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Heparin Mapping Using Heparin Lyases and the Generation of a Novel Low Molecular Weight Heparin

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Seven pharmaceutical heparins were investigated by oligosaccharide mapping by digestion with heparin lyase 1, 2, or 3, followed by high performance liquid chromatography analysis. The structure of one of the prepared mapping standards, ΔUA-Gal-Gal-Xyl-*O*-$CH_2CONHCH_2COOH$ (where ΔUA is 4-deoxy-α-L-*threo*-hex-4-eno-pyranosyluronic acid, Gal is β-D-galactpyranose, and Xyl is β-D-xylopyranose) released from the linkage region using either heparin lyase 2 or heparin lyase 3 digestion, is reported for the first time. A size-dependent susceptibility of site cleaved by heparin lyase 3 was also observed. Heparin lyase 3 acts on the undersulfated domains of the heparin chain and does not cleave the linkages within heparin's antithrombin III binding site. Thus, a novel low molecular weight heparin (LMWH) is afforded on heparin lyase 3 digestion of heparin due to this unique substrate specificity, which has anticoagulant activity comparable to that of currently available LMWH.

Introduction

Heparin (Figure 1) is a highly sulfated, linear polysaccharide consisting of repeating uronic acid (1 → 4)-D-glucosamine disaccharide subunits. Variable substitution of its disaccharide subunits with *N*-sulfo, *O*-sulfo, and *N*-acetyl groups gives rise to the extremely complex sequences of heparin. The uronic acid is most frequently an α-L-idopyranosyl uronic acid (IdoA[a]) but can be a β-D-glucopyranosyl uronic acid (GlcA), with or without a 2-*O*-sulfo group (IdoA2S and GlcA2S). The 2-deoxy-2-amino β-D-glucopyranose (GlcN) residue may be unsubstituted, modified with *N*-acetyl group (GlcNAc), or most commonly with an *N*-sulfo group (GlcNS). The GlcN residue is most commonly substituted with a 6-*O*-sulfo group (GlcNS6S and GlcNAc6S) but only rarely with a 3-*O*-sulfo group (GlcNS3S and GlcNS3S6S). Of all of these possible disaccharide subunits, IdoA2S-(1 → 4)-GlcNS6S is the most abundant in heparin. This disaccharide accounts for up to 90% of the total disaccharide units in heparins from bovine lung and up to 70% of the total disaccharide units in heparins from porcine intestine.[1]

Heparin is found primarily in the granules of connective-tissue type mast cells, where it is biosynthesized as an intracellular proteoglycan, serglycin, to which multiple heparin polysaccharide chains are covalently attached. Following their biosynthesis, the heparin proteoglycan polysaccharide chains and core protein are cleaved by an endoglucuronidase and proteases to give polydisperse mixtures of smaller heparin glycosaminoglycan and peptidoglycan chains that are stored in the cytoplasmic secretory granules of mast cells as non-covalent complexes.[1,2]

Heparin has a wide range of important biological activities due to its ability to interact with a large number of proteins.[3,4] As a clinical anticoagulant, heparin has been one of the most effective and most widely used drugs since its introduction in 1926. Heparin is unique for being one of the first biopolymeric drugs, one of a few polydisperse microheterogenous drugs, and one of the only carbohydrate-based drugs.[1,2] Low molecular weight heparins (LMWHs) are a group of heparin-derived anticoagulant/antithrombotic agents that were developed at the end of the 20th century. The introduction of LMWHs primarily resulted from our improved understanding of the molecular basis for coagulation. It was believed that a short heparin chain, containing an antithrombin III (AT) binding site but unable to accommodate thrombin binding, would prevent the formation of a ternary heparin-AT-thrombin, affording a more selective anticoagulant/antithrombotic agent. LMWHs are prepared through controlled chemical and enzymatic depolymerization of heparin. Most of the resulting chains are too small to accommodate thrombin in a ternary complex, and thus, inhibit the coagulation cascade primarily through coagulation factor Xa, which interacts directly with AT bound to a specific pentasaccharide sequence. This AT-binding sequence, -GlcNAc6S-GlcA-GlcNS**3S**6S-IdoA2S-GlcNS6S-, has a central 3-*O*-sulfo group containing glucosamine

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[a] Abbreviations: Ac, acetyl; APS, ammonium persulfate; aPTT, activated partial thromboplastin time; AT, antithrombin III; ΔUA, 4-deoxy-α-L-*threo*-hex-4-eno- pyranosyluronic acid; dp, degree of polymerization; EDTA, ethylenediamine tetracetic acid; ESI, eletrospray ionization; FT, Fourier transform; Gal, galactopyranose; GlcA, β-D-glucopyranosyl uronic acid; GlcN, 2-deoxy-2-amino-β-D-glucopyranose; HMQC, heteronuclear multiple-quantum coherence; HPLC, high performance liquid chromatography; IdoA, α-L-iduropyranosyl uronic acid; LMWH, low molecular weight heparin; MS, mass spectrometry; NMR, nuclear magnetic resonance; 1D, one-dimensional; PAGE, polyacrylamide gel electrophoresis; S, sulfo; SAX, strong anion exchange; Ser, serine; TEMED, *N,N,N′,N′*-tetramethylenediamine; 2D, two-dimensional; UV, ultraviolet; Xyl, xylopyranose.

