High-Performance Liquid Chromatographic/Mass Spectrometric Studies on the Susceptibility of Heparin Species to Cleavage by Heparanase

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ABSTRACT

Heparanase is an endo-β-D-glucuronidase that cleaves the heparan sulfate chains of heparan sulfate proteoglycans and is implicated in angiogenesis and metastasis. With the aim of establishing a simple and reliable method for studying the susceptibility of heparin/heparan sulfate oligosaccharides to be cleaved by heparanase, an on-line ion pair reversed-phase high-performance liquid chromatographic/electrospray ionization mass spectrometric method was set up. The method works in the micromolar range of concentration and does not require derivatization of the substrate or of the products. It is based on mass identification of oligosaccharide fragments generated by heparanase and their quantification with reference to an internal heparin disaccharide standard. Substrates were (1) the synthetic pentasaccharides GlcNNS,6S−GlcA−GlcNNS,3S,6S−IdoA2S−GlcNNS,6S−OMe (AGA*IAM)Q1 and GlcNNS,6S−GlcA−GlcNNS,6S−IdoA2S−GlcNNS,6S−OMe (AGAIAx1), corresponding to the heparin/heparan sulfate active site for antithrombin, and to the same sequence devoid of the 3-O-sulfate group in the central glucosamine, respectively; and (2) two natural heparin octasaccharides containing the AGA*IA sequence in different locations along the chain. The two pentasaccharides exhibited a higher susceptibility to heparanase cleavage with respect to the octasaccharides. The commercial availability of AGA*IAM makes it an ideal substrate to determine the specific activity of heparanase preparations. The present method could also be used for rapid screening of potential heparanase inhibitors.

KEYWORDS: Heparanase, heparin oligosaccharides, cleavability by heparanase, mass spectrometry.

Heparan sulfate proteoglycans (HSPGs) are macromolecules consisting of a core protein to which linear heparan sulfate (HS) chains are covalently O-linked. They are found ubiquitously as components of plasma membrane of a variety of cells; in addition, HSPGs are major constituents of the extracellular matrix (ECM), where they play not only a structural role contributing to the maintenance of ECM integrity and insolubility, but mediate several important physiological functions. Most of the biological properties of...
HSPGs are associated with their HS chains, which typically consist of repeating hexuronic (either D-glucuronic or L-iduronic acid) and D-glucosamine disaccharide units modified at different positions by various degrees of O-sulfation and N-sulfation or N-acetylation, yielding characteristic patterns of alternating unmodified and highly modified regions separated by transition segments. By binding to a multitude of proteins, such as growth factors, cytokines, and chemokines on the cell surface and in ECM, the HS side chains of HSPGs modulate important physiological and pathological processes, including morphogenesis, tissue repair, inflammation, vascularization, and cancer metastasis.

Heparanase is an endo-β-D-glucuronidase that specifically cleaves HS and hence participates in degradation and remodeling of the ECM, also favoring the release of HS-bound biological mediators. The enzyme catalyzes the hydrolytic cleavage of the glycosidic bond between glucuronic acid and glucosamine residues, yielding fragments of variable size, typically ranging from 10 to 20 sugars. Heparanase activity has long been detected in several cell types and tissues and, importantly, its overexpression has been reported to correlate with metastatic potential of tumor cells in several studies. In fact, heparanase activity also is involved in neovascularization, inflammation, and autoimmunity, associated with migration of vascular endothelial cells and activated cells of the immune system. Recently, the upregulation of heparanase in an increasing number of primary human tumors has been reported, correlated with reduced postoperative survival, increased lymph node and distal metastasis, and increased microvessel density. All of these studies account for the clinical relevance of heparanase and justify its attractiveness as a therapeutic agent. Attempts to inhibit the heparanase enzymatic activity were initiated more than 20 years ago. However, only with the availability of recombinant human heparanase and the establishment of high-throughput screening methods have a variety of inhibitory molecules been developed, including antibodies, peptides, modified non-anticoagulant species of heparin, and several other polyanionic molecules, such as laminaran sulfate, suramin, and PI-88.

