Characterization of Heparin Oligosaccharides Binding Specifically to Antithrombin III Using Mass Spectrometry[†]

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ABSTRACT: Heparan sulfate (HS) is a sulfated glycosaminoglycan attached to a core protein on the cell surface. Protein binding to cell surface HS is a key regulatory event for many cellular processes such as blood coagulation, cell proliferation, and migration. The concept whereby protein binding to HS is not random but requires a limited number of sulfation patterns is becoming clear. Here we describe a hydrophobic trapping assay for screening a library of heparin hexasaccharides for binders to antithrombin III (ATIII). The hexasaccharide compositions are defined with their building block content in the following format: (ΔHexA:HexA:GlcN:SO_3:Ac). Of five initial compositions present in the library, (1:2:3:6:1), (1:2:3:7:1), (1:2:3:7:0), (1:2:3:8:0), and (1:2:3:9:0), only two are shown to bind ATIII, namely, (1:2:3:8:0) and (1:2:3:9:0). The use of amide hydrophilic interaction (HILIC) liquid chromatography−mass spectrometry permitted reproducible quantitative analysis of the composition of the initial library as well as that of the binding fraction. The specificity of the hexasaccharides binding ATIII was confirmed by assaying their ability to enhance ATIII-mediated inhibition of Factor Xa in vitro.

Heparin and heparan sulfate (HS)[1] are a subclass of a heterogeneous family of anionic glycans called glycosaminoglycans. Heparin and HS are made of the repeating disaccharide units HexAβ/α4GlcNα4. The biosynthetic process is initiated by addition of GlcNAc to a xylosyl linker tetrasaccharide attached to a serine residue of a core protein (*1–3*). The glycan chain is elongated through addition of GlcAβ4GlcNAcα4 disaccharide units (*4, 5*). The polysaccharide chain is further modified by different enzymes while attached to the core protein. In an orderly fashion, the first modification is N-deacetylation/N-sulfation of GlcNAc (*6–12*) followed by C-5 epimerization of GlcA to IdoA (*8, 13–15*), 2-O-sulfation of IdoA (*16*), and 6-O-sulfation of GlcN (*17*). Because one modified disaccharide serves as a substrate to the next enzyme, the deacetylation, sulfation, and epimerization tend to cluster in the same region. The modifying enzymes work only on a fraction of available modification sites, and hence, the disaccharide compositions of heparin and HS are very heterogeneous. To date, 23 different disaccharides have been identified (*18*). Although heparin and HS have similar biosynthetic pathways, they markedly differ in the extent of the modifications that they undergo. Heparin, which is synthesized exclusively in mast cells and cleaved from its core protein, undergoes extensive modifications such that more than 85% of the GlcNAc residues are N-deacetylated and N-sulfated and more than 70% of the GlcA is converted to IdoA. By contrast, HS is found in all tissues, remains attached to the core protein, and is less modified than heparin. HS is composed of highly sulfated domains resembling heparin called NS domains and less sulfated domains where GlcNAc remains acetylated called NA domains. The alternation of NS and NA domains makes HS more heterogeneous in sequence than heparin (*19*).

One of the most important features of heparin and HS is the ability to interact with a wide array of proteins. More than 100 heparin binding proteins have been discovered, and the number is increasing (*20*). These proteins are involved in a wide range of physiological processes, including blood coagulation, cell proliferation, differentiation and migration, cell−cell adhesion, inflammation, and pathogen invasion (*21*). Heparin and HS exert a regulatory role on these processes by modulating the activity of the proteins with which they interact. A growing body of evidence suggests that binding of proteins to heparins is not random but requires defined sequences within the heparin chain. On the basis of the knowledge of heparin structure whereby sulfo and carboxyl groups are arranged at defined intervals and in a specific orientation, the general paradigm is that a protein requires a minimal structure that displays optimal functional group arrangement for specific interactions. The specificity of these interactions can be restrictive, meaning that one or a few structures are able to bind the protein. Conversely, some proteins can exhibit more relaxed criteria such that different patterns of substitutions are tolerated (*18*).

Heparin binding to antithrombin III (ATIII) constitutes the paradigm of a restrictive binding. ATIII belongs to the serpin

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[1] Abbreviations: Ac, acetate; HS, heparan sulfate; HexA, hexuronic acid; ΔHexA, unsaturated hexuronic acid; GlcN, glucosamine; GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc, *N*-acetylglucosamine; ATIII, antithrombin III; FGF, fibroblast growth factor; SEC, size exclusion chromatography; dp, degree of polymerization; HILIC, hydrophilic interaction chromatography; RIC, reconstructed ion chromatogram; LC/MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization.

