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Fluorescent Tagged Heparan Sulfate Precursor Oligosaccharides to Probe the Enzymatic Action of Heparitinase I

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Abstract

Heparitinase I, a key lyase enzyme essential for structural analysis of heparan sulfate (HS), degrades HS domains which are undersulfated at glucuronyl residues through an elimination mechanism. Earlier studies employed viscosimetric measurements and electrophoresis to deduce the mechanism of action of heparitinase I and two other related lyases, heparitinase II and heparitinase III. However, these findings lack molecular evidence for the intermediates formed and could not distinguish whether the cleavage occurred from the reducing end or non-reducing end. In this report, 2-aminoacridone (2-AMAC) labeled HS precursor oligosaccharides of various sizes were prepared to investigate the mechanism of heparitinase I mediated depolymerization using sensitive and quantitative methodologies. Furthermore, fluorescence (2-AMAC) tagging of heparan sulfate precursor oligosaccharides allowed us to distinguish fragments that result from cleavage of the substrates at various time intervals and sites further away from the reducing and non-reducing ends of oligosaccharide substrates. This study provides first direct molecular evidence for a predominantly random endolytic mechanism of elimination of HS precursor oligosaccharides by heparitinase I. This robust strategy can be adapted to deduce the mechanism of action of other heparitinases and also to deduce structural information of complex heparan sulfate oligosaccharides of biological importance.

Keywords

AMAC-labeling; heparan sulfate; heparitinase; heparosan oligosaccharides; mass spectrometry

Introduction

Heparan sulfate (HS) proteoglycans regulate a number of physiological and pathological functions including development, morphogenesis, angiogenesis, and immune response.[1;2]. HS and heparin polysaccharides are characterized by repeating disaccharide units of glucosamine α (1–4) linked to uronic acid (GlcA/IduA). The first known enzyme that degrades

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Supporting Information Available. Complete LC-MS spectra of various oligosaccharides ranging from DP2-AMAC to DP18-AMAC and HPLC-fluorescence chromatograms of digestion mixtures of DP8-AMAC are provided.

heparin was originally isolated from *Flavobacterium heparinum* by Korn and Payza.[3] Linker *et al.* further characterized a number of enzymes that depolymerize heparan sulfate from heparin-induced cultures of *F. heparinum*. [4;5] Subsequently, several studies reported isolation, purification, cloning and recombinant expression of heparitinase I (EC 4.2.2.8), heparitinase II (no EC numbering), and a heparinase (heparitinase III; EC 4.2.2.7). [6;7;8;9; 10;11] The discovery of heparitinases (I, II and III) and their substrate specificity eventually helped to resolve the structure and function of antithrombin III (ATIII) binding heparin and HS anticoagulants. [12;13]

The substrate specificity of all three heparitinases is well established using a number of substrates including heparin, completely desulfated N-sulfated-heparin (CDSNS-heparin), completely desulfated N-acetyl-heparin (CDSNAC-heparin), HS, chondroitin sulfate, heparosan polysaccharides (both N-acetylated and N-sulfated), and C-2/C-6 sulfated heparosan polysaccharides. [8;14;15] All of these studies have lead to the following conclusions; i) heparitinase I cleaves HS polysaccharides containing glucuronyl residues, linked to either N-acetyl or N-sulfated glucosamine units and thus heparin is a poor substrate; ii) heparitinase II cleaves GlcA(2S)/IduA- α (1-4)-GlcNS linkages; iii) heparitinase III (also known as heparinase I) cleaves sulfated iduronyl residues. Heparitinases have extensively been used in sequencing and deducing the structure of heparin oligosaccharides and also used to determine HS disaccharide composition of various cellular origins. [16;17] The mechanism of depolymerization of heparitinase II and III has been well studied by measuring of viscosity of heparin containing solutions. [18] Heparitinase III has been shown to act predominantly in a processive exolytic fashion on heparin oligosaccharides using mass spectrometric technique. [19] On the other hand, heparitinase II has been shown to cleave both heparin and HS in a non-random endolytic manner. [20] The action pattern of heparitinase I on heparan sulfate, which contains both highly sulfated, epimerized NS domains and non-sulfated, less epimerized NA domains, is suggested to be endolytic based on viscosimetric measurements and gradient PAGE analysis. [21] However, current methods lack sensitivity, quantitative estimation, and structural evidence for intermediates formed upon enzymatic action of heparitinases. Furthermore, there is no report yet, investigating the mode of action of heparitinase I on size defined HS precursor oligosaccharides to obtain direct molecular evidence. A number of sensitive mass spectrometric methods have been developed to obtain detailed structural information about heparin like disaccharides and oligosaccharides. [22;23;24;25;26]

