Published in final edited form as:

Anal Chem. 2011 October 15; 83(20): 7815–7822. doi:10.1021/ac201498a.

Competitive Inhibition of Heparinase by Persulfonated Glycosaminoglycans: A Tool to Detect Heparin Contamination

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Abstract

Heparin and the low molecular weight heparins are extensively used as medicinal products to prevent and treat the formation of venous and arterial thrombi. In early 2008, administration of some heparin lots was associated with the advent of severe adverse effects, indicative of an anaphylactoid-like response. Application of orthogonal analytical tools enabled detection and identification of the contaminant as oversulfated chondroitin sulfate (OSCS) was reported in our earlier report. Herein, we investigate whether enzymatic depolymerization using the bacterially derived heparinases, given the structural understanding of their substrate specificity, can be used to identify the presence of OSCS in heparin. We also extend this analysis to examine the effect of other persulfonated glycosaminoglycans (GAGs) on the action of the heparinases. We find that all persulfonated GAGs examined were effective inhibitors of heparinase I, with IC_{50} values ranging from approximately 0.5–2 $\mu\text{g/mL}$. Finally, using this biochemical understanding, we develop a rapid, simple assay to assess the purity of heparin using heparinase digestion followed by size-exclusion HPLC analysis to identify and quantify digestion products. In the context of the assay, we demonstrate that less than 0.1% (w/w) of OSCS (and other persulfonated polysaccharides) can routinely be detected in heparin.

Heparin and its derivatives, including the low molecular weight heparins (LMWH), have critical importance and enjoy widespread use as prophylactic and therapeutic agents. Recently, the U.S. Food and Drug Administration identified the fact that certain lots of heparin were associated with adverse side effects including labored breathing, nausea, vomiting, excessive sweating, and rapidly falling blood pressure that in some cases result in life-threatening shock and even death.^{1–3} Initial structural analysis of suspect heparin lots

did not identify elevated levels of typical biological impurities, including protein, lipids, and DNA.⁴ Therefore, to determine the nature and extent of potential contamination, multiple analytical tools were employed to analyze suspect heparin lots.⁵ An orthogonal analytical approach, employing 1D and 2D NMR spectroscopy, enzymatic digestion, and LC-MS was able to definitely identify the contaminant as oversulfated chondroitin sulfate (OSCS). The presence of OSCS within heparin was unexpected and raised the question of whether additional highly sulfated polysaccharides could also be contaminants. To address this question, a systematic study examined structural signatures for persulfonated glycosaminoglycans, including dermatan sulfate, heparin, and so-called side-stream products, using NMR, both mono- and bidimensional, and CE.⁶ These studies identified the fact that each persulfonated component, if present, would have a set of unique structural signatures. Finally, mass balance efforts on lots containing OSCS indicated that other persulfonated polysaccharides were not present in suspect lots of 2008.⁷ Nevertheless, the potential for their introduction is possible, especially given that they likely have similar biological activities as OSCS.⁸

Advanced analytical technologies, including most notably multidimensional NMR, have demonstrated the ability to detect a wide range of potential sulfonated polysaccharides if present within heparin,⁶ since initial efforts to detect OSCS in heparin employ a variety of strategies, including HPLC,^{9,10} bioassays,¹¹ and spectroscopy.^{12,13} However, development of additional, orthogonal assays that can rapidly and sensitively detect the presence of potential contaminants, if present, is warranted. To this end, there have been several reports of potential methods, including ion exchange HPLC,¹⁰ quantitative capillary electrophoresis,¹⁴ inhibition of Taq polymerase in a real-time PCR,¹⁵ a fluorescent receptor array,¹⁶ and use of potentiometric polyanion sensors.¹⁷