Several assays for evaluating heparanase activity have been developed, and are based on measurement of the cleavage of its substrates, heparin, or HS species, coincubated with the enzyme. Most of these methods require labeling of substrates, either with a radioisotope using a chemical or biosynthetic procedure, or fluorescent molecules. In most cases, separation of degraded products from noncleaved substrates by gel chromatography or ultrafiltration, or as an alternative, immobilization of the substrate on a solid surface, is required. In any case, all of these methods are time consuming and often yield only semiquantitative results.

Two of the reported methods do not require substrate labeling. A recent method, based on an enzyme-linked immunosorbent assay procedure, has an important diagnostic value. It is a highly sensitive and reliable method addressed to the detection of heparanase protein in tissue extract and body fluids, but it is not informative on its enzymatic activity. The second method, described in a patent, uses fondaparinux, a synthetic pentasaccharide corresponding to the active site for antithrombin (AT) as heparanase substrate: the measure of its residual anti-Xa activity, in the presence or in the absence of inhibitors, provides an indirect evaluation of the heparanase activity.

As an alternative to the described procedures, a novel sensitive method for measuring heparanase activity was developed that does not require any substrate derivatization. The method exploited an on-line ion pair reversed-phase, high-performance liquid chromatography/electrospray ionization ion trap mass spectrometry (IP-RP-HPLC/ESI-MS) assembly to identify mass peaks of heparin oligosaccharides generated by heparanase hydrolysis of a simple oligosaccharidic substrate and to quantify the residual substrate with reference to an internal standard (a heparin disaccharide). MS, combined with modern separation methodologies, offers a powerful and versatile tool for identification and quantification of oligosaccharides even in the absence of chromophoric groups. With respect to traditional analytical methods, it provides many advantages such as low sample consumption and high sensitivity. The method was set up using as a substrate the synthetic pentasaccharide fondaparinux, hereafter indicated as AGA*IAM, represented by the structure GlcNNS,6S–GlcA–GlcNNS,3S,6S–IdoA2S–GlcNNS,6S–OMe (Fig. 1B). Previous studies indicated that heparanase cleaves the glucuronic linkage in the AT-binding sequence when it is part of an octasaccharide fragment, and studies parallel to the present one confirmed that linkage is susceptible to cleavage by the enzyme also in AGA*IAM. As reference standard, a heparin disaccharide (IdoA2S–aM6S), where aM6S is a 6-O-sulfated anhydromannitol residue, was used. The method was also tested with three different oligosaccharides: the synthetic pentasaccharide AGAIA (Fig. 1A), differing from AGA*IAM because of the absence of the 3-O sulfate group on the central glucosamine, and the two natural heparin octasaccharides octa A (ΔU–GlcNNAc,6S–GlcA–GlcNNS,3S,6S–IdoA2S–GlcNNS,6S–IdoA2S–GlcNNS,6S) and octa B (ΔU2–GlcNNAc,6S–IdoA–GlcNNAc,6S–GlcA–G–GlcA–GlcNNS,3S,6S–IdoA2S–GlcNNS,6S), where ΔU is a 4,5-unsaturated uronic acid and ΔU2S is a 2-O-sulfated 4,5-unsaturated uronic acid. The two octasaccharides contain the active AT-binding sequence (AGA*IA) located in different areas along the chain.
Given that these octasaccharides have a natural origin, the first glucosamine residue of the AT-binding sequence bears a N-acetyl group instead of a N-sulfate group.  