This report describes fluorescent labeling of heparosan oligosaccharides with 2-aminoacridone and size separation of 2-AMAC labeled oligosaccharides of various sizes up to DP54-AMAC using high performance liquid chromatography-size exclusion (HPLC-SEC) for the first time. Homogeneous, size fractionated 2-AMAC tagged oligosaccharides were utilized in the current study to determine the mode of action of heparitinase I. The relative quantitation of all the possible intermediates by fluorescence-HPLC and their molecular characterization by liquid chromatography-mass spectrometry (LC-MS) unambiguously suggested random endolytic action of heparitinase I on DP20-AMAC and DP8-AMAC HS precursor oligosaccharides.

Materials and Methods

Preparation and partial digestion of K5 heparosan polysaccharide was performed as reported in the literature. [27] Heparitinase I was cloned and expressed following a published procedure. [11] All chemicals including 2-aminoacridone, glacial acetic acid, dimethylsulfoxide, sodium cyanoborohydride, ammonium acetate, dibutylamine, triethylamine, and HPLC grade acetonitrile/water were obtained from Sigma-Aldrich, unless otherwise stated.

AMAC labeling of heparosan oligosaccharides

2-AMAC labeling of heparosan oligosaccharides was performed using a modified procedure reported for the labeling of heparan sulfate disaccharides.[28] Briefly, purified heparosan oligosaccharides were dissolved in 20 μ L of water and then reacted with 20 μ L of 0.1 M 2-AMAC in 85% DMSO/15% acetic acid and incubated at room temperature for 20 min. Then, 20 μ L of 1 M sodium cyanoborohydride was added to the reaction mixture and the reaction was allowed to continue for 18 h at room temperature. The excess cyanoborohydride was destroyed by addition of a few drops of glacial acetic acid and then the reaction mixture was dried using a centrifugal concentrator under reduced pressure. The dried reaction mixture was extracted with water and the aqueous layer containing 2-AMAC tagged oligosaccharides was finally purified on a size exclusion HPLC column as described in the following section.

HPLC-size exclusion chromatography

2-AMAC labeled heparosan oligosaccharides were purified using HPLC (Hitachi High Technologies America, Inc., Pleasanton, CA) coupled to two serially connected size exclusion columns (TSL Gel G2000SW, 7.8 mm $\times$ 30 cm, TOSOH Bioscience, LLC.). The products were eluted with ammonium acetate buffer (0.2 M, pH 5.0) at a flow rate of 0.75 mL/min and monitored with a fluorescence detector (λ_{ex} = 425 nm and λ_{em} = 520 nm). Using this method, various oligosaccharide sizes ranging from DP2-AMAC to DP54-AMAC were resolved. HPLC fractions were collected using an automated fraction collector and the desired oligosaccharide fractions were then dried under reduced pressure using the centrifugal concentrator. The quantity of each 2-AMAC labeled oligosaccharide was determined by measuring absorbance at 260 nm (ϵ = 40,000). Purified oligosaccharides were characterized by ESI-MS in the negative ionization mode as described in the following section.

High pressure liquid chromatography coupled to ESI-MS

All mass spectrometric data were acquired using high pressure HPLC coupled to a LCT Premier XE electrospray ionization time-of-flight (ESI-TOF) mass spectrometer (Waters Corporation, Milford, MA). For LC analysis, samples were separated by reverse phase ion-pairing (RP-IP) chromatography using Acquity UPLC BEH C18 1.7 μ m 2.1 $\times$ 100 mm column (Waters Corporation, Milford, MA). A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% aqueous acetonitrile (eluent B), both containing 8 mM acetic acid and 5 mM ion-pairing agent [27]. In the negative-ion mode, the instrument was calibrated using a solution of NaI (2 ng/ μ L, Sigma-Aldrich) in 1:1 water/isopropyl alcohol through the reference nozzle. Nitrogen was used as a desolvation gas as well as a nebulizer. Resolution was measured as full width at half maximum (FWHM), which was at least 5000 ppm for all spectra. Conditions for ESI-MS were as follows: cone gas flow 50 L/h, nozzle temperature 140 $^{\circ}$ C, drying gas (N_2) flow 400 L/h, spray tip potential 2.8 kV, nozzle potential 50 V. Negative ion spectra were generated by scanning the range of m/z 200–2500. The spectra were analyzed using MassLynx V4.1 software.