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(serine protease inhibitors) superfamily of proteases (*22*). It reacts with most of the serine proteases of the coagulation cascade to inactivate them by forming 1:1 covalent complexes through interaction between a specific reactive bond on the inhibitor and the active site of the serine protease (*23, 24*). This inhibition process is enhanced severalfold by the binding of heparin that induces a conformational change in ATIII (*25*). A heparin pentasaccharide is the minimal sequence required to bind ATIII, enhancing its inhibitory activity toward Factor Xa (*26–30*). The sequence of this pentasaccharide is GlcNAc6S-GlcA-GlcNS3S-IdoA-GlcNS. The reducing end and the internal GlcN are invariably N-sulfated, while that at the nonreducing end can be either N-acetylated or N-sulfated. The requirements of the functional groups attached to the saccharide residues have been studied in detail. The reducing end and internal *N*-sulfate groups (*31*), the 6-*O*-sulfate groups at the nonreducing end (*32*), and the distinguishing 3-*O*-sulfate (*30, 33, 34*) on the central GlcN are required for binding and biological activity.

In contrast to the stringent ATIII binding requirement, the binding of growth factors to HS appears to be more relaxed. Fibroblast growth factors (FGFs) are the most studied heparin binding proteins after ATIII. The minimal heparin structure binding to FGF2 is a pentamer that contains one or more *N*- and 2-*O*-sulfates with no 6-O-sulfation requirement (*35*). FGF1 binding to HS requires five to seven monosaccharide units comprising critical 2-*O*-, *N*-, and 6-*O*-sulfate groups (*36*).

Despite the improved understanding that specific heparin and HS sequences mediate the modulation of a growing number of proteins, carbohydrate structures have been elucidated only for a limited number of cases. The heterogeneous and polydisperse nature of heparin and HS and the absence of methods for amplifying or producing them are the main limiting factors to the acquisition of structural data. Most of the structural analysis methods make use of the available chemical or enzymatic degradation procedures followed by electrophoretic, chromatographic, or mass spectrometric detection (*37–39*). These methods are best applied to purified heparin oligosaccharides in producing unambiguous structural assignments. However, in most cases, binding sequences consist of a distribution of related structures in heterogeneous mixtures. Liquid chromatography techniques have been used to separate and hence simplify the heterogeneity of starting oligosaccharide mixtures. Anion exchange chromatography (SAX) separates oligosaccharides on the basis of their charge density (*40, 41*). However, its requirement of high salt concentrations makes its on-line coupling to mass spectrometric analysis very difficult. Ion pair reverse phase chromatography is another chromatographic system that separates oligosaccharides on the basis of size, sulfation content, and isomerization in relatively simple isomeric mixtures (*42, 43*), yet the use of amines as ion pairing agents can contaminate the mass spectrometer rendering it unavailable for subsequent analysis of different classes of compounds before extensive instrument cleaning. In addition, this method requires optimization of separation conditions depending on the class of compounds analyzed.

In this work, we describe a method that enables the screening and quantitative analysis of a library of heparin/HS oligosaccharide for epitopes that are able to bind proteins of interest. To optimize and test the screening technique, a readily available and cost-effective model system for a heparin binding protein has been used, namely ATIII. However, the same principle can be extrapolated to any heparin binding protein. The advantage of this method is its ability to isolate binders in binary or ternary complexes using a small quantity of protein. Quantitative analysis of binders was undertaken using a HILIC LC−MS platform that separates oligosaccharides on the basis of polarity. Although this system does not separate isomeric compositions efficiently, it has the advantage of resolving heparinoid-derived oligosaccharides on the basis of size, sulfation, and acetylation content, properties that dictate the overall polarity of an oligosaccharide. Furthermore, the conditions used in this chromatography system are compatible with the separation of a large range of oligosaccharide sizes without further optimization.

MATERIALS AND METHODS

Materials. Porcine intestinal mucosa heparin (sodium salt, 182 USP/mg) was purchased from Sigma-Aldrich (St. Louis, MO). Heparin lyase I from *Flavobacterium heparinum* was from Ibex (Montreal, QC). Antithrombin III was a generous gift from GTC Biotherapeutics (Framingham, MA). Amide 80 packing material was obtained from TOSOH Bioscience LLC (Montgomeryville, PA). Actichrome heparin (anti-FXa) was purchased from American Diagnostica Inc. (Stamford, CT). Δ-Disaccharide standard (Di4S) (ΔIdoA-GlcNac4S) was from V-laboratories (Convington, LA). Arixtra ($C_{31}H_{53}N_3O_{49}S_8$) was purchased from Organon Sanofi-Synthelabo LLC (West Orange, NJ).