In addition to the above methods, there has been some suggestion that the heparinases, bacterially derived enzymes that can be used to degrade heparin, may present another strategy toward the detection of OSCS and other persulfonated polysaccharides.^{5,18} To this end, we and others have defined the biochemistry of the heparinases, including their substrate specificity, cofactor requirements, and kinetic parameters.^{19–23} The heparinases specifically cleave the glucosamine (1 → 4) uronsyl linkage present in heparin/HS and absent from other glycosaminoglycans, including chondroitin sulfate, dermatan sulfate, and hyaluronic acid. As such, we reasoned use of one or more of the heparinases could be used to detect the presence of nonheparin impurities or contaminants in heparin preparations. Indeed, exhaustive digestion of suspect heparin lots results in a reduction of di-, tri-, and tetrasaccharide products,^{5,18} consistent with the fact that OSCS is refractory to enzymatic digestion.

In this study, we systematically examined the effect of persulfonated polysaccharides, including OSCS, over(per-)sulfonated dermatan sulfate (OSDS), and over(per-)sulfonated heparin (OSHP), on the enzymatic activity of the heparinases, in the attempt to devise a specific, sensitive assay to detect the presence of contaminants in heparin. We find that persulfonated polysaccharides are efficient inhibitors of the enzymes, especially heparinase I, and inhibit the enzymes in a competitive, reversible manner. We also provide a structural rationale for the observed inhibitory effect of persulfonated glycosaminoglycan (GAG) substrates on heparinase I by constructing a three-dimensional model of this enzyme with different substrates using the recently solved enzyme–substrate cocrystal structure.²⁴ Utilizing this understanding, as well as a detailed structural analysis, we devise a rapid, simple assay that can be used to screen heparins for the presence of persulfonated polysaccharides, including OSCS. As compared to other assays, we demonstrate in the present study that exploiting the enzymatic activity and specificity of the heparinases enables the detection of <0.1 wt % of persulfonated polysaccharides in heparin.

MATERIALS AND METHODS

All commercial reagents and kits were used directly without further purification. Heparin and heparan sulfate (HS) were obtained from Celsus Laboratories. Heparinases I, II, and III were expressed and purified from *E. coli* as described previously.^{21,25} Persulfonated GAG materials were obtained from R. Linhardt. The molecular weight for OSCS was found to be ~16 kDa, consistent with and on the basis of previous information.²⁶ The GAG assay kit was obtained from Kamiya Biomedical Company (Cat No. BP-004). TSK SW 3000 columns (G3000SW), 3-(*N*-morpholino) propanesulfonic acid (MOPS), disodium monohydrogen phosphate, sodium dihydrogen phosphate, and calcium acetate were obtained from Sigma-Aldrich.

Assay Conditions

In general, we employed enzymatic reaction conditions that have been previously optimized by us and others for the enzymatic action of heparinases I–III.¹⁹

Heparinase I—Assays were performed at 30 °C. When measuring the enzymatic activity as a function of heparin concentration, heparin concentrations were varied from 0 to 4 mg/mL at a fixed calcium (acetate) concentration of 5 mM in 100 mM MOPS, pH 7.0.

Heparinase II—The temperature for all enzymatic activity measurements was kept constant at 35 °C. With heparin as the substrate, the reaction is carried out at a concentration of 4 mg/mL in 50 mM sodium phosphate buffer, pH 7.3. With 2 mg/mL heparan sulfate, the reaction is measured in 50 mM sodium phosphate buffer, pH 6.9.

Heparinase III—All assays were performed with heparan sulfate at a concentration of 2 mg/mL in 50 mM sodium phosphate, pH 7.6. The temperature for enzymatic activity measurements was kept constant at 35 °C.

General Experimental Procedure to Measure the K_m Values for Heparinases I and II

Different amounts of heparin/HS substrate solution in a 1.6 mL eppendorf tube were allowed to equilibrate to the recommended temperature as noted above. Then, 1 mL of substrate (heparin or HS) solution (1 mg/mL) was placed into a quartz cuvette and inserted into a thermostatted compartment. Freshly prepared, diluted heparinase I/III (0.025 μ M) or heparinase II (0.2 μ M) was added using a 10 μ L pipet and mixed well immediately. After mixing, the change in absorbance was measured as a function of time for a maximum of 60 s to measure (V_o). Measurements were made every 3 s, monitoring at a wavelength of 232 nm. Apparent K_m values for each enzyme were measured through nonlinear fitting of the V_o vs initial substrate concentration (S_o) plot.