**METHOD**

Analyses of kinetics of enzymatic hydrolysis were performed by incubating 100 mg of AGA*IAM (66 nmol) with variable amounts (0.5 to 5.0 mg) of recombinant human heparanase (kindly provided by Professor Israel Vlodavsky) at 37°C in 20 mM ammonium acetate + 2 mM Ca(OAc)₂ + 1 mM β-mercaptoethanol (pH 5.8) at a final volume of 125 μL, corresponding to an oligosaccharide concentration of 0.52 nmol/μL. Five microliters of the incubation mixture was taken, at different times (0, 2, 10, 30, and 60 minutes and 2, 4, 8, 12, 16, and 24 hours), diluted 40 times with 10 mM ammonium acetate containing 31 pmol/μL of reference standard, IdoA₂S/C₀₆S disaccharide, and treated with formic acid 0.025% to disrupt possible oligosaccharide-protein complexes. Twenty microliters of each step of digestion (originally containing 0.4 mg of pentasaccharide) were analyzed by IP-RP-HPLC/ESI-MS. The chromatographic separation was performed on a 3-μm Prontosil Hypersorb Q₅ reversed-phase C18 column (4.6 × 250 mm), by eluting with a linear gradient from 100% eluent A (MeOH/H₂O 20/80) to 100% eluent B (MeOH/H₂O 70/30), both in 5 mM dibutylammonium acetate (DBA), at a flow rate of 0.3 mL/min. Mass spectrometric analyses were performed on an Esquire 3000 Plus electrospray ion trap (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source working in negative ion mode in the mass range from m/z 400 to 1000. Sample ionization was obtained using the optimized MS conditions of spray voltage and capillary temperature (3166 V and 350°C, respectively).

Calibration of the mass spectrometer was performed using an ES tuning mix solution (Agilent Q₆ acetonitrile solution) according to a standard procedure. Data were processed by the DataAnalysis software (Version 3.0, Bruker Daltonik Q₇). To verify the possible quantitative application of the method, a calibration curve was built up with different AGA*IAM concentrations (0 to 25 pmol/μL) in the presence of a constant amount of reference standard. Following IP-RP-HPLC/ESI-MS analysis, the area of AGA*IAM signals calculated from each total ion chromatogram was normalized with respect to the area of the signal of the internal standard. As shown in Fig. 2, a good linear correlation of experimental points was found for substrate concentrations in the range 0 to 15 pmol/μL. For each experiment of kinetics of enzymatic hydrolysis, calibration curves with each of the oligosaccharides employed as heparanase substrates were set up. Because of the high sensitivity of the technique, such an internal calibration is important to exclude any possible interference due to even minor variations of instrumental conditions.

For AGAI₄M and the two natural heparin octasaccharides octa A and octa B, the kinetics of enzymatic hydrolysis were performed as described above, by incubating substrates with the heparanase amount (2.3 μg)
found sufficient to induce total degradation of 66 nmol AGA*IAM in 1 hour.

RESULTS AND DISCUSSION
Preliminary experiments of kinetics of enzymatic hydrolysis with heparanase of synthetic pentasaccharides AGA*IAM and AGAIAM were performed in infusion, by injecting the digestion mixture directly into the ESI spectrometer, without any purification/fractionation step. Good ESI spectra and reliable quantitative data were obtained. Nevertheless, the study of octasaccharides as heparanase substrates required a refinement of the procedure. In fact, ESI spectra of large and highly sulfated oligosaccharides are very complex, because of several factors: (1) each molecule is detected in a multiple-charge state; (2) the sulfate and carboxylate groups form stable adducts with metal cations to varying degrees; (3) the longer the oligosaccharide, the higher is the tendency that sulfate groups are lost during the ionization step. As a consequence of the complex peak profiles, it often is difficult to obtain reliable molecular mass information. To overcome the problem of signals overcrowding in mass spectra, and especially of ion suppression in the ion source, a chromatographic separation step of oligosaccharide species in the heparanase digestion mixtures was introduced.

In IP-RP-HPLC, the retention of analytes is influenced by ionic interactions between the positive surface potential generated by the amphiphilic ions adsorbed to the stationary phase (e.g., DBA ions) and the negative charges of the carboxylate/sulfate groups of heparin/HS oligosaccharides. By increasing the concentration of an organic modifier such as methanol, amphiphilic ions are desorbed from the stationary phase, resulting in elution of the retained analytes. A proper choice of type and concentration of ion-pairing reagent enables the efficient on-line coupling of IP-RP-HPLC to ESI-MS in the analysis of heparin/HS oligosaccharides.36

