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Specificity Studies on the Heparin Lyases from *Flavobacterium heparinum*†

Umesh R. Desai, Hui-ming Wang, and Robert J. Linhardt*

Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

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ABSTRACT: An understanding of the substrate specificity study of the heparin lyases (heparinase and heparitinases) is crucial for elucidation of the sequence of heparin and heparan sulfate. Four chemically modified heparins have been used to study the substrate specificity of the three heparin lyases. These modified heparins inlcude the *N*- and *O*-desulfated and then specifically *N*-sulfated or *N*-acetylated derivatives of heparin and a modified heparin containing L-galactopyranosyluronic acid residues. These chemically modified heparins were degraded to various extents by the three heparin lyases. Differences in degree of sulfation have profound impact on the ease of cleavage of glycosidic linkages. Heparin lyase I (EC 4.2.2.7) is selective in cleaving highly sulfated polysaccharide chains containing linkages to 2-*O*-sulfated α-L-idopyranosyluronic acid residues. Heparin lyase III (EC 4.2.2.8) cleaves linkages that have reduced density of sulfation and that contain β-D-glucopyranosyluronic acid residues. The ability of heparin lyase III to act on linkages to unsulfated α-L-idopyranosyluronic acid residues is observed for the first time. Heparin lyase II (no assigned EC number) demonstrates an unparalleled, wide specificity for substrates comprised of linkages containing both α-L-idopyranosyluronic and β-D-glucopyranosyluronic acid residues. Heparin lyase II can also act on substrates containing linkages to unnatural α-L-galactopyranosyluronic acid residues. The high level of specificity of heparin lyase I makes it particularly suitable for use in the sequencing of heparin and heparan sulfate, while caution must be excercised in using heparin lyases II and III to sequence heparin and heparan sulfate because of their relatively broad specificity.

Heparin and heparan sulfate are sulfated, polydisperse, microheterogeneous, linear copolymers of alternating 1→4-linked 2-deoxy-2-aminoglucopyranose and hexopyranosyluronic acid residues. Although heparin has been used as an anticoagulant for the past 5 decades, its precise structure remains unknown. Much of the biological activity of heparin and heparan sulfate [for reviews, see Linhardt and Loganathan (1989) and Gallagher et al. (1986)] stems from the polyelectrolyte nature of these polysaccharides. The correlation of biological activity of these polymers with their structure requires the sequencing of these biopolymers.

Heparin lyases, isolated from *Flavobacterium heparinum* (Payza & Korn, 1956), also known as *Cytophaga heparina* (Christensen, 1980), are important members of a class of enzymes called polysaccharide lyases (EC 4.2.2.*n*) that depolymerize certain acidic polysaccharides (Linhardt et al., 1986). Until recently heparin lyase I (heparinase, EC 4.2.2.7), was the only heparin lyase that had been purified to homogeneity (Yang et al., 1985). Heparin lyase II (heparitinase II, no assigned EC number) and heparin lyase III (heparitinase I, EC 4.2.2.8) have recently been purified to homogeneity in our laboratory (Lohse & Linhardt, 1992).

Enzymatic degradation studies of the intact heparin and heparan sulfate polymers and identification of resulting disaccharide products have been the principal modes of studying substrate specificities of heparin lyases. Studies by a number of researchers (Linker & Sampson, 1960; Hovingh & Linker, 1970; Perlin et al., 1971) demonstrated that the principal cleavage site by heparin lyase I is →4)-α-D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→ and by heparin lyase III is →4)-α-D-GlcNp2S(or 2Ac)6S(or 6OH)-(1→4)-β-D-GlcAp(1→).[1] The primary specificity of heparin lyase II was shown to be broad based by its ability to degrade heparin, heparan sulfate, and chemically modified heparins (Nader et al., 1990; McLean et al., 1985; Moffat et al., 1985, 1991). Heparin lyase II acts at linkages to either α-L-idopyranosyluronic or β-D-glucopyranosyluronic acid residues (Linhardt et al., 1990). The only other lyase capable of similar catalytic diversity is chondroitin ABC lyase (EC 4.2.2.4) from *Bacterioides* sp. or *Protease vulgaris* (Linhardt et al., 1986a).