General Experimental Procedure to Measure the IC_{50} of Persulfonated GAG Preparations

Different amounts of persulfonated GAG (either OSCS, persulfonated dermatan sulfate [OSDS], or persulfonated heparin [OSHP]) were added to a constant amount of heparin/HS substrate solution (1 mg/mL) in a 2 mL eppendorf and incubated at the recommended temperature to allow for equilibration. Then, 1 mL of the substrate (heparin or HS) plus persulfonated GAG preparation was placed into a quartz cuvette and allowed to sit in a thermostatted compartment for at least 5 min. Freshly prepared, diluted heparinase I/III (0.025 μ M) or heparinase II (0.2 μ M) was added using a 10 μ L pipet and mixed well prior to measurement of the absorbance change with time as above. Initial velocity for each sample was measured and plotted against the concentration of exogenously added persulfonated GAG. From this plot, an IC_{50} value (concentration at which the enzyme activity has been

inhibited by 50%) was calculated. Subsequently, the inhibition constant, K_i , calculated in mass per unit volume, was determined on the basis of the standard equation:

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[S]_0}{K_m}\right)}$$

using the measured values for IC_{50} and K_m for the enzyme, persulfonated GAG pair. In the case of heparinase I/OSCS, to examine mode of inhibition, a Line-Weaver Burke plot analysis was performed, measuring enzyme velocity in the presence of OS CS at various initial substrate concentrations. The plot of $1/v$ against $1/[S]$ can then be used to describe the nature of inhibition (i.e., competitive or noncompetitive inhibition).

$$\frac{1}{v} = \frac{(K_m + [S])}{V_{\max} \times [S]}$$

where v is the reaction velocity, K_m is the Michaelis–Menten constant, $V_{\max}$ is the maximum reaction velocity, and $[S]$ is the substrate concentration. Because only initial kinetic measurements were taken, $[S] \approx [S]_0$, and therefore, the initial concentration of substrate was used.

Analysis of Heparinase I Crystal Structure

To enable a structural understanding of the observed biochemical data, the crystal structure of heparinase I in complex with a dodecasaccharide (PDB 3INA) was used as a starting point.²⁴ Heparinase I in 3INA is a H151A-inactive mutant; therefore, this was first reversed using homology modeling. The dodecasaccharide (UroA-(2S)1-4Glc(NS, 6S)1-4IdoA(2S)1-54Glc(NS,6S)) in 3INA contains the preferred IdoA at +1 and makes optimal contacts needed for catalysis. In the next step, a suitable substrate having a GlcA in place of the preferred IdoA (+1) was obtained and docked to heparinase I. The latter was obtained from the cocrystal of heparin-like pentasaccharide and antithrombin III (PDB: 1AZX). The pentasaccharide in 1AZX is Glc(2S,3S,6S)-1-4GlcA-1-4Glc(2S,3S, 6S)1-4IdoA(2S)1-4Glc(2S,3S, 6S). The pentasaccharide from 1AZX was docked to heparinase I initially by superposing the atoms of the GlcA with the IdoA (+1) atoms found in the dodecasaccharide. Only the portion corresponding to -1, +1, and +2 subsites of the substrate were used. Following this, the new enzyme–substrate model was minimized using the Discover module in Insight II. The potentials for all structures were assigned using DISCOVER force field using default parameters. The minimization was constrained by forcing the backbone atoms of the enzyme and the substrate to remain fixed. The refined model was then subject to 500 iterations of steepest descent minimization without including charges and 500 iterations of conjugate gradient minimization including charges to obtain the final model complex of heparinase I and GlcA (+1) carrying heparin. The dodecasaccharide from 3INA was modified to ensure that 2-O and 3-O positions of all iduronic acids and 2-O, 3-O, 6-O, and N positions of all glucosamines were sulfated. Only the subsites corresponding to -2, -1, +1, and +2 positions were considered. Following this, the complex was minimized using the same protocols mentioned above. The structure of OS CS was obtained from PDB 1OFM. The OS CS sequence in the crystal structure 3GalNAc(4S)1-4GlcA-1-3GalNAc(4S) was modified to include additional sulfate groups where possible. The resulting substrate (3GalNAc(4S,6S)1-4GlcA(2S, 3S)1-3GalNAc(4S,6S)) was then docked to heparinase I by placing the atoms of the GlcA subunit in place of the IdoA(+1) atoms found in the dodecasaccharide in 3INA. The orientation was manually adjusted to eliminate unfavorable steric contacts with the amino