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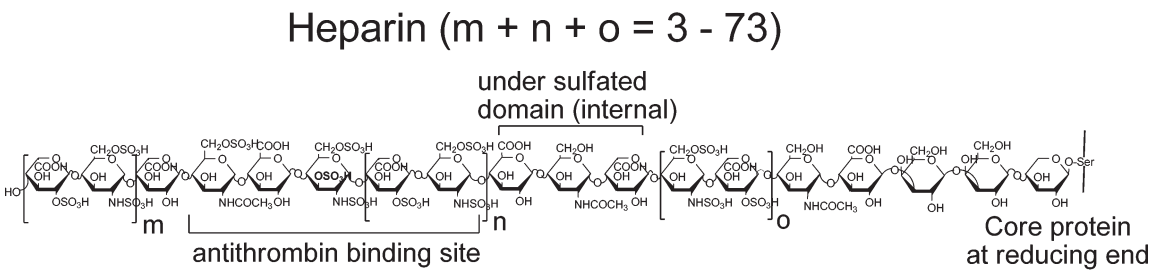

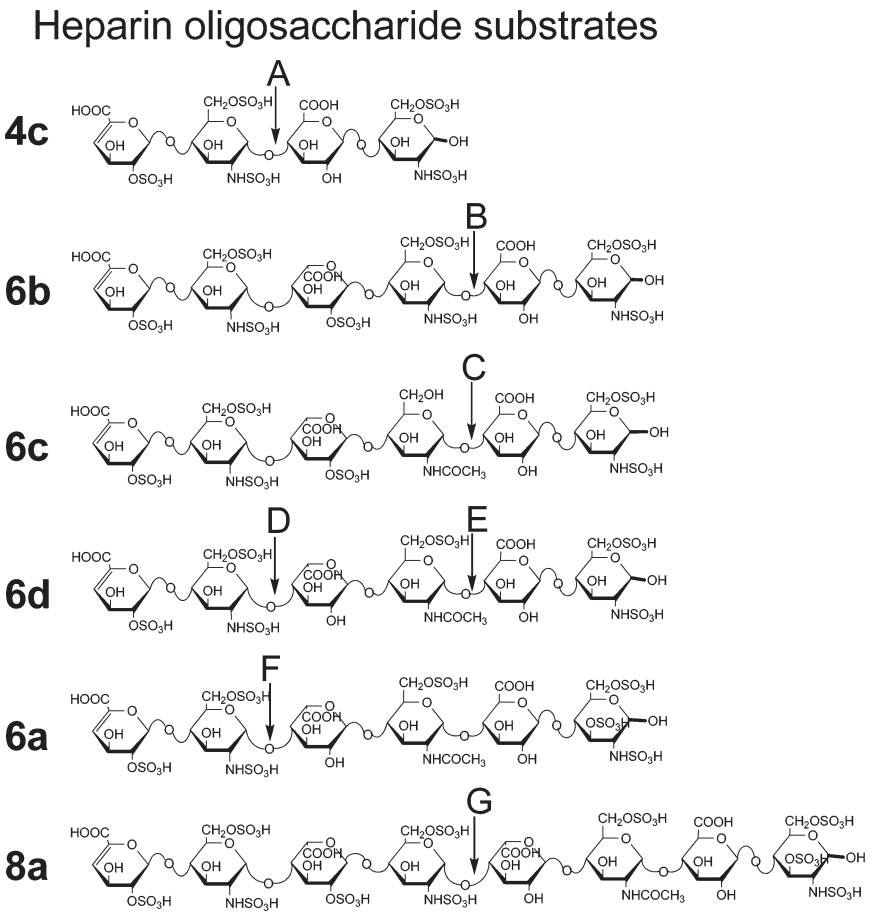

Figure 1. Schematic representation of heparin and heparin-derived oligosaccharides. Heparin is polydisperse with chains of molecular weight 5000−40000 Da with a degree of polymerization from dp20 to dp160 ($n + m + o$ = 3−73). Two under sulfated domains (< 2 sulfo groups per disaccharide unit), indicated in the internal portion of the chain, are heparin lyase 3 cleavable. The linkages labeled with A in **4c**, C in **6c**, D and E in **6d**, and F in **6a**, can be digested by heparin lyase 3. The linkages labeled B in **6b** and G in **8a**, each connected at their nonreducing end to a fully sulfated (six sulfo groups) tetrasaccharide residue, are resistant to heparin lyase 3.

residue.[5] Despite the factor Xa selectivity of LMWHs (anti-Xa/anti-IIa activity of 4−8), their clinical value comes primarily from their enhanced *subcutaneous* bioavailability and improved pharmacodynamics.[2]

The current production of pharmaceutical heparin involves isolation of the raw heparin from porcine intestine and its multistep purification.[6] As a result of a major heparin contamination crisis, which has led to the death of over 100 patients, there has recently been an increased interest in the analysis and structural evaluation of pharmaceutical heparin.[6] Improved analytical techniques and methods are required for such purposes.

Heparin lyases isolated from *Flavobacterium heparinum* also known as *Pedobacter heparinus* are important members of a class of enzymes called polysaccharide lyases (Enzyme Commission (EC) # 4.2.2) that depolymerize specific acidic polysaccharides.[7−9] Previous studies demonstrated that the site in heparin at which heparin lyase 1 acts is → 4)-α-D-GlcNS6S-(1 → 4)-α-L-IdoA2S-(1→.[10,11] The site at which heparin lyase 3 acts in heparan sulfate, a glycosaminoglycan structurally related to heparin but with lower sulfation, is →4)-α-D-GlcNAc/GlcNS/GlcNS6S-(1 → 4)-α-L-IdoA/β-D-GlcA-(1→.[10−13] Heparin lyase 2 has a wider range of specificities acting on both heparin and heparan sulfate. It acts at linkages containing either (1 → 4)-α-L-iduronic acid or (1 → 4)-β-D-glucuronic acid residues and accommodates many additional modifications of these polysaccharides as well.[10−14] It is generally accepted that tetrasaccharides containing the 3-*O*-sulfo glucosamine reducing end moiety are resistant toward the heparin lyase 1, 2, and 3.[15,16] This has posed some problems

for completely converting heparin and heparan sulfate to disaccharide products. In addition to the specificity studies, the action patterns of these heparin lyases have been extensively studied.[17] But how these action patterns are impacted by substrate size and sequence microheterogeneity is not well understood,[18] limiting the development of these enzymes as powerful analytical tools for heparin structure determination.[19,20]