Using defined oligosaccharide substrates, Rice and Linhardt (1989) showed heparin lyase I also tolerates 3-*O*-sulfation and/or 6-*O*-sulfation on the α-D-2-deoxy-2-aminoglucopyranose residue. Studies using heparin lyase II on defined oligosaccharide substrates agreed with the results from the polymeric substrate studies of Moffat et al. (1991). This study also demonstrated that heparin lyase III could tolerate a 6-*O*-sulfate group on the α-D-2-deoxy-2-aminoglucopyranose moiety, in contrast to reports by Nader et al. (1990).

The analysis of substrate specificity of the heparin lyases has been intriguingly difficult due to the many contrasting observations made by researchers in this field. Some of these anomalous observations are probably the result of contaminating impurities in preparations of the heparin lyases being

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* To whom correspondence should be addressed.

[1] Abbreviations: CDSNAc, completely desulfated *N*-acetylated heparin; CDSNS, completely desulfated *N*-resulfated heparin; CNDSNAc, completely *N*-desulfated *N*-acetylated heparin; GalAHep, α-L-galactopyranosyluronic acid containing heparin; IdoAp, α-L-idopyranosyluronic acid; GlcAp, β-D-glucopyranosyluronic acid; GalAp, α-L-galactopyranosyluronic acid; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; S, sulfate; IdoAp2S, α-L-idopyranosyluronic acid 2-sulfate; GlcNp2S, α-D-2-deoxy-2-sulfamidoglucopyranose; GlcNp2S6S, α-D-2-deoxy-2-sulfamidoglucopyranose 6-sulfate; GlcNp2Ac, α-D-2-deoxy-2-acetamidoglucopyranose; GlcNp2Ac6S, α-D-deoxy-2-acetamidoglucopyranose 6-sulfate; PAGE, polyacrylamide gel electrophoresis; SAX, strong anion exchange; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; UV, ultraviolet; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; 2D, two dimensional; TSP, sodium (trimethylsilyl)pentanoate-2,2,3,3,4,4-d_6; S/N, signal to noise ratio; COSY, *J*-correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy.

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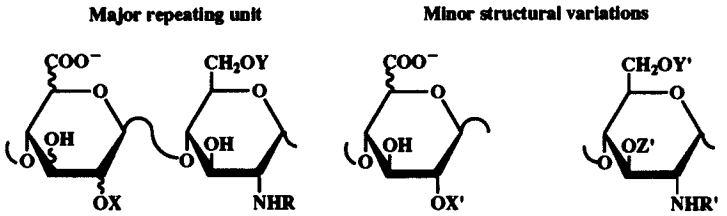

Polysaccharide	Major repeating unit	Minor structural variations	
Heparin	IdoAp; X = SO_3^- Y = SO_3^- R = SO_3^- (60-80%)	GlcAp; X' = H IdoAp; X' = H	Y' = H or SO_3^- R' = Ac or SO_3^- Z' = H or SO_3^-
Heparan sulfate	GlcAp; X = H Y = SO_3^-; R = Ac (35-45%) Y = H; R = SO_3^- (35-45%)	IdoAp; X' = H	Y' = H or SO_3^- R' = SO_3^- or Ac Z' = H or SO_3^-
CDSNAc	IdoAp; X = H Y = H R = Ac (90%)	GlcAp; X' = H IdoAp; X' = H or SO_3^-	Y' = H or SO_3^- R' = SO_3^- or Ac Z' = H or SO_3^-
CDSNS	IdoAp; X = H Y = H R = SO_3^- (90%)	GlcAp; X' = H IdoAp; X' = H or SO_3^-	Y' = H or SO_3^- R' = SO_3^- or Ac Z' = H or SO_3^-
CNDSNAc	IdoAp; X = SO_3^- Y = SO_3^- R = Ac (90%)	GlcAp; X' = H IdoAp; X' = H or SO_3^-	Y' = H R' = SO_3^- or Ac Z' = H or SO_3^-
GalAHep	GalAp; X = H Y = SO_3^- R = SO_3^- (75-85%)	GlcAp; X' = H IdoAp; X' = H or SO_3^-	Y' = H or SO_3^- R' = SO_3 or Ac Z' = H or SO_3^-