Construction of the Heparin Hexasaccharide Library. Partial digestion of 100 mg of porcine intestinal mucosa heparin (Sigma-Aldrich) was performed in 1 mL of 100 mM ammonium acetate containing 0.1 mg/mL BSA at 37 °C. Heparin lyase I (150 milliunits) was added in 6 h intervals until the digestion reached 30% completion, determined using the absorbance at 232 nm. The digestion was applied to a preparative size exclusion chromatography (SEC) column (170 cm × 1.5 cm; Bio-Rad, Hercules, CA) packed with polyacrylamide gel (Bio-Gel, P-10, Fine; Bio-Rad) running at an ionic content of 200 mM ammonium bicarbonate with a flow rate of 40 μL/min (*44*). Fractions containing heparin dp6 were combined and desalted by dialysis using a 100 Da cutoff.

Binding Assay. ATIII (1 nmol) was incubated with heparin dp6 (100 nmol) for 20 min in 100 mM ammonium acetate. The mixture was applied to a Superdex-75 PC 3.2/30 column (Amersham Biosciences, Piscataway, NJ) coupled to a Beckman (Fullerton, CA) System Gold 118 solvent module and the effluent monitored at 232 nm. The degassed mobile phase (100 mM ammonium acetate and 3% acetonitrile) was supplied at a rate of 40 μL/min. The protein fraction was collected and applied to a C18 reverse phase cartridge (MacroSpin Columns, Silica C18 Vydac, 50–450 μL, Harvard Apparatus) after column conditioning as advised by the manufacturer's protocol. The bound complex was washed three times with 250 μL of 200 mM ammonium acetate, and the remaining bound sugars were eluted with 250 μL of 2 M ammonium acetate. All experiments were performed in triplicate, and the data are reported as averages with the standard deviation.

Table 1: Hexasaccharide Compositions of a 30% Heparin Digest[a]

z	observed *m/z*	calculated *m/z*	error (ppm)	ΔHexA	HexA	GlcN	SO_3	Ac
−2	725.5406	725.5414	−1.1	1	2	3	5	1
−2	765.5241	765.5198	5.6	1	2	3	6	1
−2	805.4923	805.4982	−7.3	1	2	3	7	1
−2	744.5175	744.5145	4.0	1	2	3	6	0
−2	784.4913	784.4929	−2.1	1	2	3	7	0
−2	824.4711	824.4713	−0.3	1	2	3	8	0
−3	575.9641	575.9641	0.1	1	2	3	9	0

[a] The table includes only the masses of the nonadducted compositions. Abbreviations: ΔHexA, unsaturated hexuronic acid; HexA, hexuronic acid; GlcN, glucosamine; SO_3, sulfate; Ac, acetyl.

Liquid Chromatography−Mass Spectrometry (LC−MS). The heparin hexasaccharides were analyzed in the negative electrospray mode using an Amide-80 hydrophilic interaction chromatography (HILIC) system on-line with an Applied Biosystems QSTAR Pulsar-I (Q-ToF) mass spectrometer. The column was packed in house into 250 μm internal diameter × 15 cm silica tubing using 5 μm Amide-80 beads. The column was connected to a Waters (Milford, MA) nanoaquity chromatograph operating in the gradient mode with solvent A [50 mM formic acid (brought to pH 4.4 with ammonium hydroxide)] and solvent B (95% acetonitrile and 5% A). The compounds were eluted from the column with a gradient starting at 95% B and descending to 40% B over a period of 60 min. The dp6 library was analyzed by injection of 150 pmol of oligosaccharides. By comparison of peak areas of the starting material and the bound fraction, the amount of bound fraction analyzed was estimated to be 5 pmol. Arixtra was used to optimize detection of heparin oligosaccharides. All runs were repeated in triplicate, and mass range was calibrated with respect to 1 pmol of Di4S internal standard.

Actichrome Biological Activity Assay. The high-salt elution fraction obtained from the binding assay was dialyzed using a 100 Da cutoff and assayed for its biological activity using the actichrome heparin (anti-FXa) kit (American Diagnostica Inc.). The manufacturer's protocol was customized by scaling down the recommended volumes 10-fold, leading to a total reaction volume of 102.5 μL.