The saccharide composition of heparin is usually established by disaccharide analysis and nuclear magnetic resonance (NMR) spectra.[21,22] A number of chemical and enzymatic methods are available for the cleavage of heparin at specific linkages. Heparin lyase cleavage of heparin affords more restricted cleavage than chemical methods due to enzymatic specificity.[10,11] The presence of rare 3-*O*-sulfo glucosamine residues and the lyase sensitivity of adjacent sites pose additional difficulty in structural analysis. While the linkage to the nonreducing side of a 3-*O*-sulfo glucosamine residue is resistant to all of the heparin lyases,[15,16] the linkage to the reducing side of this residue is extremely sensitive to heparin lyases 1 and 2.[18] As a result of this specificity, exhaustive depolymerization of heparin chains with heparin lyase 1 and 2 generates oligosaccharides that contain 3-*O*-sulfo glucosamine residues at their reducing end but lack intact AT pentasaccharide binding sites.[18] The different specificities of heparin lyase 1, 2, and 3 suggest their application as reagents for oligosaccharide mapping.

The goal of oligosaccharide mapping is to separate and analyze oligosaccharide products to assess the distribution of susceptible linkages within the intact heparin chain. Heparin oligosaccharide mapping with heparin lyase 1 has been previously used to provide a molecular fingerprint of the polymeric structure and reveals detailed structural information for its sequence determination and provides oligosaccharide products for further structural analysis.[23−25] In the current study, seven pharmaceutical heparins have been examined by oligosaccharide mapping with heparin lyase 1, 2, and 3. While it is generally accepted that heparin lyase 3 does not cut heparin, we have discovered that it does indeed act on heparin, offering a new means for the preparation of a novel LMWH.

Materials and Methods

Materials. Seven pharmaceutical sodium porcine intestinal heparins originating from Europe, U.S., and China were obtained for the study with anticoagulant activities ranging from 195 to 209 IU/mg. Sodium heparin used for standard preparation was obtained from porcine intestinal mucosa (176 units/mg, Celsus Laboratories, Cincinnati, OH). Low molecular weight heparin was from Pfizer (New York, NY). Cloning, expression, and purification of the recombinant heparin lyase 1 (Enzyme Commission (EC) # 4.2.2.7), heparin lyase 2 (no EC # assigned), and heparin lyase 3 (EC # 4.2.2.8) from *F. heparinum* were performed essentially as described in previous studies.[26−28] Electrophoresis-grade acrylamide, *N*,*N*′-methylene-bis-acrylamide, sucrose, glycine, ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), bromophenol blue, and polyacrylamide gels (Bio-Gel P2 and P10) were from Bio-Rad (Hercules, CA, USA). Sodium chloride, boric acid, disodium salt of ethylenediaminetetraacetic acid (disodium EDTA), phenol red, azure A, and alcian blue were from Fisher (Pittsburgh, PA, USA). Deuterium oxide (99.99%) was from Sigma Aldrich (Saint Louis, Missouri, USA).

Heparin Mapping. Each individual heparin sample (4.0 mg) was dissolved in 4.0 mL of 50 mM, pH 7.5, sodium phosphate buffer and was incubated in a 30 °C water bath with heparin lyase 1 (1.5 IU, activity against heparin), heparin lyase 2 (350 mIU, activity against heparin), or heparin lyase 3 (5.0 IU activity against heparan sulfate). The reaction completion was monitored by taking out small amount of aliquots of the reaction mixture for polyacrylamide gel electrophoresis (PAGE) analysis (see below). The same amount of each heparin lyase was added to the reaction at each 12 h interval for another two times for an exhaustive digestion on the substrates. Reactions were finally quenched by heating in a 100 °C water bath for 10 min.

Heparin lyase digested heparin samples were analyzed by PAGE using a mini-gel apparatus (Bio-Rad, Hercules, CA). Each sample (5 μL) was mixed with 5 μL of 50% (w/v) sucrose and loaded into a stacking gel of 5% (w/v total acrylamide) and fractionated on a 15 or 22% resolving gel. Electrophoresis was performed at 200 V for 30 min for 15% gel or 80 min for 22% gel. Gels were fixed and stained with 0.5% (w/v) alcian blue in 2% (v/v) acetic acid and destained with methanol−water 50% (v/v). Undigested heparin samples were used as a negative control on the 15% PAGE gel.

SAX-HPLC analysis of heparin lyase digestion products were performed on a Shimadzu LC-10Ai LC system equipped with an SPD-20A ultraviolet−visible (UV) detector using a 4.6 × 250 mm Waters Spherisorb S5 SAX column. A two-segment gradient elution was achieved using mobile phase A (water, pH 3.5, adjusted with HCl) and mobile phase B (2.0 M NaCl, pH 3.5, adjusted with HCl) at a flow rate of 1.0 mL/min. The seven heparin lyase-digested heparin samples were loaded onto the column in a concentration of 10 μg/10 μL and washed with 0% to 60% B over 60 min. The elution was monitored at 232 nm wavelength. Analyses were all performed in triplicate.