FIGURE 1: Structures of heparin, heparan sulfate, and chemically modified heparins. The numbers in parentheses represent the porportion of major repeating unit in the polydisperse heterogeneous mixture.

studied. The possibility of enzymatic digestion conditions affecting heparin lyase substrate cannot be disregarded. The use of defined oligosaccharide substrates, as an approach to define the heparin lyase specificity originated by our research group (Linhardt et al., 1990), is also not without difficulties. First, these oligosaccharides are often poor substrates and their enzymatic breakdown requires forcing conditions. Second, defined oligosaccharide substrates having linkages that infrequently occur in heparin and heparan sulfate are difficult to prepare in quantities sufficient to carry out complete specificity studies.

The present paper adopts the approach used by Moffat et al. (1991) which relied on chemically modified heparins (Figure 1) to probe the different sites of heparin lyase II action. In combination with gel electrophoresis based oligosaccharide mapping (Linhardt et al., 1990) and capillary electrophoresis based disaccharide analysis (Ampofo et al., 1991), the substrate specificities of all three heparin lyases have been studied. The results presented expose some new complexities that need to be consider when using these lyases for heparin and heparan sulfate sequence analysis.

EXPERIMENTAL PROCEDURES

Materials

Heparin, sodium salt, was obtained from Hepar (porcine mucosal, lot ST 82261, 145 units/mg). Heparan sulfate, sodium salt (porcine intestine, ORG 553), was a gift from Organon, Oss, The Netherlands. Completely desulfated *N*-acetylated heparin (CDSNAc), completely desulfated *N*-resulfated heparin (CDSNS), and completely *N*-desulfated *N*-acetylated heparin (CNDSNAc) were obtained from Seikagaku American Inc., Rockville, MD. Disaccharides **1–8** were from Grampian Enzymes, Scotland. These disaccharides are now also available from Sigma Chemical Co., St. Louis, MO. Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (no assigned EC number), and heparin lyase III (heparitinase, EC 4.2.2.8) were purified to apparent homogeneity from *F. heparinum* (Lohse & Linhardt, 1992). The enzymatic activities of the three heparin lyases were measured under the same conditions used for the substrate specificity studies. Heparin lyase I had enzymatic activity of 130 units/mg (1 unit of heparin lyase activity is defined as 1 μmol of product formed per minute using 1 mg/mL substrate at the appropriate temperature) on heparin. Heparin lyase II had enzymatic activity of 19 units/mg on heparin and 36.5 units/mg on heparan sulfate. Heparin lyase III had enzymatic activity of 63.5 units/mg on heparan sulfate. Dialysis membranes of defined molecular weight cutoffs (MWCO) were from Spectrum Medical Industries, Los Angeles, CA. Syringe filters (0.2 and 0.45 μm, type HA) were from Millipore, Bedford, MA. HPLC was performed on dual constametric II pumps connected through a gradient mixer from LDC, Milton Roy, Riveria Beach, FL. A fixed-loop injector 7125 from Rheodyne, Cotati, CA, and variable-wavelength UV-5 detector from ISCO, Lincoln, NE, were used. Strong-anion-exchange (SAX) HPLC was carried out using a Spherisorb (4.6 × 250 mm, 5-μm particle size) column from Phase Separations, Norwalk, CT. UV spectroscopy used a Shimadzu Model UV-160 spectrophotometer equipped with a thermostated cell. A 32-cm vertical slab gel unit (SE620) was supplied by Hoefer Scientific Instruments, San Francisco, CA. Tris and SP-Sephadex C-50 were from Sigma. Acrylamide and *N,N'*-methylenebis(acrylamide) (Electran grade) were obtained from BDH Limited, Poole, England. Bio-gel P-2 (fine) and Coomassie dye were from Bio-Rad, Richmond, CA. Capillary electrophoresis was performed with a Dionex Capillary Electrophoresis System from Dionex Corp., Sunnyvale, CA. Electrophoresis grade sodium borate and boric acid were from Fischer Scientific, Fair Lawn, NJ. All other chemicals were reagent grade.