acids of the enzyme active site. The model complex then was minimized using the protocols mentioned above.

HPLC Analysis of over Sulfated GAG after Enzymatic Digestion Using Heparinase as Enzyme

Prior to HPLC analysis, ~1 mg of heparin or mixture of heparin and OSCS, OSDS, or OSHP were suspended in sodium acetate buffer, pH = 7.0, and incubated at 30 °C for about 10 min; subsequently, 1.5 μ L (0.35 μ M) of heparinase I was added and incubated in a water bath for varying times (from 30 min to overnight) to obtain primarily di- and tetrasaccharide products.²⁷ To determine the inhibition potential of various persulfonated GAGs, OSCS, OSDS, or OSHP was spiked into heparin, digested with heparinase I, and analyzed by HPLC. 200 μ L (~200 μ g) was injected to the HPLC, and digestion products were separated and quantified using two TSK SW3000 columns (G3000SW, L $\times$ I.D. 60 cm $\times$ 7.5 mm, 10 μ m particle size) connected in a series using an isocratic solvent system of 0.2 M sodium sulfate, pH = 5.0 with UV detection at 232 nm. To confirm peak assignments, a mixture of heparin di- and tetrasaccharide standards (Dextra Laboratories) were used to standardize the column. Three different concentrations of each persulfonated GAG (0.125%, 0.50%, and 2.2%) measured in triplicate were assessed by comparing with the control sample. Triplicate samples were prepared and assessed independently to determine and average value as well as error.

RESULTS

Differential Inhibition of Heparinases I, II, or III by Oversulfated Chondroitin Sulfate

Several studies have examined the biochemical and biophysical attributes of the heparinases.^{19,20} Broadly, the heparinases are all lyases, acting through eliminative cleavage to depolymerize polysaccharides containing 1,4-linked -D-glucuronate or -L-iduronate residues and 1,4-linked 2-(sulfo)amino-2-deoxy-6-(sulfo)-D-glucose residues. The result of enzymatic action is the formation of terminal 4-deoxy- -D-gluc-4-enuronosyl groups at the nonreducing end of the newly formed chain; this moiety is UV active and can be readily monitored ($\lambda_{\text{max}} = 232$ nm). In terms of their substrate specificity, heparinase I cleaves primarily the highly sulfated regions of heparin/HS at 2-O sulfated uronic acids; heparinase III cleaves primarily undersulfated regions of heparin/HS at unsulfated uronic acids. Heparinase II has the broadest substrate specificity of the enzymes. As such, heparinases I and II extensively depolymerize heparin whereas heparinase III does not.²⁸ The measured k_{cat} for heparinase I is approximately 90 s⁻¹ and that of heparinase III is ~80 s⁻¹; as such, these enzymes are equally “fast”.^{19,21,23,25} Heparinase II, on the other hand, is a slower enzyme, with an approximately 10-fold lower k_{cat} .^{19,21,29}

