et al., 1994; Kobayashi et al., 1997; Li et al., 1997; Shvorak et al., 1997; Habuchi et al., 1998).

The diversified biological functions of HS are likely attributed to their unique sulfated saccharide sequences. Although the detailed mechanism for regulating the biosynthesis of HS with a defined saccharide sequence is unknown, it has been speculated to be related to the presence of various isoforms of each class of HS biosynthetic enzyme. Indeed, HS N-deacetylase/N-sulfotransferase, 3-O-sulfotransferase, and 6-O-sulfotransferase are present in multiple isoforms, and each isoform is believed to recognize the saccharide sequence around the modification site to generate a specific sulfated saccharide sequence (Ishihara et al., 1993; Cheung et al., 1996; Aikawa and Esko, 1999; Liu et al., 1999b; Habuchi et al., 2000; Aikawa et al., 2001). This activity regulates the concentration and distribution of specific recognition motifs on the cell surface. For example, HS modified by 3-O-sulfotransferase isoform 1 (3-OST-1) binds to antithrombin (AT) with anticoagulant activity (Liu et al., 1996), whereas the HS modified by 3-O-sulfotransferase isoform 3 (3-OST-3) binds to herpes simplex 1 envelope glycoprotein D to serve as an entry receptor for herpes simplex virus 1 infection (Shukla et al., 1999).

Heparin is the most commonly used anticoagulant drug. Heparin and HS have very similar disaccharide compositions, except that heparin has a greater content of iduronic acid and a higher number of sulfates per polysaccharide chain (Lindahl et al., 1998). The HS- and heparin-involved anticoagulation mechanisms have been studied extensively. It is now known that HS and heparin interact with AT, a serine protease inhibitor, to inhibit the activities of thrombin and factor Xa in the
blood coagulation cascade (Rosenberg et al., 1997). Anticoagulant-active HS (HS\textsuperscript{act}) and heparin contain one or multiple AT binding sites per polysaccharide chain. This binding site is a pentasaccharide with a structure of \(-\text{GlcNS}(\text{or Ac})\text{6S-GlcA-GlcNS3S(\text{6S})-IdoA2S-GlcNS6S-}\) (where GlcN is glucosamine, IdoA is iduronic acid, GlcA is glucuronic acid, S is sulfate, and Ac is acetate). The 3-O-sulfation of glucosamine to generate GlcNS3S(6S) is a critical modification that results in the formation of HS\textsuperscript{act}. HS 3-O-sulfotransferase 1 (3-OST-1) (EC 2.8.2.23) is the critical enzyme that forms the AT binding site (Liu et al., 1996; Shworak et al., 1997).

Because there are few techniques to separate and characterize HS oligosaccharides, the relationship between the saccharide sequences and diversified biological function remains obscure. Despite the recent progress in purifying and sequencing HS oligosaccharides, the available techniques for completely characterizing HS structures are still inadequate. It is important to note that a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) technique was developed to analyze HS oligosaccharides (Juhasz and Biemann, 1994; Rhomberg et al., 1998). This approach affords high sensitivity and enhanced throughput (Venkataraman et al., 1999; Shriver et al., 2000). The HS oligosaccharides, however, are not observed directly. Instead, this method necessitates formation of a complex between the HS and a basic peptide prior to analysis. Hence, direct structural information via tandem MS is not available; only the mass difference between the complex and free peptide is observed. Moreover, quantitative analysis has not been reported with this method.

Electrospray ionization mass spectrometry (ESI-MS) techniques have attracted attention for analysis of HS and chondroitin sulfate oligosaccharides because they provide high mass accuracy, structural information, and the ability to quantify the analyte (Chai et al., 1998; Kim et al., 1998; Desaire and Leary, 2000; Yang et al., 2000). Those reported approaches utilize microscale, forced-flow ESI, at flow rates of 5–20 µl/min and sample concentrations of 5–200 µM. Thus, each analysis consumes about 40–400 pmole of oligosaccharide. Unfortunately, the application of ESI-MS for analyzing biologically active HS oligosaccharides has been hindered due to high sample consumption.