Methods

Preparation of Substrates. A uniform, sodiated heparin was prepared by dialyzing (MWCO 3500 at 4 °C) heparin

(approximately 20 mg in 100 mL of water) first against 1 M sodium chloride (10 volumes), followed by dialyzing against distilled, deionized water (3 × 1000 volumes). After dialysis the heparin was syringe-filtered (0.2 μm) into a preweighed vial, freeze dried, and stored desiccated over anhydrous calcium chloride for 2 days. The heparin sample was then carefully weighed, and the appropriate volume of distilled, deionized water was added to obtain a 20 mg/mL stock solution. A similar procedure was followed to prepare heparan sulfate stock solution. The chemically modified heparins CDNS, CNDSNAc, and CDSNAc were used as obtained. Stock solutions at 20 mg/mL concentration were prepared similarly but without dialysis. A 20 mg/mL stock solution of GalAHep polymer was prepared as previously described (Desai et al., 1993).

Treatment of Substrates with Lyases. A number of conditions for enzymatic depolymerization by heparin lyases are reported. These include the ethylenediamine–acetate buffer system (Nader et al., 1990), the sodium acetate–calcium acetate buffer system (Ototani et al., 1983), and the sodium phosphate buffer system (Linhardt et al., 1990; Moffat et al., 1991; Turnbull & Gallagher, 1988). While all these conditions are suitable, the optimum conditions, elucidated by Lohse and Linhardt (1992) for the enzymatic digestions by the three enzymes, were used. The reactions were performed to completion in 24 h at 30 °C (heparin lyases I and II) and 43 °C (heparin lyase III). The substrate (200 μg) in 5 mM sodium phosphate containing 200 mM sodium chloride (100 μL) at pH 7.0 was treated with heparin lyase (3 milliunits), and the digestion was run for 24 h. The reactions were terminated by addition 30 mM hydrochloric acid, resulting in a solution at pH 3.0. To remove the enyzme, the reaction mixture was poured onto an SP-Sephadex microcolumn (100-μL total gel volume) preequilibrated with hydrochloric acid at pH 3.0. The column was washed with hydrochloric acid (2 mL, pH 3.0), and the eluent was adjusted to pH 7.0 with sodium hydroxide (0.01 N) and freeze dried.

Analysis of Polymeric Substrates and Their Digested Mixture. The depolymerized, freeze-dried samples of heparin, heparan sulfate, and chemically modified heparins were reconstituted at 1 mg/mL concentration. The final UV absorbance value of the enzymatic digestion mixture was measured at 232 nm in 30 mM hydrochloric acid solution ($\epsilon_M \approx 5500$; Linhardt et al., 1988). The molecular weight of the disaccharide resulting from the major repeating unit of the polysaccharide chain (see Figure 1) was used to calculate the moles of product formed. The percentage of linkages cleaved was calculated on the basis of the expected UV absorbance from the known amount of polymeric substrate (100 μg) assuming complete depolymerization of substrates to disaccharides.

The enzymatic digestion mixtures were analyzed by linear gradient polyacrylamide gel electrophoresis (PAGE). The linear gradient resolving gels (12–22% total acrylamide) were prepared as described by Al-Hakim et al. (1990). A linear gradient was formed between the glass plates (32 cm × 16 cm × 0.75 cm) from the bottom (maximum gel concentration 22%) to the top (minimum gel concentration 12%). Polysaccharide samples (40 μL) containing approximately 50% (w/v) sucrose and a trace quantity of Phenol Red were carefully layered onto bottom of the wells with a microsyringe. Marker samples (10 μL) containing trace quantities of Bromophenol Blue and Phenol Red in 50% (w/v) sucrose were also run on each gel. Samples on a single gel were subjected to electrophoresis at a constant voltage of 400 V for approximately 8 h. Heat was dissipated using a heat exchanger with circulating tap water (10–15 °C). Polysaccharides were visualized by staining with Alcian Blue followed by silver staining as described previously (Rice et al., 1987; Al-Hakim & Linhardt, 1991).