Figure 3A shows the total ion chromatograms (TICs) recorded on samples at three different times of incubation of AGAIAM with heparanase. At time 0, the peaks of substrate and reference standard were detected. After 10 minutes, the intensity decrease of the AGAIAM signal was accompanied by the appearance of hydrolysis products, the disaccharide GlcNNS,6S/C0 GlcA and the trisaccharide GlcNNS,6S/C0 IdoA2S/C0 GlcNNS,6S. After 1 hour, the substrate signal was no more detectable, indicating that the enzymatic reaction reached completion. The decrease of intensity of the AGAIAM signal with respect to the standard signal provides the measure of susceptibility to heparanase cleavage. Oligosaccharide species were identified on the basis of mass values in the corresponding ESI mass spectra. In Fig. 3B, the ESI-MS full scan spectra of each ion component of TIC at 10 minutes of incubation are reported. A series of peaks corresponding to adducts of the molecular ion with the ion-pairing reagent (DBA) and/or Na are observed. In addition, peaks with double-negative charge and others due to fragments generated by loss of sulfate groups can occur during ionization, as in the case of trisaccharide and pentasaccharide. However, given that the enzymatic cleavage was measured on the basis of TIC, neither cation adduction nor desulfation affected quantitative evaluation.

The TICs recorded after three different times of incubation with heparanase of the AGAIAM are shown in Fig. 4. At time 0, more precisely a few seconds after the addition of the enzyme, peaks corresponding to digestion products (i.e., the disaccharide GlcNNS,6S/C0 GlcA and the trisaccharide GlcNNS,6S/C0 IdoA2S/C0 GlcNNS,6S) were observed, together with signals of substrate and standard. After 2 minutes of incubation, almost all the substrate was degraded and the increase of the signals of products was recorded. No more substrate was detectable after a 10-minute incubation.

The kinetics of hydrolysis by heparanase of the two natural octasaccharides, octa A and octa B, are shown in Figs. 5 and 6, respectively. Their low susceptibility to heparanase hydrolysis is indicated by TICs at the three
Figure 3  Kinetics of hydrolysis by heparanase of GlcNS,6S\textbar GlcA\textbar GlcNS,3S,6S\textbar IdoA2S\textbar GlcAOMe (AGA\*IA). (A) Ion pair reversed-phase, high-performance liquid chromatography separation of digestion products at three different times of incubation: total ion chromatograms (TICs) detected by negative electrospray ionization ion trap mass spectrometry (ESI-MS) are shown. Peaks of standard, substrate, and its hydrolyzed di- and trisaccharide fragments are indicated. (B) ESI-MS full-scan spectra of each oligosaccharide peak of the TIC after 10 minutes of incubation: for major peaks, m/z values and molecular ion compositions are given. The number between brackets indicates the loss of sulfate groups. Std, standard; DBA, dibutylammonium acetate.

Figure 4  Kinetics of hydrolysis by heparanase of GlcNS,6S\textbar GlcA\textbar GlcNS,3S,6S\textbar IdoA2S\textbar GlcAOMe (AGA\*IA). Ion pair reversed-phase, high-performance liquid chromatography separation of digestion products at three different times of incubation: total ion chromatograms detected by negative electrospray ionization ion trap mass spectrometry are shown. Peaks of disaccharide standard, substrate, and its hydrolyzed fragments are indicated. Std, standard.

Figure 5  Kinetics of hydrolysis by heparanase of octasaccharide A. Ion pair reversed-phase, high-performance liquid chromatography separation of digestion products at three different times of incubation. Legend as in Fig. 4\textsuperscript{Q11}. Std, standard; octa, octasaccharide.
most representative times of incubation. For octa A, after only 1 hour a consistent decrease of substrate signals was detected, together with the appearance of signals of the digestion products (i.e., the trisaccharide $\Delta U$-GlcN$_{\text{N6S}}$-GlcA and the pentasaccharide GlcN$_{\text{N5S6S}}$-IdoA$_2$-GlcN$_{\text{N6S}}$-IdoA$_2$-GlcN$_{\text{N1S6S}}$-GlcN$_{\text{N1S6S}}$). The complete degradation of octa A was observed after 4 hours of incubation. The slowest kinetics of heparanase hydrolysis was exhibited by octa B, which required 24 hours to be totally degraded. Regarding the order of elution of its digestion products (Fig. 6), the shorter retention time of the pentasaccharide fragment $\Delta U_2$-GlcN$_{\text{N6S}}$-IdoA-GlcN$_{\text{N6S}}$-GlcA with respect to the trisaccharide fragment GlcN$_{\text{N5S6S}}$-IdoA$_2$-GlcN$_{\text{N6S}}$ is attributable to its lower degree of sulfation. For AGA*IAM, all the components of TICs of AGA*IAM, octa A, and octa B were assigned on the basis of their ESI mass spectra (data not shown).