Heparitinase I depolymerization of DP20-AMAC and DP8-AMAC

Substrates, DP20-AMAC (23 μ g) and DP8-AMAC (10 μ g), were depolymerized with 0.2 mU of heparitinase I (EC 4.2.2.8) in 100 μ L of 40 mM ammonium acetate buffer (pH 7.4) containing 3.3 mM CaCl_2 at 37 $^{\circ}$ C. Aliquots of 14 μ L sample from the reaction mixture was withdrawn at 1, 5, 10, 20, 30, 60 min and 36 hr intervals, boiled at 100 $^{\circ}$ C for 1 min to inactivate the enzyme and centrifuged to remove any pellet formed. All the aliquots were diluted to 100 μ L with deionized water prior to HPLC analysis. The percentage fluorescence intensities reported in table I and table II are an average of two independent depolymerization experiments.

Results

Preparation of 2-AMAC labeled heparosan oligosaccharides

Heparosan polysaccharide was prepared from *E. Coli* K5 strain by fermentation and was subjected to controlled depolymerization with heparitinase I as described in the experimental section to obtain oligosaccharides of larger sizes in sufficient quantities. After heparitinase I digestion of heparosan polysaccharides, the reaction mixture containing various sizes of heparosan oligosaccharides was concentrated and then labeled with the fluorescent tag, 2-AMAC, in the presence of sodium cyanoborohydride.[28;29] The aqueous extract of the reaction mixture was subjected directly to HPLC-SEC analysis using two sequentially connected size exclusion columns. The elution of the 2-AMAC labeled heparosan oligosaccharides from size exclusion column was monitored with the aid of a fluorescence detector. 2-AMAC labeled oligosaccharides were excited at 425 nm and the emission fluorescence was monitored at 520 nm. It was observed that 2-AMAC labeled oligosaccharides were retained more strongly than the corresponding unlabeled oligosaccharides. This unusually high retention time of 2-AMAC labeled oligosaccharides is possibly due to the non-specific hydrophobic interaction of aromatic part of the 2-AMAC with packing material of the size exclusion columns. However, it is noteworthy that this interaction is highly beneficial and facilitated the separation of 2-AMAC labeled oligosaccharides. In fact, we were even able to resolve 2-AMAC labeled 54 mer (DP54-AMAC) for the first time using size exclusion column (Figure 1). Size fractionated 2-AMAC labeled heparosan oligosaccharides were analyzed by mass spectrometry to confirm their purity and identity.

Mass spectrometric characterization of AMAC labeled heparosan oligosaccharides

In order to confirm the purity of 2-AMAC labeled oligosaccharides, DP2-AMAC to DP20-AMAC oligosaccharides were separated using high-pressure Aquity C18 column and then analyzed by ESI-MS in the negative ionization mode. As expected, higher molecular weight oligosaccharides showed multiple ion peaks in the negative ionization mode (Figure 2). DP2-AMAC appeared as a single molecular ion at m/z 572.17 corresponding to $[M-H]^{1-}$, DP4-AMAC gave both singly and doubly charged molecular ions at m/z 951.29 and 475.13 corresponding to $[M-H]^{1-}$ and $[M-2H]^{2-}$ (see supporting information for complete range of MS spectra). 2-AMAC labeled hexasaccharide appeared as a major peak at m/z 664.70 corresponding to $[M-2H]^{2-}$ and as a minor peak at m/z 1330.44 corresponding to $[M-H]^{1-}$ whereas the 2-AMAC-labeled octasaccharide showed peaks at m/z 854.25 $[M-2H]^{2-}$ and 589.16 $[M-3H]^{3-}$. Triple and quadruple charged molecular ion peaks for AMAC-labeled DP18 and DP20 oligosaccharides appeared at m/z 1201.0 & 900.5 and 1327.39 & 995.29 corresponding to $[M-3H]^{3-}$ and $[M-4H]^{4-}$, respectively. The ESI-MS spectra of selected oligosaccharides from DP2-AMAC to DP20-AMAC are shown in figure 2 (see supporting information for complete range of MS spectra). These mass spectrometric data confirmed both the purity of 2-AMAC labeled heparosan oligosaccharides and their identity.