Nano-electrospray ionization mass spectrometry (nESI-MS) has shown promise for analyzing proteins and other biomolecules because of its reduced flow rate and sample consumption (Wilm and Mann, 1996; Bahr et al., 1997). These advantages make it a choice method for the study of peptides in the low fmole range. Therefore, we have adapted nESI-MS for HS analyses with high sensitivity. In this manuscript, we report that nESI-MS was used to analyze HS pentasaccharides at a concentration as low as 50 nM. Based on our estimation, this approach consumes 3–30 fmole (× 10\textsuperscript{15} mole) to obtain a spectrum with a signal-to-noise ratio greater than 5. In addition, we found a linear relationship between the relative response of the molecular ion and the concentration of the analyzed 3-OH pentasaccharide, suggesting that nESI-MS can be utilized as a sensitive approach to quantify HS oligosaccharides. We used nESI-MS to quantify a concentration of 3-O-sulfated pentasaccharide that was prepared from enzymatic modification. The result is very similar to the estimation from \textsuperscript{[35S]}radioactivity.

Results

Analysis of HS disaccharide standards by nESI-MS

Given that nano-electrospray provides high sensitivity for analyzing peptides, proteins, and oligonucleotides, we employed this technique to analyze HS oligosaccharides. Six HS disaccharide standards were utilized. The structures of those disaccharides are shown in Figure 1. (The disaccharides and pentasaccharides are likely present in the ammonium salt form before the analyses by nESI-MS under our conditions. However, we predominantly observed the molecular ions in the free acid forms. The observation is consistent with the previously reported results [Chai et al., 1998; Desaire and Leary, 2000]. To maintain clarity for MS presentation, we listed the disaccharides and pentasaccharides in free acid forms.) All six disaccharides yielded relatively simple spectra, which are shown in Figure 2 (A to F). The nonsulfated disaccharide, \(\Delta\text{UA-GlcNAc}\), was observed as a singly charged ion \([M-1H]\textsuperscript{+} at \(m/z\) 378 (Figure 2A). Mono-, di-, and trisulfated disaccharides were ionized in multiple charge states (Figure 2B to F). It should be noted that \(\Delta\text{UA-GlcNAc}\) was chosen as an internal standard for the subsequent quantitative analysis. The selection was based on the fact that \(\Delta\text{UA-GlcNAc}\) predominantly shows a single charge state at \(m/z\) 378, which is easily identifiable in the mixture of oligosaccharides.

Analysis of HS disaccharides with nESI-MS using an MS/MS technique

Using MS/MS, we can distinguish isomeric disaccharides. A similar approach was previously reported to identify other glycosaminoglycan-derived disaccharides by using fast-atom bombardment MS (Lamb et al., 1992). The MS/MS spectra of two isomeric standards \(\Delta\text{UA-GlcNS6S}\) and \(\Delta\text{UA2S-GlcNS}\) (Figure 1B, structures D and E) show nearly identical dissociation patterns with the predominant exception being the ion at \(m/z\) 168.5, which is unique to \(\Delta\text{UA-GlcNS6S}\) (Figure 3A,B). Natural abundance isotopic labeling technique allows us to identify the ion at \(m/z\) 168.5 as a doubly charged ion containing two sulfate groups as described below. (This technique compares products from ions that have the same elemental formula but differ in the composition of \(3\text{S}\) and \(3\text{S}\) isotopes. A disulfated molecular ion is flanked by an isotope peak 2 Da higher, at 8.4% abundance, provided that a heavy isotope of sulfur, \(3\text{S}\), is 4.21% abundant in natural sources, and isotope peaks with two sulfate groups contain one \(3\text{S}\) and one \(3\text{S}\) atom. The molecular ion and its isotope peak should dissociate and give the identical product ions with molecular mass differed by 2 Da.) Figure 3C shows the traces of the product ions that were obtained by dissociation of the isolated \([M-2H]\textsuperscript{2-} precursor ion, \(\Delta\text{UA-GlcNS6S}\) at \(m/z\) 247.5 (lower trace) and its isotope peak at \(m/z\) 248.5 (upper trace). Based on the separation of those product ions, we determined the number of sulfate groups at \(m/z\) 168.5 by the following rules: Product ions with no sulfate groups have singlet peaks that align in both traces; monosulfated product ions have doublet peaks in the upper trace; disulfated product ions have nonaligned singlet peaks differing by one \(m/z\) unit for a doubly charged ion. The spacing between the singlet peaks in the two traces (Figure 3C) is one \(m/z\) unit. Hence, it is a doubly charged fragment with two S atoms. Thus, the ion at \(m/z\) 168.5 is derived from cleavage of the glycosidic linkage between the rings as described from
other reports (Thomsann et al., 2000; Wheeler and Harvey, 2000), with two charges retained on the GlcNS6S moiety.