The susceptibility to cleavage by heparanase was measured through the measurement of intensity of residual substrate signals with respect to the reference standard versus time. This susceptibility for the four oligosaccharides (Fig. 7) is in the following order: AGA*IAM $>$ AGA*IAM $>$ octa A $>$ octa B.

The pentasaccharide AGA*IAM turned out as the best substrate for heparanase among those tested in the present work. In agreement with previous findings, 3-O-sulfation of the central glucosamine residue in this sequence hinders cleavage by the enzyme slightly. However, AGA*IAM still remains a good substrate, confirming a previous report 33 that AGA*IA is cleaved even when it is not part of larger oligosaccharides. Both octa A and B appear to be poor substrates in comparison with the pentasaccharides. The presence of a $N$-acetyl group instead of $N$-sulfate group on the glucosamine residue flanking the glucuronic acid residue could explain in part the different degree of cleavability of the two octasaccharides with respect to AGA*IAM. In addition, it is likely that products of cleavage of octa A and B act as heparanase inhibitors by competing with the intact substrates. In fact, both the nona-sulfated pentasaccharide and the hexa-sulfated trisaccharide, generated from octa A and octa B, respectively, could interact with the basic clusters of the heparin/HS binding regions of the enzyme, 39 interfering with binding of the intact substrate.

CONCLUSIONS

The on-line IP-RP-HPLC/ESI-MS technique offers a simple, sensitive, and reliable method for detecting the susceptibility to cleavage by heparanase of heparin/HS oligosaccharides. The commercial availability of AGA*IAM makes it an ideal substrate to determine the specific activity of heparanase preparations instead of other methods 33 using HS or HSPG extracted from animal organs. Moreover, measurement of the extent of cleavage of AGA*IAM could be performed in the presence of putative heparanase inhibitors, permitting a rapid evaluation of potential inhibitors of the enzyme. With respect to a previously described method, 33 which also uses AGA*IAM as heparanase substrate and quantifies its residual amount by measuring the anti-Xa activity, the present method offers the following advantage: by performing a direct measurement of the residual substrate, the potential problem of interference of additional proteins (i.e., AT) is overcome with the use of heparanase inhibitors. 38

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kindly providing recombinant human heparanase, AGAIA_M and AGA^IAM (fondaparinux), respectively. This work was supported by European Commission grant QL.K3-CT-2002–02049.

**ABBREVIATIONS**

| AGA^IAM | GlnNS,6S – Gln β GlcNS,3S,6S – GlnNS,3S,6S – IdoA2S – GlnNS,6S – GlnNS,6S – OMe |
| aM6S | 6-O-sulfated anhydromannitol |
| AT | antithrombin |
| DBA | dibutylammonium acetate |
| ECM | extracellular matrix |
| ESI-MS | electrospray ionization-MS |
| GlcA | d-glucuronic acid |
| GlcNac | N-acetyl-D-glucosamine |
| GlcNac,6S | N-acetylated, 6-O-sulfated GlcN |
| GlcNNac,3S,6S | N,3,6-O-trisulfated GlcN |
| GlcNNac,6S | N-acetylated, 6-O-sulfated GlcN |
| HPLC | high-performance liquid chromatography |
| HS | heparan sulfate |
| HSPG | HS proteoglycans |
| IdoA | 1-iduronic acid |
| IdoA2S | 2-O-sulfated IdoA |
| IP-RP-HPLC | ion pair reversed phase HPLC |
| MS | mass spectrometry |
| TIC | total ion chromatogram |
| ΔU | 4,5-unsaturated uronic acid |
| ΔU2S | 2-O-sulfated, 4,5-unsaturated uronic acid |

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