Capillary electrophoresis was used to identify the disaccharides formed by the heparin lyase degradation of the polysaccharides. A new capillary (69-cm total length, 75-μm internal diameter) was activated by sequentially washing with phosphoric acid (0.1 M, 500 μL), sodium hydroxide (0.5 M, 1 mL), distilled, deionized water (37 °C, 500 μL), and run buffer (1 mL). The buffer consisted of 10 mM sodium borate containing 50 mM boric acid at pH 8.8, and the analysis employed detection at 232 nm. The samples (1 μg/μL), prepared in deionized water, were injected into the capillary using hydrostatic pressure. Each injection used approximately 14 nL of the sample. Electrophoresis was performed at a constant voltage of 20 130 V for a period of 30 min. Data points were sampled at a rate of 10 Hz. A dedicated computer system (ACI Model I) by Dionex performed the retention time analysis and integration of the electropherograms.

NMR Spectroscopy of Polysaccharides and Disaccharide 9. All NMR experiments were performed using either a Bruker WM360 or a Bruker AMX600 (Spectrospin AG, Switzerland) spectrometer at the operating frequency of 360 or 600 MHz for proton, and 90 or 150 MHz for carbon, respectively. The samples of chemically modified heparins (10 mg) were dissolved in distilled, deionized water (500 μL) and filtered through a 0.45-μm membrane. The solution was lyophilized and the resulting solid exchanged in ^2H_2O (99.9% ^2H) by repeated lyophilization. After three exchanges, the solid was dissolved in ^2H_2O (99.996% ^2H, 500 μL) for NMR experiments. The ^1H NMR experiments were conducted at 298 K with a digital resolution of 0.246 Hz/point and a 30° pulse. A line broadening factor of 0.244 Hz was used prior to Fourier transformation. The HO^{2}H signal was suppressed with a low power pulse applied over a period of 1.5 s. The ^{13}C spectra were acquired with a 45° pulse using proton–noise decoupling with decoupling power set at 2 W. Typically 90 000 scans were acquired for a good S/N ratio. The TSP signal was set at 0 ppm as the reference for ^{13}C NMR spectra. The structure elucidation of disaccharide **9** was carried out using a combination of 2D NMR techniques such as *J*-correlated spectroscopy (COSY) and dipolar correlated spectroscopy (NOESY). These pulse programs utilized standard Bruker software. Typically the data were acquired using 1024 data points in the *f*2 dimension and 256 data points in the *f*1 dimension, and Fourier transformed onto a 1K×1K data matrix using a sine bell window function.

RESULTS AND DISCUSSION

The discovery of numerous new biological activities for heparin and heparan sulfate has resulted in increased activity in the field of acidic polysaccharide sequencing. While the classical sequencing techniques used for proteins and nucleic acids have not yet been applied to carbohydrates, oligosaccharide mapping relying on heparin lyases has been widely used. The use of enzymes in sequencing is an attractive approach because of the mild conditions, the absence of chemical contaminants complicating analysis, and the presumed high specificity of enzymes.

Although the substrate specificity of the heparin lyases has been under investigation for a long time, complications have arisen due to confusing enzyme nomenclature and the wide variety of experimental conditions used in different studies. Studies of the enzymatic degradation of heparin and heparan sulfate have also been performed using enzyme preparations