Preparation and Characterization of Oligosaccharide Standards. Heparin (400 mg in 50 mM, pH 7.4, sodium phosphate buffer) was exhaustively digested by heparin lyase 1 (20 IU, activity against heparin) in the same conditions described above. Buffer salts and disaccharide components within the product mixture were removed by chromatography on a 100 × 5.0 cm Bio-Gel P10 column eluted at 1.2 mL/min with 0.2 M NaCl in distilled water. The remaining oligosaccharide mixture was desalted on a 100 × 2.0 cm P2 column and lyophilized. The resulting mixture was fractionated on a 20 × 250 mm semipreparative strong anion exchange (SAX)-high performance liquid chromatography (HPLC) column (Waters Spherisorb S5) eluted with a salt gradient (see below) over 60 min at a flow rate of 4.0 mL/min with absorbance detected at 232 nm. Individual peaks were desalted on a Bio-Gel P2 column and were further purified by repeated separation on the same SAX-HPLC column. Oligosaccharide standards produced by heparin lyase 2 digestion were prepared in an identical manner. The purity of each of the oligosaccharides prepared from HPLC was determined to be >95% pure by analytical SAX-HPLC and PAGE analysis. The structures of these standards were characterized by HPLC-ESI-MS, and one-dimensional (1D) and two-dimensional (2D) NMR experiments.[18] High resolution mass spectra were obtained for all new compounds using ESI-Fourier transform-mass spectrometry (FT-MS) on an LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).[29]

Specificity of Heparin Lyase 3. Each heparin substrate (20 μg in 100 μL of pH 7.4, 50 mM sodium phosphate buffer) was incubated with an excessive amount of heparin lyase 3. The reaction was incubated in a 30 °C water bath for 12 h. The reaction was quenched by heating at 100 °C and the product was lyophilized before further analysis. The oligosaccharide products were then redissolved and analyzed on a 22% PAGE gel at 200 V for 70 min, and were subsequently subjected to analytical SAX-HPLC (see above) using the oligosaccharide standards for composition assignment.

Action Patterns of Heparin Lyase 1 and Heparin Lyase 2. The action patterns of heparin lyases 1 and 2 were examined using substrates of heparin-derived oligosaccharides with a size range from degree of polymerization (dp) 8 to dp16 that have been prepared by heparin lyase 1 partial digestion of porcine intestinal

mucosa heparin.[18] In these experiments, 50 μg of each oligosaccharide substrate was dissolved in 200 μL of pH 7.4, 50 mM sodium phosphate buffer, and 1.0 μL of heparin lyase 1 or heparin lyase 2 (0.01 mU/μL) was added to each reaction. The reactions were incubated in a 30 °C water bath, and 40 μL aliquots were removed at time points of 10 min, 20 min, 30 min, 1 and 12 h each. Aliquots were immediately heated in a 100 °C water bath to stop the reaction, and were then dried by lyophilization and redissolved in 20 μL of deionized distilled water. A 2 μL portion of each sample was analyzed by electrophoresis on a 22% PAGE gel. The remaining 18 μL of each sample was subjected to analytical SAX-HPLC. Undigested oligosaccharides were used as 0 min control.

LMWH Generated by Heparin lyase 3 Digestion. Heparin sodium (20 mg from Celsus Laboratories) was incubated with 10 IU (activity against heparan sulfate) heparin lyase 3 for 12 h at 25 °C. A second 15 IU heparin lyase 3 was then added for another 12 h incubation to exhaustively digest the substrates. The reaction was terminated in 100 °C water bath for 10 min, and the precipitant was removed by centrifugation at 10000*g* for 30 min. The product was reconstituted in 16% (w/w) NaCl and precipitated by 80% (w/w) methanol.[30] Residual sodium chloride was removed using a 3000 molecular weight cut off (MWCO) membrane centrifuge concentrator. The sample was then dried by lyophilization.

The amount of LMWH in the lyophilized sample was determined by micro carbazole assay.[31] The LMWH was analyzed by electrophoresis on a 15% PAGE gel at 200 V for 30 min. The gel was fixed and stained with 0.5% (w/v) alcian blue in 2% (v/v) acetic acid and destained with distilled water. The gel was scanned, digitized, and analyzed by the software UN-scan-it gel (Silk Scientific, Orem, UT). The molecular weight of the resulting LMWH was calculated based on the heparin oligosaccharide standards and was compared to that of the intact heparin and a commercial LMWH from Pfizer.[32,33] 1D NMR experiments were performed at 800 MHz (Bruker Avance 800) for digestion completion evaluation. Negative control was made by adding thermally inactivated heparin lyase 3 to the same amount of heparin substrate. A coagulation analyzer (ACL 8000, Beckman Coulter, Fullerton, CA) was used to determine antifactor Xa, antifactor IIa (thrombin), and activated partial thromboplastin time (aPTT) activity following previously published protocols.[34]

Results and Discussion

Heparin Mapping. Heparin oligosaccharides mixtures were prepared by exhaustive digestion using heparin lyases 1, 2, and 3, respectively. PAGE and SAX-HPLC analyses of these oligosaccharide mixtures show that heparin lyase 2 digestion resulted in the most complete digestion (95%) of the glycosidic linkages to uronic acid cleaved (Table S1, Supporting Information). In contrast, heparin lyase 3 resulted in the least complete (5%) digestion (Table S1, Supporting Information). Most of the oligosaccharides contained in the heparin lyase 3 digestion product mixture were in a size larger than degree of polymerization 16 (dp16) (Figure S1, Supporting Information), suggesting that only a small percentage of heparin linkages were cleaved. Heparin lyase 1 afforded an intermediate level of heparin digestion (75%) leaving some (13% in molar percentage) undigested tetrasaccharides and a small quantity of undigested hexasaccharides within the products (Table S1, Supporting Information).