RESULTS

To prepare the oligosaccharide library, porcine intestinal mucosa heparin was subjected to partial enzymatic digestion. The digestion mixture was fractionated by preparative SEC and the dp6 fraction collected. A hexasaccharide represents the minimal lyase-generated oligosaccharide that contains an ATIII binding sequence and hence was used as the library to screen for ATIII binders. The library was incubated with ATIII and the protein fraction separated from the unbound oligosaccharides by SEC. The benefit of an initial SEC step is realized in a situation where isolating a complex of more than one protein in a ternary complex with an oligosaccharide may be necessary (*45*). The collected protein fraction that contains free protein and protein bound to sugars was applied to a reverse phase cartridge, and the unspecific binders were washed away with a low salt concentration (*46–48*). The specifically bound sugars were eluted using a high salt concentration, and the composition distribution of the eluted sugars was analyzed and quantified by LC−MS. The liquid chromatography system uses an amide-silica as the stationary phase. Oligosaccharides are bound to and eluted from this stationary phase with a HILIC mechanism (*49*). Oligosaccharides are retained on the basis of hydrogen bonding interactions between the amide functionality and the polar hydroxyl groups. When a high to low organic gradient is used, Amide-80 separates oligosaccharides in time on the basis of their polarity and offers the advantage of reproducibility that allows quantification. This stationary phase has been used for LC−MS of chondroitin sulfate and dermatan sulfate oligosaccharides (*50, 51*) as well as *N*-glycans (*52, 53*) but has never been applied to heparinoids.

The oligosaccharide compositions of the hexasaccharide library are listed in Table 1. The abbreviation of each composition is given in the following format: (ΔHexA:HexA:GlucN:SO_3:Ac). Seven different compositions are observed in two categories. The acetylated compositions are (1:2:3:5:1), (1:2:3:6:1), and (1:2:3:7:1). The nonacetylated compositions are (1:2:3:6:0), (1:2:3:7:0), (1:2:3:8:0), and (1:2:3:9:0). Table 1 gives the *m/z* values only for the unadducted species with error values ranging from −7.3 to 5.6 ppm on the instrument used. This error range represents typical performance in the negative ion spray mode of heparinoids. Up to four ammonium adducts per ion were observed (data not shown). In all cases, two charge states were observed, the doubly and triply negatively charged ions.

One issue that complicates the analysis of heparin/HS oligosaccharides is the loss of sulfate that occurs during the ionization step. The extent of in-source loss of sulfate was determined by studying the ionization properties of a sulfated standard, Arixtra. This octasulfated pentasaccharide methyl glycoside with a known composition ($C_{31}H_{53}O_{49}S_8$) and a theoretical molecular mass of 1506.9513 Da is very pure. Its chemical properties make it a good candidate for mimicking the behavior of heparin hexasaccharides and hence allow estimation of the degree of sulfate loss that occurs in source. Figure 1 shows the mass spectrum of Arixtra summed from an LC−MS data set where the doubly charged and triply charged ammonium-adducted species are detected. Both the doubly charged (*m/z* 752.4685) and the triply charged (*m/z* 501.3105) ions show fragmentation products generated by loss of one or two sulfate groups. Quantitative analysis of sulfate loss estimates the degree of fragmentation to be 16.0 and 4.2% for one and two sulfates, respectively. Since the Arixtra mass spectrum shows negligible loss of two sulfates, the analysis below focuses on loss of a single sulfate. The degree of sulfate loss is difficult to model because it depends on the concentration and charge state.

The advantage of the chromatography step prior to mass spectrometric detection is that it enables correction for the in-source sulfate losses. Figure 2 shows the reconstructed

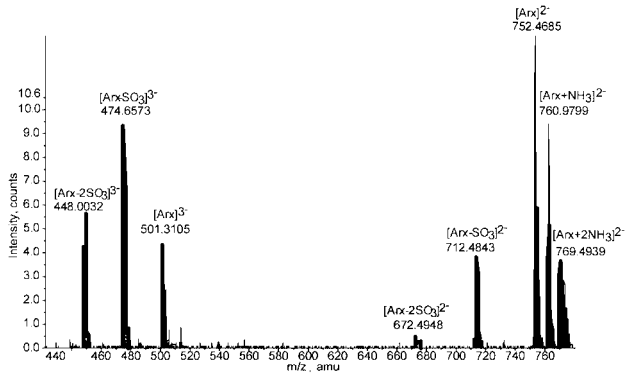

FIGURE 1: Negative ESI-MS of Arixtra. The figure shows the LC−MS spectrum of Arixtra. The doubly (*m*/*z* 752.4685; 0.15 ppm) and triply charged (*m*/*z* 501.3105; 1.3 ppm) ions are shown. Arixtra undergoes loss of one and two sulfates as revealed by the peaks at the lower *m*/*z* values.