Mode of action of heparitinase I on size defined AMAC labeled oligosaccharides

Heparitinase I preferentially acts on substrates containing GlcA-GlcNAc(NS) disaccharide units. Unlike other two lyases, heparitinases II and III, the mode of action of heparitinase I is not well studied. Heparosan oligosaccharides contain GlcA residues and therefore are good substrates for heparitinase I. The mode of action of heparitinase I was investigated with two well-characterized 2-AMAC labeled heparosan oligosaccharides, DP20-AMAC and DP8-AMAC, which carry the fluorescence tag at the reducing end. The progress of the lyase reaction was monitored very conveniently using HPLC system coupled to a fluorescence detector. Furthermore, fluorescence detection is much more sensitive than measuring UV absorption at 232 nm at distinguishing the generation of $\Delta^{4,5}$ unsaturated glucuronyl residues at the non-reducing end of the intermediate products of depolymerization, and therefore only a small

quantity of substrate is required for monitoring the lyase action. Another important advantage of fluorescence tagging is that there is a mass difference of 194 amu between reducing and non-reducing end oligosaccharide intermediates, i.e. fluorophore carrying and non-carrying oligosaccharide intermediates, produced during enzymatic depolymerization. This information allowed us to establish the mechanism of action of heparitinase I unequivocally and provided first direct molecular evidence for intermediates formed upon digestion of heparosan oligosaccharides.

2-AMAC labeled DP20 oligosaccharide (25 μ g) was incubated with heparitinase I (0.2 mU) at 37 °C. Aliquots were withdrawn from the reaction vial at various time intervals, 1, 5, 10, 20, 30, 60 min, of enzymatic reaction and at the end of the incubation, and were quenched immediately by heating them at 100 °C in boiling water for 1 min. These samples were diluted with 100 μ l deionized water and analyzed by size exclusion HPLC. As shown in figure 3, peaks corresponding to different oligosaccharide intermediates are observed even at 1 min reaction time. Since the fluorescent tag is attached to the oligosaccharide at the reducing end, more quantitative information about the substrate, disaccharide end products and intermediates can be obtained. The fluorescence intensities of various oligosaccharide intermediates carrying 2-AMAC tag is presumed to be proportional to their relative concentration in the reaction mixture at any given time because of their structural similarity. Analysis of the fluorescence intensities of intermediate oligosaccharides at 1 min reaction time revealed that all possible 2-AMAC labeled oligosaccharide intermediates were found with nearly equal intensity, except DP2-AMAC (Figure 3b). However, as the reaction proceeded, the intensities of higher molecular weight intermediates like DP18-AMAC and DP16-AMAC and that of lower molecular weight intermediate DP4-AMAC steadily increased. These findings may suggest that smaller oligosaccharides are less preferred substrates for heparitinase I with DP4-AMAC being the least preferred intermediate due to the presence of both the open chain conformation of the reducing end glucosamine residue and the hydrophobic 2-AMAC derivative at the reducing end. The poor substrate preference of 2-AMAC labeled DP4 for heparitinase I is also reflected by the fact that the formation of DP2-AMAC was observed only after 5 min of initial reaction time. Complete depolymerization of all the labeled oligosaccharide intermediates into DP2-AMAC was achieved only after 36 hours of incubation period. The ratio of percentage intensities of various intermediates at different reaction time points are listed in the table I.

In order to find the size dependent/independent depolymerization, we digested a lower molecular weight oligosaccharide DP8-AMAC (10 μ g) with heparitinase I under similar reaction conditions as that of DP20-AMAC. The relative percentages of fluorescence intensities of the peaks obtained from DP8-AMAC digestion are listed in the table II. Analysis of the fluorescence intensities of the intermediates at different time points has shown that DP4-AMAC is a major intermediate until the complete disappearance of the starting substrate, DP8-AMAC. This higher intensity of DP4-AMAC, as observed in the DP20-AMAC digestion, is possibly due to poor substrate specificity of this molecule toward heparitinase I for the reasons described in the previous section. Furthermore, the comparison of disappearance of substrates DP20-AMAC and DP8-AMAC upon heparitinase I digestion as a function of time suggests that the initial rate of digestion of DP20-AMAC is faster than that of DP8-AMAC (Figure 4), even though the number of possible cleavage sites are nine and three for DP20-AMAC and DP8-AMAC, respectively. This reaffirms that heparitinase I has increased preference for higher oligosaccharide sizes and thus, DP20-AMAC is more preferred than DP8-AMAC.