Disaccharides have a relatively small size and are only slightly charged, causing an inadequate fixation by the staining solution alcian blue and hence difficultly in observing them on the PAGE gel (Figure S1, Supporting Information).[35] Therefore, the oligosaccharide mixtures prepared using individual lyase were further analyzed by SAX-HPLC, and the major oligosaccharide peaks in the chromatogram were assigned using heparin oligosaccharide standards (Figure 2). Reference standards used to characterize the heparin lyase 1 digestion products were prepared directly from a large-scale heparin lyase 1 digestion of heparin (from Celsus). The most prominent peak in the chromatogram of heparin lyase 1 digested heparin was the trisulfated disaccharide **2d** (ΔUA2S-GlcNS6S). Tetrasaccharide **4d** (ΔUA2S-GlcNS6S-IdoA2S-GlcNS6S), which is composed of two units of **2d** and is known to be cleavable to heparin lyase 1, was also present in the "completely" digested product mixture. Compared with previous studies,[23] the percentage of **4d** was greatly reduced due to the large excess of heparin lyase 1 used in the current study. Smaller substrates such as tetrasaccharides bearing heparin lyase 1 sensitive linkages are reportedly more difficult to digest.[36] Evaluation of the digestion completeness was made by monitoring the UV absorbance at 232 nm versus time. This is apparently too imprecise a measure to detect the small change in UV absorbance occurring when minor resistant oligosaccharides are digested. **4c** (ΔUA2S-GlcNS6S-GlcA-GlcNS6S) also showed resistance to heparin lyase 1 digestion and was the major tetrasaccharide product. **6a** (ΔUA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S), a hexasaccharide with 3-*O*-sulfo glucosamine residue at its reducing end, was the major hexasaccharide product.

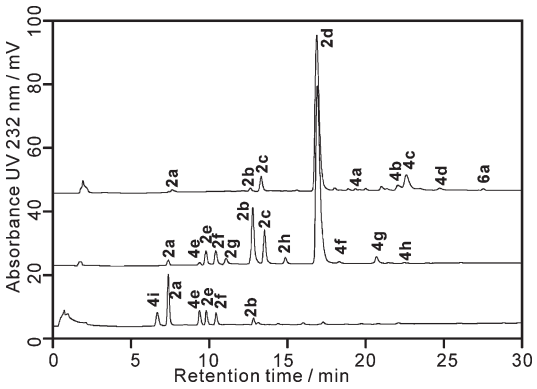

Figure 2. Chromatograms of heparin lyases produced oligosaccharides for heparin mapping. SAX-HPLC chromatograms are (a) heparin lyase 1 treated heparin; (b) heparin lyase 2-treated heparin; and (c) heparin lyase 3 treated heparin. The structures determined for each peak are **2a** ΔUA-GlcNAc, **2b** ΔUA-GlcNS6S, **2c** ΔUA2S-GlcNS, **2d** ΔUA2S-GlcNS6S, **2e** ΔUA-GlcNS, **2f** ΔUA-GlcNAc6S, **2g** ΔUA2S-GlcNAc, **2h** ΔUA2S-GlcNAc6S, **4a** ΔUA2S-GlcNS-IdoA2S-GlcNS, **4b** ΔUA2S-GlcNS6S-IdoA2S-GlcNS, **4c** ΔUA2S-GlcNS6S-GlcA-GlcNS6S, **4d** ΔUA2S-GlcNS6S-IdoA2S-GlcNS6S, **4e** ΔUA-Gal-Gal-Xyl-*O*-$CH_2CONHCH_2COOH$, **4f** ΔUA-GlcNAc6S-GlcA-GlcNS3S, **4g** ΔUA-GlcNAc6S-GlcA-GlcNS3S6S, **4h** ΔUA-GlcNS6S-GlcA-GlcNS3S6S, **4i** ΔUA-Gal-Gal-Xyl-*O*-Ser, **6a** ΔUA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S.

Heparin lyase 2 digestion afforded a greater number of disaccharide products than did heparin lyase 1, cleaving both tetrasaccharides **4c** and **4d**. Interestingly, the three tetrasaccharide products, **4f** (ΔUA-GlcNAc6S-GlcA-GlcNS3S), **4g** (ΔUA-GlcNAc6S-GlcA-GlcNS3S6S), and **4h** (ΔUA-GlcNS6S-GlcA-GlcNS3S6S), observed in the heparin lyase 2 product mixture, each had a 3-*O*-sulfo group containing glucosamine residue at their reducing end. These 3-*O*-sulfo group containing tetrasaccharides are absent in the heparin lyase 1 digestion chromatogram, demonstrating that heparin lyase 1 cannot act on higher oligosaccharides such as **6a** to convert it into **4g** and **2d**.[18] These observations are consistent with