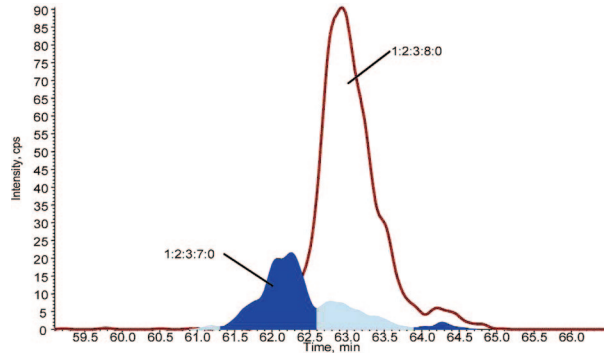

FIGURE 2: Fragmentation of (1:2:3:8:0) into (1:2:3:7:0). The RIC of the (1:2:3:7:0) composition is superimposed over that of the (1:2:3:8:0) composition. Three peaks can be identified in the RIC of (1:2:3:7:0) at respective retention times of 62.25, 62.65, and 64.25 min. The peaks at 62.65 and 64.25 min overlap totally and follow the profile of the (1:2:3:8:0) composition. Hence, they are considered loss of sulfate products of (1:2:3:8:0). During the calculation of relative abundance, the highlighted integrated areas under these two peaks are added to the area under the RIC of (1:2:3:8:0). The RIC shown here represents a composition and all its adducts.

ion chromatogram (RIC) for compositions (1:2:3:7:0) (shaded) and (1:2:3:8:0) from LC−MS of a heparin hexasaccharide fraction. The (1:2:3:7:0) composition is observed as three peaks at respective approximate elution times of 62.25, 62.65, and 64.25 min. The (1:2:3:8:0) composition is observed as two peaks at elution times of 62.65 and 64.25 min. The extent to which the (1:2:3:8:0) composition undergoes sulfate loss is shown by the area of the (1:2:3:7:0) composition at the same retention time. Therefore, the area of the (1:2:3:8:0) peak was corrected for sulfate loss by adding that of the coeluting (1:2:3:7:0) peak (light blue at 62.65 min). Similarly, the peak at 64.25 min in (1:2:3:8:0) coeluted with (1:2:3:9:0), and hence, its area was used to correct for sulfate loss of this latter composition (data not shown). This example illustrates the method used to correct for sulfate losses on the basis of chromatographic elution times.

The RICs for separation of the library of hexasaccharides are shown in Figure 3. For both the nonacetylated and acetylated compositions, the general trend of the elution profile is that the retention time increases with an increase in sulfation content. The column resolves (1:2:3:7:0), (1:2:3:8:0), and (1:2:3:9:0) for the nonacetylated hexasaccharides and (1:2:3:6:1) and (1:2:3:7:1) for the acetylated ones. However, (1:2:3:6:0) and (1:2:3:5:1) show complete

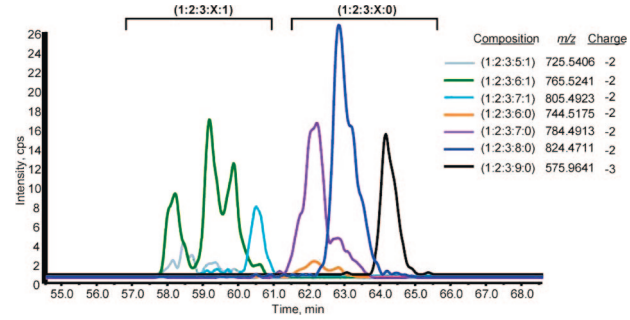

FIGURE 3: Amide-80 chromatographic separation of the different heparin hexasaccharides. The Amide-80 separates the heparin hexasaccharides according to their polarity. The acetylated category, (1:2:3:*X*:1), elutes at a higher organic content of the gradient compared to the unacetylated category, (1:2:3:*X*:0). Within each category, the general trend is such that the more sulfated compositions display higher retention times. This figure shows the reconstructed ion chromatograms of the nonadducted compositions.

overlap with (1:2:3:7:0) and (1:2:3:6:1), respectively. Hence, the total overlap seen between (1:2:3:6:0) and (1:2:3:7:0) on one hand and (1:2:3:5:1) and (1:2:3:6:1) on the other means that former compositions are loss of sulfate products of the latter.