Quantitative analysis of 3-OH pentasaccharides by nESI-MS
We then conducted an analysis of a chemically synthetic pentasaccharide (3-OH pentasaccharide, for structure see Figure 1C) by using nESI-MS. Figure 4A shows the spectra of 3-OH pentasaccharide at the concentrations of 5 μM and 50 nM. As expected, 3-OH pentasaccharide at 5 μM shows a triply charged ion [M-3H]+ at m/z 475 and a strong quadruply charged ion [M-4H]+ at m/z 356 (Figure 4A). We found that this method can detect a concentration as low as 50 nM
Fig. 2. Mass spectra of HS disaccharides. (A) is ΔUA-GlcNAc, (B) is ΔUA-GlcNS, (C) is ΔUA-GlcNAc6S, (D) is ΔUA-GlcNS6S, (E) is ΔUA2S-GlcNS, and (F) is ΔUA2S-GlcNS6S.

Fig. 3. Product ion spectra of isomeric disaccharides. (A) MS/MS of ΔUA-GlcNS6S, [M-2H]^2+ at m/z 247.5. (B) MS/MS of ΔUA2S-GlcNS, [M-2H]^2+ at m/z 247.5. The star highlights the difference between the two spectra, the doubly charged ion at m/z 168.5. (C) shows the overlay of dissociation of the molecular ion and the isotope peak of ΔUA-GlcNS6S. The shift in mass, from 168.5 to 169.4, indicates that the ion contains two sulfate groups (see text for explanations). (D) shows the structure of m/z 168.5, the GlcNS6S portion of the disaccharide.
Structural analysis of heparan sulfate

50 nM 3-OH pentasaccharide

5 µM 3-OH pentasaccharide

Fig. 4. Quantitative analysis of 3-OH and 3-O-sulfated pentasaccharides. (A) shows mass spectra of the 3-OH pentasaccharide at concentrations of 50 nM (top) and 5 µM (bottom). The [M-4H]4– and [M-3H]3– regions of the 50 nM spectrum have been amplified fivefold. (B) shows the calibration curve obtained for the 3-OH pentasaccharide. The filled-in circles represent 3-OH pentasaccharide data points. The filled-in triangle represents the point for the 3-O-sulfated pentasaccharide. Each data point represents the average of two determinations. The error bars indicate the range.

We also investigated the relationship between the intensity of the molecular ions and the concentration of the analyzed 3-OH pentasaccharide. To normalize the results among the determinations, we mixed various concentrations of 3-OH pentasaccharide with 15 µM of the nonsulfated disaccharide (ΔUA-GlcNAc) prior to analysis. We observed that the 3-OH pentasaccharide showed both triply charged and quadruply charge states at m/z = 475 and m/z = 356, respectively, whereas ΔUA-GlcNAc appears as the singly charged ion at m/z = 378. (Representative spectra are shown in Figure 4A.) For each determination, the data were summed for 1 min to acquire the best spectrum during a 5-min scan, and the relative ratio of the sum of the peak areas of m/z 356 and m/z 475 (for the 3-OH pentasaccharide) and the peak area of m/z 378 (for ΔUA-GlcNAc) was calculated. The relative intensity of the 3-OH pentasaccharide was then plotted against its concentration (Figure 4B). A reasonable linear curve was observed (R2 = 0.98), and the results suggest that nESI-MS can be utilized for quantitative analysis of HS pentasaccharides.