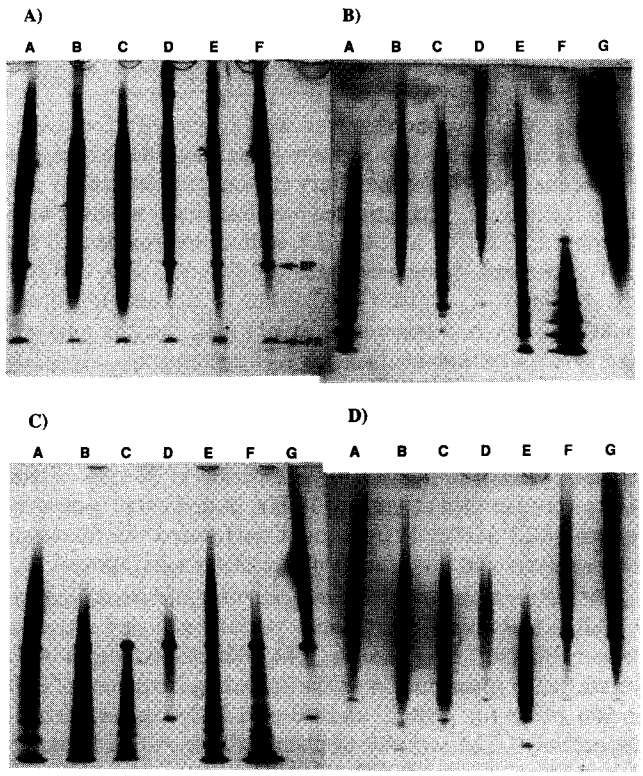

FIGURE 2: Gradient PAGE with alcian blue/silver staining of lyase digested polymeric substrates. Section A shows untreated polymeric substrates, B shows heparin lyase I (EC 4.2.2.7) digested polymeric substrates, C shows heparin lyase II (no assigned EC number) digested polymeric substrates, and D shows heparin lyase III (EC 4.2.2.8) digested polymeric substrates. The corresponding lane assignments are as follows: A, GalAHep; B, CNDSNAc; C, CDSNS; D, CDSNAc; E, heparan sulfate; F, heparin; and G, in sections B, C, and D, undigested heparin as control.

of varying homogeneity. The present work eliminates these potential problems. Homogeneous preparations of the three heparin lyases are used, and these have been established to correspond to commercial preparations (Linhardt et al., 1990). The optimized conditions for the enzymatic degradation have also been applied in this study (Lohse & Linhardt, 1992).

Heparin, heparan sulfate, and chemically modified acidic polysaccharides (Figure 1) were used to determine the importance of polymer substitution and functional groups on substrate specificity. The chemical composition of the heparin and heparan sulfate used in this study has been fully analyzed by oligosaccharide mapping (Linhardt et al., 1990) and by using ^{1}H and ^{13}C NMR spectroscopy. Completely desulfated *N*-acetylated heparin (CDSNAc), completely desulfated *N*-resulfated heparin (CDSNS), and completely *N*-desulfated *N*-acetylated heparin (CNDSNAc) were subjected to NMR spectroscopic analysis. The ^{1}H NMR spectra of these modified heparins were found to be similar to that of heparin, suggesting the retention of backbone structure in the chemical modification. The ^{13}C NMR analysis suggests that greater than 90% functional transformation from starting heparin to the modified substrates has occurred. In addition, the ^{13}C NMR spectrum of CDSNAc and CNDSNAc indicates the proportion of β-D-glucopyranosyluronic acid residues to α-L-idopyranosyluronic acid residues to be about 1:10. A modified polymer (GalAHep, Figure 1), containing α-L-galactopyranosyluronic acid residues, was prepared following the method of Jaseja et al. (1990). The ^{1}H NMR spectrum of this modified polymer suggested that approximately 85% of the total uronic acid residues had been chemically converted to α-L-galactopyranosyluronic acid residues (Desai et al., 1993).

The chemically modified heparins, heparin, and heparan sulfate were depolymerized using the standard protocol with the three heparin lyases, and the depolymerized mixture was examined by gradient PAGE (Figure 2) and UV spectroscopy (Table I). Heparin was a good substrate for heparin lyase I (Figure 2B, lane F), leading to formation of disaccharides and oligosaccharides. Heparin lyase I acted on heparan sulfate and GalAHep to a limited extent, but failed to depolymerize any of the other chemically modified heparins Figure 2B, lanes B–D). The low final UV absorbance values (Table I) for heparin lyase I acting on the chemically modified heparins is probably due to the failure of chemical methods used to prepare these modified heparins to effect the total desired transformation, thereby retaining a small proportion of heparin's major structural repeating unit (Figure 1). These observations strongly suggest that both *O*- and *N*-sulfation are required for cleavage by heparin lyase I. Heparan sulfate is a poor substrate for heparin lyase I, consistent with the known presence of rare sequences having the structure →4)-α-D-GlcNp2S(1→4)-α-L-IdoAp2S(1→ (Habuchi et al., 1992). While GalAHep is partially degraded by heparin lyase I (Figure 2B, lane A), SAX-HPLC analysis of the products formed showed that only glycosidic linkages, which escaped modification [i.e., →4)-α-D-GlcNp2S6S(1→4)-α-L-Idop2S-(1→] in the chemical synthesis of GalAHep, are cleaved (Desai et al., 1993).