In the RIC of (1:2:3:6:1) (*m*/*z* 765.5241), three peaks can be observed at different retention times, 58.0, 59.2, and 59.8 min. This profile was reproducible over three replicates. The comparison of the *m*/*z* spectra of each of these peaks separately shows the following. The major peak in the spectra of the peaks at 59.2 and 59.8 min was the expected one at *m*/*z* 765.5241 and may represent structural isomers that are differentially retained on the Amide-80 column. Surprisingly, in the spectrum of the peak at 58.0 min, the major peak was at *m*/*z* 765.0260. This latter mass is consistent with the glycosylamine corresponding to the (1:2:3:6:1) composition with an error of −2.3 ppm. Hence, the RIC of the (1:2:3:6:1) composition shown in Figure 3 is in fact tracing two different compositions, the (1:2:3:6:1) and its corresponding glycosylamine. The conversion into a glycosylamine derivative and the presence of multiple isomers both contribute to the multiple-peak elution profile of the (1:2:3:6:1) composition. Glycosylamine formation is a general phenomenon for acetylated heparin oligosaccharides resulting from the buffer conditions used in the preparative SEC step and has not been observed for nonacetylated compositions (*54*).

The method was used to correct the relative abundances of the different compositions in the heparin dp6 library. Figure 4A shows the compositions identified and their relative abundances. Five different hexasaccharide compositions are generated after partial digestion, namely, (1:2:3:6:1), (1:2:3:7:1), (1:2:3:7:0), (1:2:3:8:0), and (1:2:3:9:0). The nonacetylated hexasaccharides with eight and nine sulfates are the most abundant compositions, together accounting for 68.1% of the total generated hexasaccharides.

Analysis of the high-salt elution fraction (termed bound fraction) by LC−MS shows that when the initial library is subjected to the workup protocol, it undergoes drastic compositional change. The bound hexasaccharides are the nonacetylated and the most highly sulfated ones, namely, (1:2:3:8:0) and (1:2:3:9:0) (Figure 4B). The quantification of the relative abundances of the bound compositions reveals that (1:2:3:8:0) accounts for 44.4% while (1:2:3:9:0) repre-

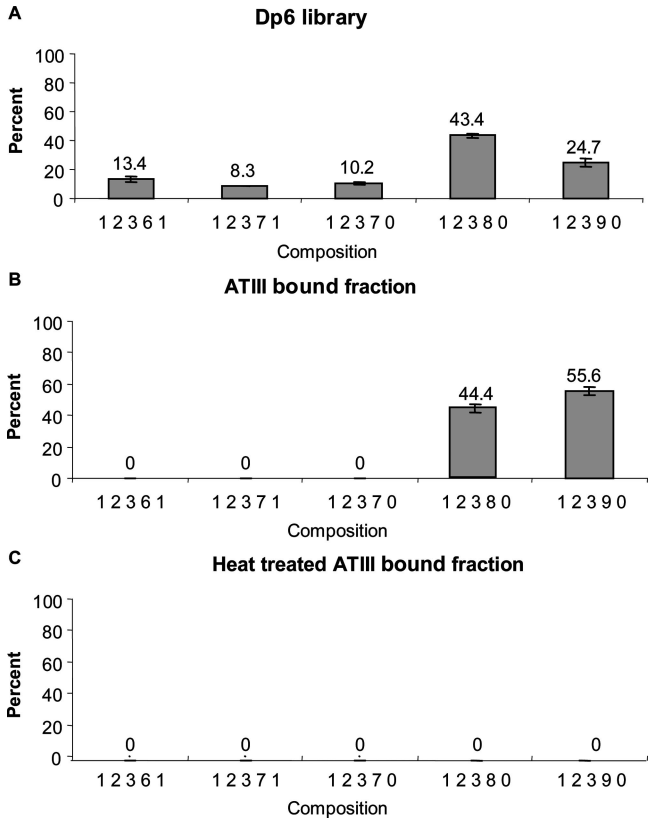

FIGURE 4: Quantification of the relative abundances of heparin hexasaccharide. (A) Relative abundances of the different hexasaccharides present in the dp6 library. Analysis was done in triplicate. (B) Relative abundances of the different hexasaccharides present in the bound fraction (2 M ammonium acetate elution fraction). Analysis was done in triplicate. (C) Relative abundances of the different hexasaccharides present in the bound fraction of a heat-inactivated ATIII. Analysis was done in triplicate.