Analysis of 3-O-sulfated pentasaccharide

We decided to test our method by quantifying a sample of biologically active HS oligosaccharides, prepared from an enzymatic modification of the 3-OH pentasaccharide. The AT-binding pentasaccharide was selected in this experiment for the following reasons. First, we are capable of radiolabeling the AT-binding pentasaccharide with [35S] (3-O-sulfated pentasaccharide). Therefore, the amount of the pentasaccharide is easily estimated based on the specific [35S] radioactivity. This information was used to validate the result from a quantitative analysis by nESI-MS as described below. Second, the purification of the AT-binding pentasaccharide can be easily carried out. The binding affinity of the 3-OH pentasaccharide (non-AT-binding pentasaccharide) to AT is about 16,000-fold less than that of 3-O-sulfated pentasaccharide (AT-binding pentasaccharide) (Atha et al., 1985). Using AT-affinity chromatography combined with anion exchange-HPLC, the 3-OH and 3-O-sulfated pentasaccharides are readily separated.

The 3-O-sulfated pentasaccharide was prepared by incubating purified HS 3-O-sulfotransferase 1 and 3-OH pentasaccharide in the presence of [35S]PAPS as described under Materials and methods. The 3-O-sulfated pentasaccharide was purified by using an AT-affinity column followed by silica-based polyamine high-performance liquid chromatography (PAMN-HPLC). We confirmed that 3-O-sulfated pentasaccharide binds to AT by using affinity coelectrophoresis (data not shown).

The nESI-MS spectrum of the 3-O-sulfated pentasaccharide is shown in Figure 5. (We calculated the molecular weight of 3-O-[35S]sulfated pentasaccharide based on [35S], because [35S]sulfate represents less than 0.4% of total 3-O-sulfate.) The pentasaccharide ion current is partitioned between triply charged and quadruply charged ions [M-nH]n– at m/z 501 and 376, respectively. The deconvoluted spectrum is also shown in Figure 5, inset A. The signals at molecular mass of 1508, 1530, and 1546 correspond to the 3-O-sulfated pentasaccharide, and its sodium and potassium adducts. (The sodium and potassium adducts of 3-O-sulfated pentasaccharide were observed during the analysis by nESI-MS. However, we did not observe such high levels of salt adducts to 3-OH pentasaccharide [Figure 4A]. We believe the reason for the presence of sodium...
and potassium adducts in 3-O-sulfated pentasaccharide is likely due to incomplete desalting after the purification by PAMN-HPLC, which was eluted by 1 M potassium phosphate monobasic. It should be noted that we did not observe 3-OH pentasaccharide signals at \( m/z \) 475 and 356, suggesting that our preparation of 3-O-sulfated pentasaccharide is pure and desulfation in the source is negligible.

To further confirm the identities of the signals at \( m/z \) 501, 508, and 524, we conducted neutral loss scans. Isolated ions are sequentially admitted to a collision cell filled with an inert gas under controlled energy conditions, resulting in limited dissociation. The linkage between the sulfate and the hydroxyl group is labile, and products from a series of sulfate losses within the collision cell are common (Chai et al., 1998; Kim et al., 1998). Two quadrupoles (Q1 and Q3) were scanned in a linked method such that only ions losing the preset mass of sulfate will be detected. When the mass spectrometer was scanned in the neutral loss mode looking for oligosaccharides that lose 26.7 \( m/z \) (corresponding to the loss of the sulfate from the triply charged pentasaccharide), two major peaks at \( m/z \) 508.5 and 501.2 were detected (Figure 5, inset B). Thus, the results from neutral loss scans confirmed that the ions at \( m/z \) 501 and 508 are indeed triply charged 3-O-sulfated pentasaccharide.

Neutral Loss 26.7 \( m/z \)

(A) shows the deconvoluted spectrum wherein as many as three sodium adducts, one potassium and a single imidazole adduct may be clearly discerned. A neutral loss scan, which selectively detects loss of SO\(_3\) from a triply charged precursor, is illustrated in (B).