To understand the effect of OSCS on heparinase I activity, OSCS (1 μ g/mL) was added to various concentrations of heparin. This resulted in a dramatic shift in the observed K_{m} value for heparinase I with little to no change in the V_{max} . Taken together, measurements of initial velocity of the enzyme in the absence or presence of OSCS indicate that OSCS is a competitive inhibitor of heparin (Figure 1). To extend this analysis, plotting $1/v$ (initial reaction velocity) vs $1/[S]_0$ (initial substrate concentration) for different OSCS concentrations indicated that the lines share the same y-intercept but have different x-intercepts, confirming that OSCS is a competitive inhibitor of heparinase I. To extend this further analysis, the IC₅₀ value of OSCS was determined by increasing the amount of OSCS in a constant, saturating amount of heparin (1 mg/mL). Consistent with OSCS being a competitive inhibitor, an increase in added OSCS results in a concomitant decrease in V_0 . The IC₅₀ value and inhibition constant (K_i) of OSCS are found to be 0.5 μ g/mL (~30 nM) and 0.2 μ g/mL (~10 nM), respectively (Figure 2). This result suggests that presence of less

than 0.5 μg of OSCS per milligram of heparin (0.05% w/w) can significantly inhibit the enzymatic activity of heparinase I and be detected, making this a potentially sensitive method to detect OSCS. All the kinetics experiments to determine K_m of Hep I and IC_{50} of OSCS for Hep I were performed multiple times in different days. The K_m and IC_{50} value is exactly matched with the unit shown in Figures 1 and 2. Furthermore, two different batches of Hep I was used for the kinetic study of enzyme catalysis. The K_m of Hep I for both batches with respect to the same substrate of heparin showed a similar result.

To extend the analysis and to understand the inhibition potential of OSCS toward heparinases II and III, we completed an identical set of experiments with heparinases II and III. In the present study, with heparin as substrate for heparinase II and HS as substrate for heparinase III, we observe that OSCS is a less efficient inhibitor of heparinases II and III. The IC_{50} value and K_i for OSCS-based inhibition of heparinase II are found to be 25 $\mu\text{g/mL}$ and 4 $\mu\text{g/mL}$ ($\sim 1.5 \mu\text{M}$ and $\sim 0.3 \mu\text{M}$), respectively (Figure 3), whereas for heparinase III, the observed values are much lower, approximately 700 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ ($\text{IC}_{50} = \sim 40 \mu\text{M}$ and $K_i = \sim 15 \mu\text{M}$), respectively (data not shown). Therefore, on the basis of the above information, we find that the inhibition potential of OSCS decreases in moving from Hep I > Hep II > Hep III. This result suggests closer inspection of the inhibition of Hep I by other persulfonated GAGs to determine the best path forward in the design of a sensitive, specific quality control test.

Comparison of the Heparinase I Inhibition Using Three Different Persulfonated GAGs

Previous studies have identified the fact that other GAGs may be present in side-stream heparin, suggesting there is the potential for contamination with these GAG sources.^{6,30} Specifically, we examined the two most prevalent, OSDS and OSHP. Similar to the studies for OSCS, we determined the inhibitory potential of OSDS and OSHP. The results of these analyses are shown in Table 1. Inspection of this data and comparison to the data from Figure 2 indicates that heparinase I is potently inhibited by both OSDS and OSHP. For heparinase I, OSDS showed less, but still potent, inhibitory capacity (approximate $\text{IC}_{50} = 1.8 \mu\text{g/mL}$) compared to OSCS whereas OSHP showed very similar inhibition capacity (approximate $\text{IC}_{50} = 0.85 \mu\text{g/mL}$). Taken together, these results indicate that the activity of heparinase I is sensitive to wt % levels of ~ 0.1 – 0.2% for many persulfonated glycosaminoglycans.