sents 55.6% of the total (Figure 4B). Two experiments were conducted to rule out nonspecific binding. First, the exact binding protocol was repeated using ATIII that was heat inactivated and subsequently mixed with the heparin dp6 library to isolate binders (Figure 4C). Second, the active ATIII protein was mixed with unspecific chondroitin sulfate B hexasaccharide (data not shown). Any oligosaccharides collected in the bound fraction of these experiments will be retained to the final step of the protocol due to unspecific binding. Analysis of the bound fraction yielded by these two experiments by LC−MS did not show peaks with *m*/*z* values corresponding to hexasaccharides (Figure 4C and data not shown).

To further prove that the hexasaccharides obtained in the high-salt elution step (bound fraction) were not a result of the failure of the protocol to eliminate unspecific binders and hence prove their specific interaction with ATIII, we tested its ability to modulate ATIII activity compared to the oligosaccharides collected in the low-salt step (200 mM ammonium acetate wash fraction). The activity of the bound fraction was assayed by assessing its ability to enhance the ATIII inhibitory effect on Factor Xa using the actichrome heparin (antiFXa) kit. In this kit, the proteolytic activity of excess FXa is assayed by incubating it with a substrate yielding a chromogenic product that absorbs at 405 nm. When ATIII and heparin are added to the reaction mixture, a fraction of the FXa is neutralized and the 405 nm absorbance measured is representative of the residual FXa activity. Hence, the resulting spectrophotometric signal is inversely proportional to the heparin activity in the sample. The ability of the heparin hexasaccharide-bound fraction to inhibit FXa was assayed and compared to that of the wash fraction (Figure 5). Because the bound hexasaccharides were not quantified, their activity was compared to an excess of nonbinders (1 nmol). The hexasaccharides in the high-salt elution fraction showed significantly higher potency in triggering the ATIII-mediated inhibition of FXa compared to the wash fraction.

DISCUSSION

In this work, ATIII binding to heparin hexasaccharides is used as a model to optimize an SEC hydrophobic trapping method for consumption of a small (1 nmol) quantity of protein. A low rate of protein consumption is critical for eventual application to growth factor binding to heparin and HS. We have used a library of heparin hexasaccharides as those represent the smallest oligosaccharides containing an ATIII binding site. Compositional analysis of this library at high mass accuracy has been done after correction for the in-source loss of sulfate. The correction for sulfate loss was facilitated by using an Amide-80 HILIC LC−MS system that separates the hexasaccharides on the basis of their polar character. As the polarity of a molecule increases, it is retained longer by the column and elutes at a higher polar content of the gradient. The differential polarity of heparin oligosaccharides is mainly dictated by the number of hydroxyl, sulfate, and acetyl groups they contain. The polar character varies directly with sulfation and indirectly with acetylation. The analysis revealed five different compositions, (1:2:3:6:1), (1:2:3:7:1), (1:2:3:7:0), (1:2:3:8:0), and (1:2:3:9:0). One acetylated composition, (1:2:3:6:1), was further modified into the corresponding glycosylamine. The formation of glycosylamines results from the protocol of generation of heparin hexasaccharides. The partial heparin digestion is separated into different size fractions on a preparative SEC column running with 200 mM ammonium bicarbonate buffer. As the dp6 fractions are combined and dried, they are constantly in the presence of an increasing concentration of ammonium ions. These conditions are known to favor the formation of glycosylamines (*55, 56*). From our practical experience with working with heparin, we have observed the formation of glycosylamine derivatives only with acetylated heparin oligosaccharides. The tendency of formation of these derivatives varies inversely with the number of sulfates on an oligosaccharide. This might explain why other acetylated hexasaccharides with a higher degree of sulfation do not undergo conversion to glycosylamines.

The analysis of the results using the novel Amide-80-based LC−MS method has permitted accurate quantification of the results obtained. With the complexity of a heparin sample, direct quantification of the different compositions by static negative nano-electrospray ionization (ESI) MS is a difficult task due to the irreproducibility of the spectra obtained. Addition of the liquid chromatography component prior to the MS step offered the advantage of reproducibility for the quantification of the different compositions of heparin hexasaccharides. The degree of reproducibility over triplicate experiments ranged between 0.03 and 2.7%.