Mass spectrometric analysis of heparitinase I mediated depolymerization of DP20-AMAC and DP8-AMAC

The depolymerized intermediate oligosaccharides, resulting from the digestion of DP20-AMAC and DP8-AMAC by heparitinase I, carrying a fluorescent tag at the reducing end was

monitored by size exclusion HPLC-coupled to a fluorescence detector. However, oligosaccharide intermediates resulting from endolytic cleavage of 2-AMAC labeled substrates at the non-reducing end could not be evaluated by the fluorescence method because these cleaved intermediates lack 2-AMAC labeling. However, this limitation has been overcome by the mass spectrometric analysis of the entire reaction mixture. Theoretically, if the heparitinase I enzyme were to exclusively act in an exolytic fashion from the non-reducing end, then only the underivatized DP2 (m/z 378) and other labeled oligosaccharide intermediates would be observed in the negative ion mode. On the other hand, if heparitinase I cleaved the 2-AMAC labeled oligosaccharide substrates in an endolytic manner both unlabeled, and 2-AMAC labeled oligosaccharide intermediates corresponding to DP2, DP4, DP6, etc., is predicted to be present in the MS spectrum.

The substrate DP20-AMAC was incubated with heparitinase I for 5 min and the reaction mixture was analyzed by ESI-mass spectrometry in the negative ionization mode. The peaks corresponding to DP20-AMAC oligosaccharide along with 2-AMAC tagged and unlabeled oligosaccharide intermediates dominated the MS spectrum of the reaction mixture (Figure 5A). The signals corresponding to the AMAC-labeled products are marked by alphabets and the unlabeled peaks are marked by numbers in figure 5A. MS analysis indicated that both unlabeled and 2-AMAC labeled oligosaccharides were present in the 5 min reaction mixture suggesting a random endolytic cleavage of DP20-AMAC (Table III). LC-MS analysis of 1 min reaction mixture did not reveal the presence of either DP2 or DP2-AMAC ions but it did provide molecular evidence for the presence of a number of 2-AMAC labeled and unlabeled oligosaccharide intermediates (Figure 5B). MS analysis of reaction mixtures of heparitinase I digested DP20-AMAC oligosaccharide at other time points such as 10, 20 and 30 min, also showed molecular ions corresponding to both unlabeled and 2-AMAC fluorophore carrying intermediate oligosaccharides. The reaction mixture after 36 hr incubation time showed only molecular ions at m/z 378 and 572 corresponding to DP2 and DP2-AMAC end products, respectively. However, the LC-MS spectra of cleavage products of DP20-AMAC is not quantitative due to differential ionization properties of various 2-AMAC labeled and unlabeled intermediates and presence of varying percentage of organic solvent content encountered during chromatographic separation. Thus, the purpose of these LC-MS studies is only to provide evidence for the presence of unlabeled and labeled intermediates during the enzymatic action of heparitinase I on DP20-AMAC whereas the purpose of the fluorescence HPLC is to provide quantitative data for endolytic cleavage intermediate oligosaccharides carrying a 2-AMAC tag at the reducing end.

Discussion

Heparan sulfate and heparin both belong to the same class of glycosaminoglycans containing GlcA/IduA $\alpha(1\rightarrow4)$ GlcNAc(S) disaccharides. However, HS is less sulfated, consisting of unsulfated and highly sulfated domains, whereas heparin is highly sulfated lacking domain organization. Heparitinases I, II, and III from *F. heparinum* show differential activity towards both of these molecules. For example, HS is the better substrate for heparitinase I but heparin is a good substrate for heparitinase III. Heparitinase II cleaves both HS and heparin with preference for HS. Because of their selective reactivity to GlcNAc(S) $\alpha(1\rightarrow4)$ GlcA linkage, heparitinase I has been utilized exhaustively in the identification of various growth factors binding HS sequences.[30] Furthermore, heparitinase I has potential for sequencing of HS oligosaccharides and inhibition of neovascularization.[31] Therefore, study of mode of action of this enzyme is critical for advancing our knowledge of heparanome and their functions in biological systems.