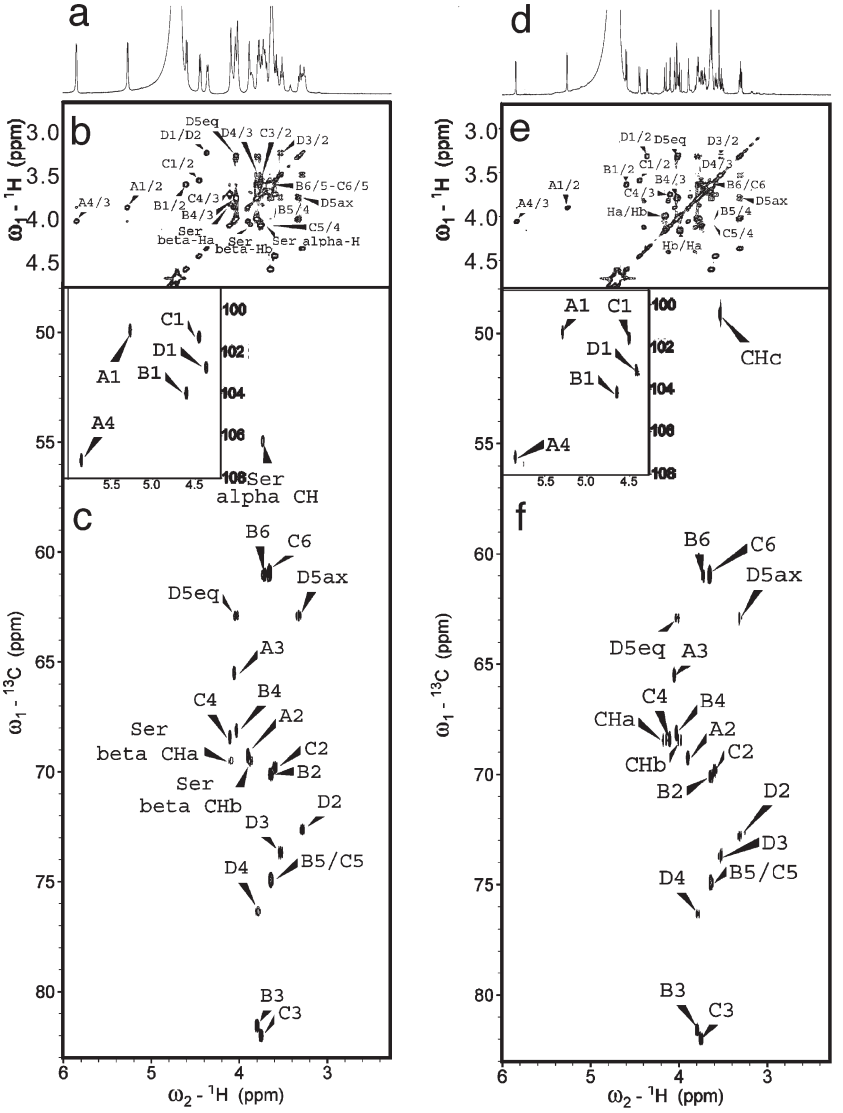

Figure 3. 1D and 2D NMR spectra of the linkage core tetrasaccharides. Spectra for **4i** are (a) 1D ^{1}H NMR; (b) ^{1}H, ^{1}H−COSY; and (c) ^{1}H, ^{13}C-HMQC (inset shows the anomeric signals). Spectra for **4e** are (d) 1D ^{1}H NMR; (e) ^{1}H, ^{1}H−COSY; and (f) ^{1}H, ^{13}C-HMQC (inset shows the anomeric signals). The structures and labeled positions can be found in Figure S4, Supporting Information.

our previous understanding of the differences in the specificity of heparin lyase 1 and 2.[10,11]

Heparin lyase 3 digested heparin released only undersulfated disaccharides containing 0, 1, or 2 sulfo groups with no 2-*O*-sulfo group in the unsaturated uronic acid residues and tetrasaccharides containing residues derived from the undersulfated core protein linkage region (Figure 2). One of the heparin lyase 3 tetrasaccharide products, **4i** (ΔUA-Gal-Gal-Xyl-*O*-Ser), has a structure associated with the core protein linkage region and has been previously reported by our group and other groups.[37−39] 1D and 2D NMR spectra (Figure 3, Table S2, Supporting Information) indicated that **4e** (ΔUA-Gal-Gal-Xyl-*O*-$CH_2CONHCH_2COOH$) had a structure similar to **4i** but is distinct from any previously reported structure. The anomeric protons A1 (5.264 ppm), B1 (4.603 ppm), C1 (4.453 ppm), and D1 (4.353 ppm) in **4e** had the same chemical shifts as **4i**. Differences in the HMQC spectra originated from two different amino residues attached to these tetrasaccharides. **4i** had the expected serine amino acid residue attached to the reducing end of the core tetrasaccharide. The alpha proton and the beta protons in the serine residue could be easily assigned in the HMQC spectrum. However, the same cross-peaks were absent in the HMQC spectrum of **4e**. Instead, the resonances of H_a (4.179 ppm), H_b (3.984 ppm), and H_c (3.533 ppm) in the HMQC spectrum for **4e** indicated an *O*-glycolylglycine residue. Assignment of the structures of **4e** and **4i** is shown in Figure S4, Supporting Information. The structures of these two compounds were further confirmed by high-resolution mass spectrometry with the molecular ion peaks $[M - H]^-$ for **4e** and **4i** detected at 746.19 and 718.20, respectively (Figure S4, Supporting Information).