The ATIII binding fraction that was eluted with high salt is composed two unacetylated compositions, (1:2:3:8:0) and

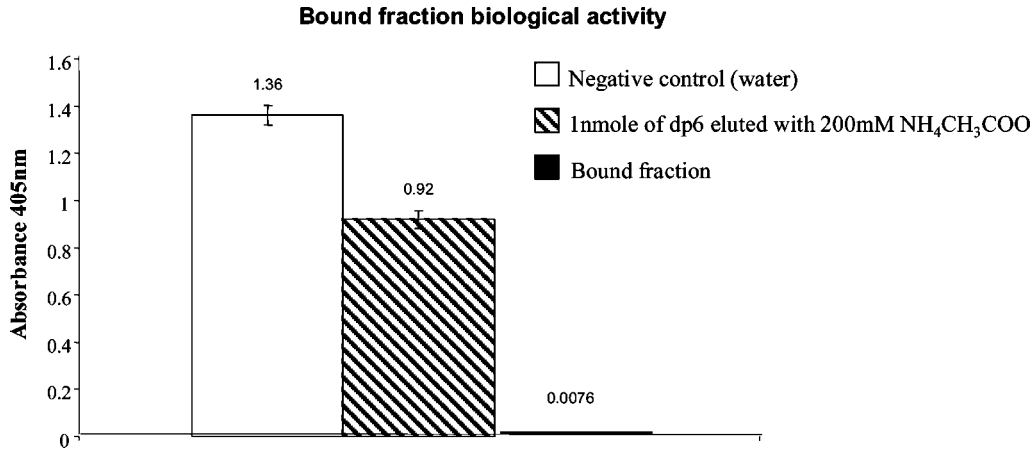

FIGURE 5: Anti-FXa activity of the bound fraction eluted with 2 M ammonium acetate. The ATIII-mediated inhibitory activity of heparin hexasaccharides on FXa was studied using a colorimetric assay. FXa is able to hydrolyze a synthetic substrate yielding a colored product detected at 405 nm. When FXa is incubated with its substrate in the presence of ATIII and heparin, its hydrolytic activity is inhibited. The 405 nm absorbance is inversely related to the anti-FXa activity of heparin used in the experiments. Experiments were conducted in triplicate.

(1:2:3:9:0). While the (1:2:3:8:0) composition represents the most abundant composition in the initial library with a relative abundance of 43.3%, the (1:2:3:9:0) composition becomes the predominant one in the bound fraction, accounting for 55.6% of the total. Original publications using a heparin hexasaccharide library generated by nitrous acid cleavage recovered binders carrying both *N*-sulfate and *N*-acetate at the nonreducing GlcN (*26*). It is now known that the nonreducing *N*-acetyl group is not essential for ATIII binding (*28*), and these results are confirmed by chemical synthesis. A synthetic pentasaccharide in which the *N*-acetyl group of the nonreducing GlcN was replaced with an *N*-sulfate has a K_d of 50 nM for ATIII (compared to a K_d of 80 nM for the N-acetylated pentasaccharide and an enhanced anti-FXa activity) (*57*). The absence of acetylated compositions in the bound fractions of this work indicates that those components, if present in the bound fraction, are under the limit of detection of the mass spectrometer used. More recently, Abzalimov et al. (*58*) have screened for ATIII binders in a heparin hexasaccharide library by spraying protein−oligosaccharide complexes. Their results corroborate ours in that only (1:2:3:8:0) and (1:2:3:9:0) bind ATIII with a higher propensity for the latter composition to bind.

The evidence that the high-salt elution fraction is specific was confirmed via several experiments. The results confirm that the devised protocol is able to eliminate any binding that is not dependent on the biological activity of ATIII or specific heparin sequence that binds ATIII. In addition, this fraction was assayed for its anti-FXa activity and this activity compared to that of the low-salt wash fraction. The observation that the high-salt fraction has significantly higher activity compared to the unspecific low-salt wash fraction strongly supports the conclusion that the (1:2:3:8:0) and (1:2:3:9:0) compositions are specific binders.

The method discussed in this work aims to solve the challenging task of identifying protein binding heparin oligosaccharides. Although ATIII is used as a model system, the method can be applied to any heparin binding protein using small quantities of protein. Some other proteins of interest belong to the growth factor family. Variation in the structures of HS on cell surfaces and the extracellular matrix is a mechanism whereby cellular responses to growth factor stimulation are modulated. This method will enable progress in the understanding of heparin/HS expression and growth factor binding.

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