Structural Rationale for Observed Inhibition

Experimental data demonstrated that the persulfonated GAGs were all competitive inhibitors of heparinases I–III, with heparinase I demonstrating the most marked effect, and that there seemed to be a slight preference for OSCS and OSHP over OSDS. The preference shown by heparinase I in the choice of uronic acid (IdoA over GlcA) at the reducing end (+1 subsite) of its heparin substrate was investigated using the cocrystal structures of heparinase I in complex with different heparin substrates.²⁴ The heparin from 1AZX docks well into the active site of heparinase I. Comparison between 3INA and the model complex reveals that the directionality of the C5 proton on the +1 subsite, which plays a critical role in the cleavage mechanism, is opposite in the two bound substrates. In the case where there is IdoA in the +1 subsite, the C5 proton faces the side chain of H151, facilitating a hydrogen bond between the N 2 of histidine and the C5 proton, thereby enabling proton abstraction (Figure 4A). The distance between the N 2 atom of H151 and the C5 proton is 2.8Å. In contrast, the C5 proton of GlcA (+1) in the model complex faces away from H151 side chain (distance equal to 5.4Å) (Figure 4B). This prohibits the necessary bond formation resulting in loss of catalytic function. In summary, heparin having GlcA at +1 has an inhibitory effect on heparinase I.

The above investigation was further extended to understand contacts between heparinase I and persulfonated GAG substrates including persulfonated heparin (contains a 2-O and 3-O sulfated iduronic acid) and persulfonated chondroitin sulfate. The modified substrate (IdoA 2S,3S-GlcNS,3S,6S-IdoA 2S,3S-GlcNS,3S,6S) docks well into the enzyme active site. From the original 3INA complex structure, both the C5 proton and the 3-O position of IdoA (+1) face the N 2 atom of H151. Analysis of the model complex shows that a sulfate group attached to the 3-O position causes the plane of H151 to shift which might disable proton abstraction from the C5 atom (Figure 4C). Thus, the presence of 3-O sulfate group on IdoA (+1) has an inhibitory effect on heparinase I. Within this context, OSCS docks well into the enzyme active site; however, the C5 of GlcA(+1) faces away from the N 2 atom of H151 (distance equal to 5.4 Å), which likely prohibits proton abstraction from OSCS (Figure 4D). Taken together, these results confirm and extend the biochemical findings, viz., OSCS, and other persulfonated glycosaminoglycans are potent inhibitors of heparinase I.

HPLC as Tool to Estimate the Inhibition Potential of over Sulfated GAG

Having developed a robust understanding of the biochemical and structural role of OSCS/OSDS/OSHP mediated inhibition of heparinase I, we sought to develop an assay that could rapidly determine whether a given heparin preparation contained one or more persulfonated compounds. On the basis of the kinetic analyses (above), persulfonated GAGs strongly inhibit heparinase I. However, a kinetic analysis alone would likely not possess the necessary specificity for the detection of persulfonated contaminants. This is because it is known that heparinase activity can be inhibited by other potential impurities, including metal ions.³¹ Therefore, to increase the specificity (and utility) of an enzyme-coupled assay to detect persulfonated GAGs, we completed HPLC profiling of samples obtained from heparinase digestion of either pure heparin (control) or a mixture of heparin and persulfonated GAG. Several types of chromatography have been used to separate and quantify enzymatic digestion products, including strong anion exchange and reverse phase HPLC. However, we reasoned that size-exclusion chromatography (SEC) would be superior to other options since the elution times of size-defined fractions can be easily benchmarked using commercially available standards. In addition, SEC is amenable to both the use of dual detector systems, such as detection by UV absorbance and refractive index. This enables not only direct detection of enzymatic products but also detection of contaminant, if present, and undigested material. Finally, an SEC method does not involve the use of complex buffer or gradient systems, making it ideally suited for a quality control environment.