The seven commercial heparin samples were digested by the three heparin lyases individually and were analyzed in triplicate by SAX-HPLC (Figure S2, Supporting Information). The assigned peaks were integrated with the relative peak areas shown in Table S1, Supporting Information. The average micromolar concentrations of the oligosaccharide compositions are presented in Figure 4. Each heparin lyase afforded a unique chromatogram containing different disaccharides and oligosaccharides, providing more structural information for each individual heparin sample than would a

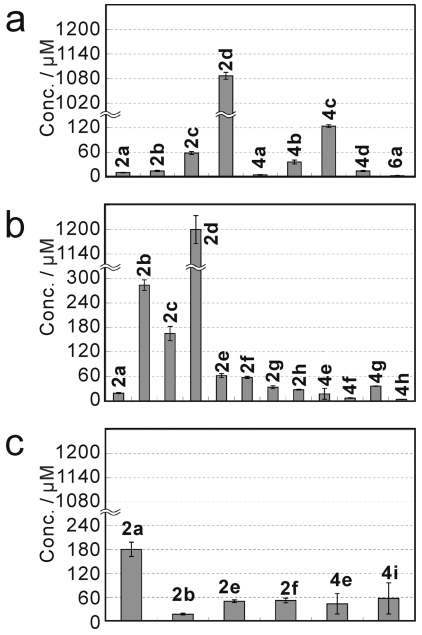

Figure 4. Heparin oligosaccharide mapping results based on the quantification of SAX-HPLC data. Heparin (M_W 12 000 Da) at 83 μM was treated with (a) heparin lyase 1; (b) heparin lyase 2; and (c) heparin lyase 3. The micromolar concentrations of each product are shown with standard deviations. The structures of these oligosaccharides can be found in Figure 2.

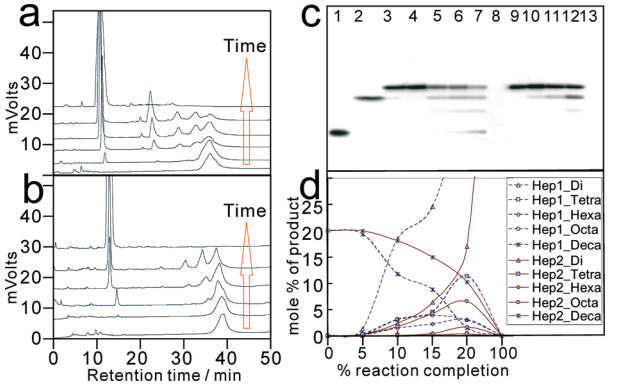

Figure 5. Heparin lyase 1 and heparin lyase 2 action pattern studies using decasaccharide **10a** as a model substrate. **10a** ΔUA2S-[-GlcNS6S-IdoA2S-]$_4$-GlcNS6S. SAX-HPLC chromatograms of a digestion time course are shown for (a) heparin lyase 1 and (b) for heparin lyase 2. The red arrows indicate increasing digestion time. PAGE (22% total acrylamide) analyses of **10a** treated by heparin lyase 1 and heparin lyase 2 are shown in (c) where lane 1 is **4d**; lane 2 is **8b** ΔUA2S-[-GlcNS6S-IdoA2S-]$_3$-GlcNS6S; lane 3 through lane 8 are **10a** incubated with heparin lyase 1 from time-point 0 to time-point 5; lane 9 through lane 13 are **10a** treated by heparin lyase 2 from time-point 0 to time-point 5; lane 8 and lane 13 are **10a** exhaustively digested by heparin lyase 1 and heparin lyase 2, respectively. The mole percent of each size product (indicated with different symbols) is plotted as a function of percent reaction completion in (d) for heparin lyase 1 (dotted lines) and heparin lyase 2 (solid lines).

simple disaccharide analysis. The relative quantities of certain oligosaccharides varied significantly between the different commercial heparin samples (Figure 4 and Table S1, Supporting Information) suggesting such analyses might be useful for quality evaluation within the heparin manufacturing process. The manufacturing process may also modify the structure of the reducing end of intact heparin resulting in a reduction in the formation of **4i** and an increase in the formation of the new structure of **4e**. The relative amount of **4e** to **4i** is on average 1−1.3 (Table S1, Supporting Information).

Specificity of Heparin Lyase 3. The specificity of heparin lyase 3 has been previously studied in our group using heparin-derived tetrasaccharides and hexasaccharides as primary substrates.[10] Heparin lyase 3 cleaves the glucosamine (1 → 4) iduronic acid/glucuronic acid linkage, where the uronic acid residue contains no 2-*O*-sulfo group. Furthermore, heparin lyase 3 acts on either heparan sulfate polysaccharide[11,17] or heparan sulfate precursor-derived oligosaccharides in a random endolytic action pattern.[40] The current study examines the heparin lyase 3 sensitivity of some previously prepared heparin-derived oligosaccharides.[18] Oligosaccharide substrates **4c** (ΔUA2S-GlcNS6S-GlcA-GlcNS6S) and **6b** (ΔUA2S-GlcNS6S-IdoA2S-GlcNS6S-GlcA-GlcNS6S) having the same internal GlcNS6S (1 → 4) GlcA linkage gave different results when treated with heparin lyase 3 under identical reaction conditions. Tetrasaccharide **4c** was cleaved, while hexasaccharide **6b** was not a substrate. Similarly, hexasaccharide **6a** (ΔUA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S) was converted by heparin lyase 3 into a tetrasaccharide and a disaccharide product, whereas octasaccharide **8a** (ΔUA2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS3S6S) bearing the same potentially cleavable linkage failed to serve as a substrate (Figure 1, Figure S3, Supporting Information). Hexasaccharides **6c** (ΔUA2S-GlcNS6S-IdoA2S-GlcNAc-GlcA-GlcNS6S) and **6d** (ΔUA2S-GlcNS6S-IdoA-GlcNAc6S-GlcA-GlcNS6S) were both digested by heparin lyase 3 (Figure S3, Supporting Information), and this result is consistent with our previous report.[10] These experiments suggest that heparin lyase 3 can cut an oligosaccharide at a linkage between glucosamine (1 → 4) unsulfated iduronic acid/glucuronic acid only when that linkage is two saccharide units away from the nonreducing end. It is likely that extra fully sulfated saccharide residues flanking the cleavage site from the nonreducing end may prevent the action of heparin lyase 3.