Structurally well characterized and homogeneous 2-AMAC labeled heparosan oligosaccharides that are amenable for monitoring the depolymerization are excellent substrate

candidates to determine the exact mode of action of heparitinase I. Thus, heparosan oligosaccharides functionalized with a 2-AMAC fluorescent tag at the reducing end was prepared from heparosan polysaccharide from K5 *E. Coli*. 2-AMAC labeled heparosan oligosaccharides up to DP54 was resolved in HPLC and the desired oligosaccharides were purified to homogeneity using size exclusion chromatography. 2-AMAC labeled heparan sulfate oligosaccharides have been used in the literature to elucidate hepatocyte growth factor/scatter factor interactions through mobility shift assay.[28] Furthermore, heparan sulfate disaccharides[32] and dextran oligosaccharides[33] have also been labeled with AMAC and analyzed by HPLC and mass spectrometry. However, the combined utility of fluorescence labeling and mass spectrometric analysis has not been demonstrated for studying mode of action of heparitinases.

Mode of enzymatic action of heparitinase I on DP20-AMAC and DP8-AMAC was monitored using fluorescence detection and the relative fluorescence intensities of intermediate products were also estimated (Table I and Table II). Analysis of the size exclusion chromatograms of the heparitinase I treated DP20-AMAC at different time intervals suggested the presence of all possible AMAC oligosaccharide intermediates with the exception of DP2-AMAC. This observation confirmed that the heparitinase I acted in a random endolytic manner. However, the slightly higher intensities of DP18, DP16, DP6-AMAC, and DP4-AMAC at early reaction times (1, 5, 10, and 20 min) may suggest a minor preference for possible exolytic cleavage. In addition, there is no preference for cleaving the substrate from either reducing end or non-reducing end, which is indicated from the percentage intensities of 2-AMAC labeled DP18 and DP16. The observed high fluorescence intensity of DP4-AMAC suggests that AMAC tagged tetrasaccharide is a poor substrate for heparitinase I. This means that the DP4-AMAC is a transient end product, which is eventually converted to disaccharides, DP2 and DP2-AMAC, after prolonged digestion time. This view is reinforced by the observation that DP4-AMAC is the major intermediate at various intervals of digestion time and DP2-AMAC is not observed until after 5 min from the reaction initiation time.

The LC-MS analysis of the reaction mixtures not only confirmed the presence of 2-AMAC labeled reducing end intermediates but also unlabeled non-reducing end oligosaccharide intermediates and disaccharides (Figure 5). The presence of negligible amount of the DP2 (m/z 387) and DP2-AMAC in addition to other unlabeled oligosaccharide peaks in the MS spectra of the reaction mixtures of early time points strongly suggests that the enzyme heparitinase I acted in a random endolytic fashion. Furthermore, the relative intensities of the extracted ion chromatograms of non-labeled intermediate fragments, for example DP14, DP10 and DP4 increased at the early periods of the lyase reaction (Figure 6). These results provide first direct molecular evidence for endolytic enzymatic action of heparitinase I on 2-AMAC labeled heparosan oligosaccharides. The higher total ion counts observed for the high molecular weight intermediates (dp14 and dp16) at 1 min reaction mixture could possibly be due to an increased organic solvent content, acetonitrile, upon LC separation. Thus, the comparison of total ion count of various intermediates at a given reaction time may not be accurate but the comparison of the same intermediate at different reaction time points may provide information that is more meaningful.

In summary, 2-AMAC labeled heparosan oligosaccharides were prepared and fractionated into individual oligosaccharides by size exclusion chromatography. Purified and homogeneous DP20-AMAC and DP8-AMAC samples were subjected to the heparitinase I digestion. The quantitation of AMAC labeled intermediate by fluorescence and characterization of the labeled and unlabeled intermediates by LC-MS unambiguously confirmed that the mode of action of the heparitinase I enzyme on heparosan oligosaccharides is random endolytic. Based on the findings reported here, it is envisioned that collective utilization of both fluorescence labeling as a reading frame and mass spectrometric techniques can be very effective in obtaining the

sequence information of structurally complex heparan sulfate polysaccharides. In addition, the nature of organization of domains and presence of critical groups essential for binding to various proteins of biological significance as well as their abundance and possibly their location relative to that of reducing end can be deduced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. HPLC-fluorescence elution chromatogram of 2-AMAC derivatized heparosan oligosaccharide mixtures separated on a size exclusion column. Heparosan polysaccharide was digested with heparitinase I in a controlled manner and then labeled with 2-aminoacridone as described in the experimental section. The samples were eluted with ammonium acetate buffer (0.2 M, pH 5.0) at a flow rate of 0.75 mL and detected using online fluorescence detector ($\lambda_{\text{ex}} = 425 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$).

Fig 2.