To standardize the analysis, a mixture of 4,5-containing diand tetrasaccharide standards were used to benchmark elution time for each of the components and to verify sytem/ column operation. An equal mass of both di- and tetrasaccharide was injected onto the HPLC, but the elution time and area under the curve showed an AUC ratio of approximately 20:80 (expected 33:67), demonstrating that the different components likely have a different molar extinction coefficient. Digestion of heparin by heparinase I for 30 min and separation of the resulting enzymatic products resulted in primarily the presence of di- and tetrasaccharides, with some hexasaccharides, as reported previously.^{32,33} Addition of increasing amounts of OSCS resulted in dramatic changes to the profile, both in terms of the quantitative amount (AUC) of di- and tetrasaccharide peaks, which were reduced substantially, as well as the appearance of longer oligosaccharide fragments (Figure 5). Notably, and as predicted from the biochemical studies, addition of 0.125 wt % OSCS resulted in a substantial drop in AUC compared to control digestions, and furthermore, increasing the amount of spiked OSCS results in a systematic reduction of the AUC for both the tetra- and disaccharide products. Thus, the LOD of the OSCS detection in heparin is found to be 0.06% of the total substrate used of the enzymatic reaction.

To test whether the inhibition is reversible, enzyme was added and the reaction was carried out overnight. The overnight reaction shows that more di- and tetrasaccharide peaks are formed compared to the reaction carried out for 30 min in the presence of OSCS (0.25%). Furthermore, addition of either OSDS or OSHP showed a similar reduction of peak AUC (Figure 6). Extrapolation of the AUC reduction for both the di- and tetrasaccharides indicates that, for OSCS, OSDS, and OSHP, the presence of approximately 2.2–2.5 wt % of persulfonated GAG results in no detectable levels of di- and tetrasaccharide products.

DISCUSSION

The heparinases have become essential tools in the analysis of heparin and heparan sulfate.³⁴ A cocktail consisting of heparinases I–III is often used for compositional analysis of heparin and heparin sulfate preparations and in conjunction with either HPLC or capillary electrophoresis has proven to be a powerful tool for the identification and quantification of heparin/HS composition.^{27,35} Furthermore, partial digestion with one or more of the enzymes has been utilized to obtain sequence information.³³ While NMR-based procedures were primarily used in the initial identification of OSCS as the primary contaminant in heparin preparations, enzymatic digestion coupled with HPLC also proved to play an important role. Since this initial report, limit digests of heparin coupled to LC-MS have been employed to identify the presence of OSCS in heparin.¹⁸

Many strategies have looked at the use of heparinases I–III as an enzyme “cocktail” followed by exhaustive depolymerization of heparin and analysis of the products by HPLC or LC-MS.³⁶ We reasoned that one could take advantage of the inherent substrate specificity of the enzymes to detect contaminants, such as OSCS in a more rapid and potentially more sensitive manner. Numerous biochemical studies have identified the fact that the heparinases, with overlapping but distinct substrate specificities, more efficiently cleave intersugar glycosidic linkages in heparin and HS, leaving intact other glycosaminoglycan linkages, including those present in chondroitin sulfate, dermatan sulfate, and hyaluronic acid.^{22,37,38} Furthermore, the presence of 3-O sulfation glucosamine is known to inhibit heparinase action,^{39,40} suggesting that persulfonated GAGs may inhibit heparinase action. Given this understanding, in the present work, we completed extensive kinetic analysis to understand the mechanism and extent of OSCS-mediated inhibition of heparinases I–III and extend this analysis to other persulfonated GAGs.

The results presented here indicate that OSCS (and by extension OSDS and OSHP) is a reversible inhibitor of the heparinases, with OSCS inhibiting heparinase I to a much greater extent (higher potency) than either heparinase II or III. Therefore, our results, presented here, demonstrate that the heparinases, especially heparinase I, can be used as an effective tool to screen heparin. Given the fact that <0.17% OSCS inhibits the enzymatic action of heparinase I and that the use of a single enzyme instead of a cocktail results in a more controlled digest, requiring less materials and potentially reducing variability, we analyzed the ability of a heparinase I digest to detect the presence of persulfonated materials.