Action Patterns of Heparin Lyase 1 and Heparin Lyase 2. At a time-point corresponding to 20% completion of heparin lyase 1 or 2 respective digestion on a decasaccharide **10a** (ΔUA2S-[-GlcNS6S-IdoA2S-]$_4$-GlcNS6S), exolytic products were 3-fold more prominent than endolytic products (Figure 5). Thus, for oligosaccharide substrates, heparin lyase 1 and 2 showed an exolytic bias in their action patterns. This observation is in contrast to the endolytic action patterns of both lyases on polysaccharide substrates.[17,41] Heparin lyase 1 has also been reported to act predominantly by an exolytic, processive mechanism, depolymerizing its substrate by cleaving linkages starting from the nonreducing end of an octasaccharide.[42] Under similar protocol, heparin lyase 2 showed an endolytic, nonrandom action pattern.[36] However, the current study shows that both heparin lyase 1 and heparin lyase 2 have a similar exolytic preference on small oligosaccharide substrates although their substrate specificities are different. These observations suggest a reason for the controversy surrounding the action pattern of these important enzymes, which needs to be further clarified by additional studies.

LMWH Produced by Heparin Lyase 3. A low molecular weight heparin was prepared by the complete heparin lyase 3 digestion of heparin. This new LMWH closely resembled the LMWHs previously reported in our laboratory.[43] Unlike

heparin lyase 1 and 2, heparin lyase 3 is incapable of cleaving linkages within heparin's AT-III binding site.[15] This property of heparin lyase 3 should make it an useful tool for the development of novel anticoagulant agents. This new LMWH had a number-averaged molecular weight (M_N) of 5300 Da, a weight-averaged molecular weight (M_W) of 7700 Da, and a polydispersity (PD) of 1.45, as determined by PAGE analysis on a 15% gel (Table S3, Supporting Information). The reaction completion of heparin lyase 3 on heparin was estimated by 1D ^{1}H NMR spectroscopy, which clearly showed the H-4 signals in unsaturated uronic acid residues (Figure S5, Supporting Information) corresponding to disaccharide and other oligosaccharides produced. The digestion completion was less than 5% ([cleaved linkages/linkages to uronic acid] × 100%) based on the integration of the H-4 signals in unsaturated uronic acid residues and all anomeric proton signals observed. The aPTT activity of this novel LMWH was comparable to a commercially available LMWH but showed a lower antifactor Xa/antifactor IIa ratio (Table S3, Supporting Information).

Under sulfated domains in heparin chains have previously been reported.[44−46] In the above specificity study of heparin lyase 3, we found that the linkage of glucosamine (1 → 4) iduronic acid/glucuronic acid, where the uronic acid residue has no 2-*O*-sulfo group, could not be acted on by the enzyme when the adjacent tetrasaccharide residues were fully sulfated (**6b** and **8a** in Figure 1). Nevertheless, the result of PAGE analysis showed that heparin was partially digested by heparin lyase 3 (Figure S5, Supporting Information). This suggests that the pharmaceutical heparin chains have internal under sulfated domains that can be cleaved by heparin lyase 3 (Figure 1). In this case, one intact heparin chain (M_W = 12 000 Da, Table S3, Supporting Information) could be cut into two shorter chains (M_W = 7700 Da). On the basis of the weight average molecular weight of the resulting LMWH product, one intact heparin chain approximately has only one internal under sulfated region that is susceptible to heparin lyase 3.

Conclusions

Pharmaceutical heparins from a variety of commercial sources were examined using heparin lyases digestion followed by HPLC analysis. The heparin mapping chart based on the quantification by HPLC is another new type of fingerprint of pharmaceutical heparins in addition to the regular NMR spectra and disaccharide analysis chromatogram. The mapping methodology may also serve as a way of evaluating and monitoring heparin manufacturing processes. The specificity of heparin lyase 3 and the action patterns of heparin lyase 1 and heparin lyase 2 have also been determined in the current study. Heparin lyase 3 has been shown unexpectedly to cleave heparin at linkages of glucosamine (1 → 4) iduronic acid/glucuronic acid with no 2-*O*-sulfo group on the uronic acid. We believe that the use of large amounts of recombinant heparin lyase 3 and sensitive analytical methods explains why we were the first to report heparin sensitivity to heparin lyase 3. The susceptibility of linkages to heparin lyase 3 is also affected by the structure and length of the adjacent saccharide residues. By using heparin lyase 3, we observe that most of the pharmaceutical heparin chains have only one internal undersulfated domain and one undersulfated domain adjacent to the core protein linkage region. Heparin lyase 3 is incapable of cleaving the AT binding site present in an intact heparin chain. Cleavage of heparin's undersulfated domains affords a novel LMWH with a similar average molecular weight as other currently available LMWHs.

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Supporting Information Available: Quantification of oligosaccharide compositions (Table S1), NMR data for oligosaccharide standards (Table S2), molecular weights (Table S3), PAGE analysis for digested heparins (Figure S1), chromatogram of digested heparins (Figure S2), analysis of heparin lyase 3 treated oligosaccharides (Figure S3), structures and mass spectra of **4e** and **4i** (Figure S4), and PAGE and NMR analysis of LMWH (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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