Fig. 5. Spectrum of the 3-O-sulfated pentasaccharide. The 3-O-sulfated pentasaccharide is derived by incubation of 3-OH pentasaccharide with 3-O-sulfotransferase. The raw data shows prominent 3- and 4-charge states. Salt adducts were minimized by the addition of imidazole. (A) shows the deconvoluted spectrum wherein as many as three sodium adducts, one potassium and a single imidazole adduct may be clearly discerned. A neutral loss scan, which selectively detects loss of SO\(_3\) from a triply charged precursor, is illustrated in (B).

Discussion

A sensitive method to analyze HS oligosaccharides is crucial to delineate the specific roles of HS and establish the relationship...
between the biological functions and sulfated monosaccharide sequences. Various mass spectral techniques for structural analysis of HS oligosaccharides have been reported. (Chai et al., 1995, 1998; Huszsz and Biemann, 1994; Millis et al., 1989; Reinhold et al., 1987) We are particularly interested in developing an MS technique capable of analyzing very small sample amounts, as the total yield of biologically active HS oligosaccharides is often limited. In this study, we optimized nESI-MS to analyze HS disaccharides and pentasaccharides. Compared to previously reported forced-flow ESI techniques, we reduced sample consumption by more than 100-fold. MALDI-MS has been used to analyze HS oligosaccharides in the 0.1 to 1 pmole range (Liu et al., 1999a; Shriver et al., 2000). Our results suggest that nESI-MS achieves very similar sensitivity to MALDI-MS and has the capability for quantitative analysis. Moreover, ESI is compatible with a variety of tandem instruments, so reliable MS/MS is possible. This technique gives us the capacity to identify signals that are indeed HS oligosaccharides by removing chemical noise and eliminates the possibility of misidentifying a signal due to a contaminant.

It should be noted that the conditions for nESI-MS are significantly milder than forced-flow ESI-MS and the ionization efficiency for nESI-MS is estimated to be 510 times higher than ESI-MS (Wilms and Mann, 1996). We used a low negative nESI voltage (–530 to 630 V) and low cone voltage (–13 V) during the analysis compared to ESI voltages used at higher flow (–2000 to 4000 V and cone voltage at around –60 V). In negative ESI, higher voltages contribute to corona discharge and thus increased chemical noise. We have noted that milder nESI-MS conditions reduce desulfation of the oligosaccharides as well. Indeed, we observed a relatively low level of desulfation during the analyses of disaccharides and pentasaccharides, except for the trisulfated disaccharide (ÁUA2S-GlcNS6S, Figure 2F). We also observed an increase in desulfation of the pentasaccharides when higher cone voltages were employed for nESI-MS. In addition, we found that adding 0.2% of imidazole is helpful to reduce the level of sodium adducts of pentasaccharides and improve sensitivity (spectra not shown). It is known that imidazole and pyridine suppress the formation of sodium/oligonucleotide adducts (Grieg and Griffith, 1995).

Like previously reported ESI-MS methods, nESI-MS is capable of quantifying HS oligosaccharides, provided that appropriate standards are available. Such a quantitative capability will play an important role in sequencing analysis of HS oligosaccharides because it is necessary to monitor the conversion yield during each sequencing step. The currently reported sequencing approaches for underivatized oligosaccharides utilize the absorbance at UV 232 nm or radioactivity (Merry et al., 1999; Venkataraman et al., 1999). However, the sensitivity at 232 nm is relatively low. Furthermore, for those samples without radiolabels or absorbance at 232 nm, neither method can be used. Our data suggest that the standard curve constructed from the 3-OH pentasaccharide could be used to estimate the amount of the 3-O-sulfated pentasaccharide. It might be essential to choose appropriate standards to generate a suitable curve for quantitative analysis of an unknown structure. Ideally, the oligosaccharides with known structures would be used to generate standard curves which project the amount of oligosaccharides with unknown structures. It is unfortunate that we are presently unable to investigate the general rules for choosing standards for quantitative analysis because well-defined HS oligosaccharides are not available.