To extend this analysis, we sought to incorporate these findings into a robust, routine quality control test. We first considered whether a kinetic assay, potentially measuring a change in absorbance at 232 nm as a function of time, a measure of enzyme activity, could be used to detect the presence of persulfonated contaminants. However, specificity of this type of assay is not assured since the presence of several components within a heparin preparation could inhibit the heparinase I enzyme, including high salt concentration.¹⁹ Therefore, to improve the specificity of the test and provide a useful quality control tool for heparin, we employed a rapid digestion of heparin with heparinase I, followed by separation and quantification of the products by SEC. The products of enzymatic digestion of heparin by heparinase I have

been defined previously,^{27,32} and we find that separation of the products provides additional information, enabling the robust and sensitive detection of persulfonated GAGs, if present. Finally, such an assay system is easily implemented and is able to broadly detect the presence of persulfonated contaminants, making it amenable to use as a screening tool for heparin and low molecular weight heparin preparations.

In summary, the results provided demonstrate that enzymatic tools can be used for quality control of heparin. By providing an additional level of specificity and an easily interpreted readout, such an assay should prove useful in the analysis of heparin and assuring its purity and identity.

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Figure 1. Comparative enzymatic activity of heparinase I with () or without () addition of 1 $\mu\text{g/mL}$ OCS.

Figure 2. Inhibition of heparinase I by OSCS. The IC₅₀ value was determined using different concentrations of OSCS from 0 to 2.1 μg/mL with the constant amount of heparin, 1 mg/mL as substrate, and heparinase I (0.25 μM) as enzyme.

Figure 3.

Measuring the ability of OSCS to inhibit heparinases II. Inhibition of heparinase II activity was measured using different concentrations of OSCS from 0 to 210 $\mu\text{g/mL}$ with a constant amount of heparin (1 mg/mL) as substrate.

Figure 4. Structural complex of heparinase I with different GAG oligosaccharides. The active site of Hep I is shown as a cartoon representation (in gray) with the side chains of the key active site residues labeled. The basic residues Lys, His, and Arg are colored blue, and Tyr is colored orange. A trisaccharide motif comprising the cleavable hexosamine–uronic acid linkage is shown in stick representation colored by element (carbon in green for hexosamine and violet for uronic acid, oxygen in red and sulfur in yellow). The C5 proton of uronic acid is shown in white. (A) Demonstration of the critical interaction of H151 with the C-5 proton of iduronic acid in the heparin substrate (black dotted line). (B) In contrast, the C-5 proton of glucuronic acid is oriented such that it faces away from H151 and hence is not favorably positioned for abstraction. (C) The presence of a 3-O sulfate on uronic acid (red dotted circle) in persulfonated GAGs causes the plane of the H151 residue to change its orientation, making it unfavorable for abstraction of C-5 proton. (D) Consistent with observation in (B), the C-5 proton of the glucuronate in OSCS faces away from H151.

Figure 5.

HPLC analysis to detect OSCS contamination in heparin. (A) Injection of a standard of 1:1 di/tetrasaccharide mixture and analysis by HPLC. The elution time of each peak and area under the curve was taken as standard representation to measure product formation after enzymatic reaction. (B) HPLC profile of clean heparin after treatment with heparinase I. (C) Different weight percents of OSCS in heparin, obtained by spiking, were subjected to digestion with heparinase I. The AUC of di- and tetrasaccharides was measured for each after a limit digestion; results are plotted as a function of weight percent of OSCS. The control digestion yielded AUCs of $42\,700 \pm 700$ for disaccharide () and $15\,200 \pm 80$ for tetrasaccharide ().

Figure 6. Detection of persulfonated GAGs by heparinase digestion and analysis by HPLC. The AUC of di- and tetrasaccharides obtained upon heparin digestion in the presence of various amounts of persulfonated GAGs, either (A) OSDS or (B) OSHP.

Table 1

Inhibition of Heparinase I by Persulfonated Glycosaminoglycans

polysaccharide ($\mu\text{g/mL}$)	% activity (+OSDS)	% activity (+OSHP)
0	100	100
0.5	68.3	72.9
1	53.7	47.6
2	48.8	37.3
4	31.7	ND ^a
5	ND	22.0

^aND = not determined.