ESI-mass spectrometric characterization of 2-AMAC labeled heparosan oligosaccharides (DP2 to DP20). A) The expanded MS spectra of the 2-AMAC labeled oligosaccharides in the negative ion mode. (1) [(DP2-AMAC) - 1H]¹⁻; (2) [(DP4-AMAC) - 2H]²⁻; (3) [(DP6-AMAC) - 2H]²⁻; (4) [(DP10-AMAC) - 2H]²⁻; (5) [(DP12-AMAC) - 3H]³⁻; (6) [(DP14-AMAC) - 3H]³⁻; (7) [(DP16-AMAC) - 4H]⁴⁻; (8) [(DP18-AMAC) - 3H]³⁻. B) Complete LC-MS spectra of (1) DP8-AMAC and (2) DP20-AMAC are shown. Inset shows the expanded version of multiply charged [M - 2H]²⁻ for DP8-AMAC and [M - 4H]⁴⁻ for DP20-AMAC.

Fig 3.

HPLC-fluorescence elution profile of heparitinase I depolymerized products of DP20-AMAC at various time points; a) 0 min, b) 1 min, c) 5 min, d) 10 min, e) 30 min, f) 60 min, and g) 36 h. Each fluorescence peak is denoted by numerical letter that corresponds to 2-AMAC labeled oligosaccharides; 1 (DP20-AMAC), 2 (DP 18-AMAC), 3 (DP 16-AMAC), 4 (DP 14-AMAC), 5 (DP 12-AMAC), 6 (DP 10-AMAC), 7 (DP 8-AMAC), 8 (DP 6-AMAC), 9 (DP 4-AMAC), 10 (DP 2-AMAC). The lyase reaction was stopped by boiling the aliquots for 1 min at 100 °C as described in the experimental section. These samples were diluted to 100 μ L and then analyzed by HPLC using a fluorescence detector (λ_{ex} = 425 nm and λ_{em} = 520 nm).

Fig 4. The rate of relative fluorescence intensity decrease of DP20-AMAC (◆) and DP8-AMAC (■) oligosaccharides upon heparitinase I digestion.

Fig 5.

The LC-MS spectra of 5 min (A) and 1 min (B) reaction mixtures of DP20-AMAC. Both the reaction mixtures were subjected to reverse phase ion-pairing (RP-IP) chromatographic separation on an Aquity (1.7 μ m, Waters Co.) C₁₈ column using dibutylamine (5 mM) as an ion-pairing agent. MS data of the LC separated samples were obtained from hyphenated ESI-TOF mass spectrometer. Unlabeled intermediate peaks identified in the negative ion mode; m/z 378.1 (1, [DP2 - 1H]¹⁻), 757.1 (2, [DP4 - 1H]¹⁻), 567.6 (3, [DP6 - 2H]²⁻), 757.2 (4, [DP8 - 2H]²⁻), 946.8 (5, [DP10 - 2H]²⁻), 1136.3 (6, [DP12 - 2H]²⁻), 883.6 (7, [DP14 - 3H]³⁻), 1009.9 (8, [DP16 - 3H]³⁻), 1136.3 (9, [DP18 - 3H]³⁻). AMAC labeled products; m/z 572.2 (a, [DP2-AMAC - 1H]¹⁻), 475.1 and 951.3 (b, [DP4-AMAC - 2H]²⁻ and [DP4-AMAC - 1H]¹⁻), 664.7 (c, [DP6-AMAC - 2H]²⁻), 854.2 (d, [DP8-AMAC - 2H]²⁻), 1043.8 (e, [DP10-AMAC - 2H]²⁻), 821.9 (f, [DP12-AMAC - 3H]³⁻), 948.2 (g, [DP14-AMAC - 3H]³⁻), 1074.6 (h, [DP16-AMAC - 3H]³⁻), 1201.0 (i, [DP18-AMAC - 3H]³⁻), 995.3 and 1327.3 (j, [DP20-AMAC -

$4\text{H}]^{4-}$ and $[\text{DP20-AMAC} - 3\text{H}]^{3-}$). Insets show the expanded MS spectra of peaks 3 and 7. MS spectrum of 1 min reaction showed negligible amount of DP2 (m/z 378.1).