In summary, nESI-MS is a sensitive tool to quantitatively analyze HS oligosaccharides. This technique will provide an alternative approach to MALDI-MS for interrogating HS oligosaccharides. Each technique has its unique strengths for analyzing HS oligosaccharides. Combining MALDI-MS and nESI-MS will be essential to characterize the structures of unknown HS oligosaccharides with greater confidence.

Materials and methods

HS disaccharide standards were obtained from Seikagaku American (Falmouth, MA). HS 3-O-sulfotransferase isoform 1 (3-OST-1) was expressed in insect SF9 cells by using baculovirus expression approach and purified as described previously (Hernaiz et al., 2000). [35S]Radiolabeled 3′-phosphoadenosine 5′-phosphosulfate ([35S]PAPS) was prepared by incubating ATP and [35S]sodium sulfate with yeast extract as described by Bame and Esko (1989). Human antithrombin is from Cutter Biological (Berkeley, CA). The chemically synthesized pentasaccharide, GlcNS6S-GlcA-GlcNS6S-IdoA2S-GlcNS6SMe (for structure, see Figure 1), is a generous gift from Dr. Maurice Petitou, Sanofi Recherche, and from Dr. Robert Rosenberg, Massachusetts Institute of Technology. Concanavalin A (ConA)-Sepharose, unlabeled PAPS and other chemicals are from Sigma.

nESI-MS analysis

A Micromass Quattro II with QH geometry and the Z-Spray source was used in these experiments. Borosilicate glass capillaries (OD 1 mm and ID 0.57 mm) were pulled on a glass capillary puller (Narishige Company, Japan). A titanium wire (99.99%, Goodfellow, Inc.) was inserted in the distal end of the glass capillary, to provide electrical contact to the solution. Negative nESI voltage was maintained between –530 V and –650 V during the analysis. To prevent desulfation in the gas phase, the absolute value of the sampling cone voltage was maintained < 13 V. Unit mass resolution was used in all MS1 scans. During MS/MS experiments, argon was used as the collision gas. Collision energy was kept low (12 eV), because desulfation is a facile gas phase process. Spectra were acquired over 300–800 m/z, at 2 s per scan, 16 pts/Da.

In the neutral loss scans, MS/MS spectra were obtained by scanning Q1 and Q3 with an offset of 26.7 or 20 m/z in their scan cycles, corresponding to the loss of sulfate from the triply or quadruply charged pentasaccharides, respectively. To maximize the sensitivity of neutral loss detection, resolution was lowered so that peak widths were ca. 1.2 Da at full width at half maximum.

Quantitative analysis of HS pentasaccharides by nESI-MS

The 3-OH pentasaccharide was diluted with a solvent containing 0.2% NH₄OH, 0.2% imidazole, and 50% acetonitrile. The internal standard, ÁUA-GlcNAc, was spiked into each sample to yield a final concentration of 15 µM. The final 3-OH pentasaccharide concentration ranged from 0.05 µM to 10 µM. Approximately 2 µl of the mixture was loaded into a pulled-glass capillary and infused for 5 min. Ion chronograms were constructed and selected from 1 min of analysis time, during which time signal response was
strongest. All peaks were integrated, and the ion intensities of the [M-3H]− and [M-4H]− peaks of the 3-OH pentasaccharide were summed, and divided by the intensity of the internal standard. Experiments were performed in duplicate, and the relative intensities were averaged together. These relative intensities were plotted against the concentration of the 3-OH pentasaccharide. To quantify the 3-O-sulfated pentasaccharide, the pentasaccharide (11,500 c.p.m./μl or 0.85 μM) was diluted with the solvent mixture as described above in the presence of 15 μM ΔUA-GlcNAc.