Fig 6. Changes in total ion count of unlabeled intermediates DP4 (◆, m/z [757.2211]¹⁻), DP10 (■, m/z [946.7653]²⁻), and DP14 (▲, m/z [883.5841]³⁻) at early reaction periods of enzymatic action of heparitinase I on DP20-AMAC. The ion counts were obtained by integrating peaks from the extracted ion chromatogram of individual species at each time points (1, 5, 20, and 30 min). The extracted ion chromatograms were analyzed from corresponding LC-MS spectra using Masslynx 4.1 software.

Scheme 1.

Heparitinase I catalyzed depolymerization of heparosan polysaccharide (A) and reductive amination of heparosan oligosaccharide (DP20) with 2-AMAC fluorophore (B).

Table 1

The fluorescence intensity ratios of DP20-AMAC and its heparitinase I depolymerized products at different time intervals. The fluorescence values are an average of two independent experiments.

Time (min)	DP20	DP18	DP16	DP14	DP12	DP10	DP8	DP6	DP4	DP2
0	100	0	0	0	0	0	0	0	0	0
1	83.6	2.7	1.9	1.5	1.4	1.5	1.6	2.7	3.5	
5	59.1	7.3	4.3	3.2	2.8	2.8	3.6	5.9	9.7	1.3
10	42.0	7.9	5.2	4.0	3.6	5.3	5.7	8.4	15.6	2.4
20	15.4	6.6	5.2	4.7	4.9	5.2	8.0	11.9	32.4	5.6
60	2.8	2.9	2.8	3.2	3.9	4.8	5.8	9.6	44.2	16.7
720	ND	ND	ND	ND	ND	ND	0.6	0.5	9.9	88.0
2160	ND	ND	ND	ND	ND	ND	ND	ND	ND	99.5

SD- standard deviation; ND- not detected.

Table II

Fluorescence intensity ratios of DP8-AMAC and its Heparitinase I depolymerized products at different time intervals. The fluorescence values provided are an average of two independent experiments.

Time (min)	Average % fluorescence intensity (SD)			
	DP8	DP6	DP4	DP2
0	100 (0)	0	0	0
1	86.1 (1.8)	5.9 (0.1)	7.6 (1.8)	0.4 (0.1)
5	64.7 (1.1)	13.5 (0.4)	19.9 (0.8)	2.0 (0.6)
10	52.7 (0.1)	18.1 (0.4)	26.8 (0.7)	2.6 (0.2)
20	31.6 (0.6)	19.3 (0.1)	43.0 (0.1)	6.1 (0.3)
30	18.3 (0.1)	16.1 (0.1)	54.4 (0.5)	11.4 (0.5)
2160	ND*	ND	ND	99.7 (0.2)

SD- standard deviation; ND- not detected.

Table III

Comparison of observed and calculated mass values (m/z) for unlabeled and 2-AMAC labeled oligosaccharides formed upon heparitinase I digestion of DP20-AMAC and DP8-AMAC.

Unlabeled oligosaccharides	Observed mass (m/z)	Calculated mass (m/z)	2-AMAC labeled oligosaccharides	Observed mass (m/z)	Calculated mass (m/z)
[DP2 - 1H] ¹⁻	378.1057	378.1037	DP2-AMAC - 1H] ¹⁻	572.1948	572.1881
[DP4 - 1H] ¹⁻	757.2211	757.2151	[DP4-AMAC - 2H] ²⁻	475.1501	475.1537
[DP6 - 2H] ²⁻	567.6594	567.6672	[DP4-AMAC - 1H] ²⁻	951.2983	951.2995
[DP8 - 2H] ²⁻	757.2211	757.2229	[DP6-AMAC - 2H] ²⁻	664.7079	664.7093
[DP10 - 2H] ²⁻	946.7802	946.7786	[DP8-AMAC - 2H] ²⁻	854.2593	854.2651
[DP12 - 2H] ²⁻	1136.3408	1136.3342	[DP10-AMAC - 2H] ²⁻	1043.8384	1043.8207
[DP14 - 3H] ³⁻	883.5985	883.5933	[DP12-AMAC - 3H] ³⁻	821.9192	821.9176
[DP16 - 3H] ³⁻	1009.9607	1009.9638	[DP14-AMAC - 3H] ³⁻	948.2980	948.2881
[DP18 - 3H] ³⁻	1136.3408	1136.3342	[DP16-AMAC - 3H] ³⁻	1074.6587	1074.6586
			[DP18-AMAC - 3H] ³⁻	1201.0334	1201.0290
			[DP20-AMAC - 4H] ⁴⁻	995.2969	995.2996
			[DP20-AMAC - 3H] ³⁻	1327.4124	1327.3995