**Preparation of 3-O-pentasaccharide**

Purified 3-OST-1 (70 ng) was mixed with 1 μg of the chemically synthesized pentasaccharide and 10 μM [35S]PAPS (7.2 × 106 c.p.m., 14,000 c.p.m./pmole) in 50 μl of the enzyme reaction buffer containing 50 mM 2-[N-morpholino]ethanesulfonic acid, 1% Triton X-100, 1 mM MgCl2, 2 mM MnCl2, and 168 μg/ml of bovine serum albumin, pH 7. The reaction was incubated at 37°C for 2 h and was terminated by heating at 100°C for 2 min. The resultant was centrifuged at 14,000 r.p.m. for 1 min to remove insoluble matter. The 3-O-[35S]sulfated pentasaccharide was purified by an antithrombin-Con A affinity column as described previously (Liu et al., 1996). Briefly, the resulting supernatant was mixed with 50 μl water and then incubated with 50 μl of a buffer, containing final concentrations of 10 mM Tris, 150 mM NaCl, 1 mM Ca2+, 1 mM Mg2+, 1 mM Mn2+, and 0.1 mg/ml of antithrombin (pH 7.5), at room temperature for 30 min. The solution was then loaded onto a 300-μl ConA-Sepharose column equilibrated with a buffer containing 10 mM Tris, 0.0004% Triton X-100, and 150 mM NaCl, pH 7.5. The gel was incubated at room temperature for an additional 30 min, washed with 3 × 1ml of a buffer containing 10 mM Tris, 0.0004% Triton X-100, and 150 mM NaCl (pH 7.5). The 3-O-sulfated pentasaccharide was eluted from the gel by using 2 × 0.5 ml of a buffer containing 10 mM Tris, 1000 mM NaCl, and 0.0004% Triton X-100 (pH 7.5). The eluted material was dialyzed against 50 mM ammonium bicarbonate using 3,500 MWCO dialysis tubing (Spectrum). Ten reactions were prepared to obtain a sufficient amount of 3-O-sulfated pentasaccharide for the analyses by nano-electrospray and affinity coelectrophoresis. Assuming one [35S]sulfate is transferred to every molecule of pentasaccharide by 3-OST-1 enzyme, a total of 3 × 107 c.p.m. of pentasaccharide, equivalent to 2.1 nmole, were prepared.

Affinity chromatography–purified 3-O-sulfated pentasaccharide was further purified by PAMN-HPLC for nano-electrospray MS analysis. The 3-O-sulfated pentasaccharide was injected onto a PAMN column (0.46 × 25 cm; Waters) equilibrated with 350 mM KH2PO4 at a flow rate of 1 ml/min. The column was then eluted with a linear gradient of KH2PO4 from 350 mM to 1000 mM in 60 min, and the concentration of KH2PO4 remained at 1000 mM for an additional 20 min at a flow rate of 1 ml/min. The eluate was collected every minute, and 5 μl of the eluate was removed to mix with 7 ml of scintillation fluid (Ecolume, ICN) to determine [35S]radioactivity on a β-counter (Packard Instrument). The 3-O-sulfated pentasaccharide emerged at 54–55 min. The fractions containing [35S]radioactivity were pooled and dialyzed against 50 mM ammonium bicarbonate using 3,500 MWCO dialysis tubing. The dialyzed sample was resolved on BioGel P-6 (0.75 × 200 cm) eluted with 0.5 M ammonium bicarbonate to further remove potential salt contamination followed by dialyzing against 50 mM ammonium bicarbonate and water.

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**Abbreviations**

3-OST-1, heparan sulfate 3-O-sulfotransferase isoform 1; AT, antithrombin; GlcA, glucuronic acid; GlcNS, glucosamine N-sulfate; GlcNS(or Ac)6S, N-sulf- or N-acetyl-glucosamine 6-O-sulfate; GlcNS4S(±6S), 6-O-sulfoglucuronic N-3-sulfate or glucosamine N-3-sulfate; IdoA2S, 2-O-sulfated iduronic acid; HS, heparan sulfate; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; nESI-MS, nano-electrospray ionization mass spectrometry; PAMN-HPLC, silica-based polyamylone high-performance liquid chromatography; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; ΔUA, Δ4,5-unsaturated uronic acid.

**References**


Structural analysis of heparan sulfate