The digestion mixtures of the polymeric substrates were also analyzed by capillary electrophoresis (CE). Capillary electrophoresis is a highly sensitive technique capable of detecting femtomole amounts of unsaturated disaccharides (Figure 3) (Ampofo et al., 1991). The CE electropherogram of the digested products of heparin degradation by heparin lyase I shows an intense peak due to disaccharide **1** as well as minor peaks corresponding to disaccharides **2-4** (data not shown). No peaks corresponding to disaccharides **5–8** were observed, suggesting a rather specific requirement of functional groups of heparin lyase I to act. The CE analysis of chemically modified heparins shows no formation of disaccharides **1–4** (data not shown), suggesting that only isolated sites are cleaved, resulted in large oligosaccharide products not analyzable by CE.

The substrate specificity of heparin lyase II has been explored in depth by Moffat et al. (1991) using size exclusion chromatography. Our studies using gradient PAGE, UV absorbance measurements, and CE for the analysis of the products formed on the heparin lyase II digestion of these modified polysaccharides confirm their results. Heparin lyase II demonstrates a wide specificity, cleaving all the chemically modified polymers, CDSNS, CDSNAc, and CNDSNAc (Figure 2C, lanes B–D, respectively). An interesting aspect of the heparin lyase II substrate specificity, not previously reported, is its ability to cleave unnatural GalAp-containing linkages. GalAHep was partially cleaved by heparin lyase II as shown by gradient PAGE (Figure 2C, lane A). The gel banding patterns were different from those observed on heparin lyase I cleavage of GalAHep. Capillary electrophoresis of the enzymatic digestion mixture showed an unusual peak at 11.2 min (Figure 4B), assignable to disaccharide **9**. Control experiments with the heparin lyase II depolymerized heparin and heparan sulfate did not show a peak with the same migration time (Figure 4C,D). The >50% proportion of disaccharide **9** found in the GalAHep digestion suggests that heparin lyase II cleaves GalAHep at the α-D-GlcNp2S6S-(1→4)-α-L-GalAp(1→ linkage. To confirm the unusual specificity of heparin lyase II, preparative SAX-HPLC was performed on a large-scale enzymatic digestion mixture and

Table I: Susceptibility of Substrate (100 μg) to the Heparin Lyase Measured by Ultraviolet Absorbance

substrate	heparin lyase I			heparin lyase II			heparin lyase III		
	A232[a]	μmol[b]	% cleavage[c]	A232	μmol	% cleavage	A232	μmol	% cleavage
heparin	0.481	0.093	57.8	0.703	0.135	84.5	0.054	0.010	6.6
heparan sulfate	0.194	0.037	18.7	0.409	0.079	39.3	0.972	0.187	93.5
CDSNAc	0.068	0.013	5.2	0.632	0.122	48.6	0.879	0.169	67.6
CDSNS	0.079	0.015	7.0	0.801	0.154	70.9	0.490	0.094	43.4
CNDSNAc	0.024	0.005	2.6	0.399	0.077	43.1	0.144	0.028	15.6
GalAHep	0.217	0.042	21.8	0.182	0.035	18.3	0.053	0.010	5.3

[a] In 30 mM hydrochloric acid. [b] Micromoles of disaccharide formed using the molecular weights of disaccharides **1** (665), **2** + **6** (533), **8** (401), **4** (461), **5** (605), and **2** (563). [c] Percent cleavage calculated on the basis of complete conversion of substrate (100 μg) to disaccharides (100 μg). See Methods.

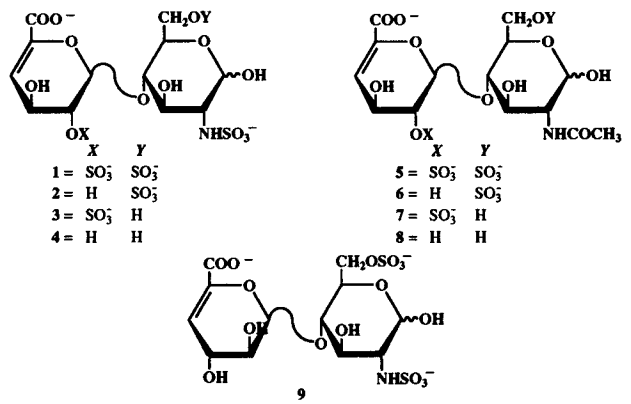

FIGURE 3: Structures of disaccharides **1**–**9**.

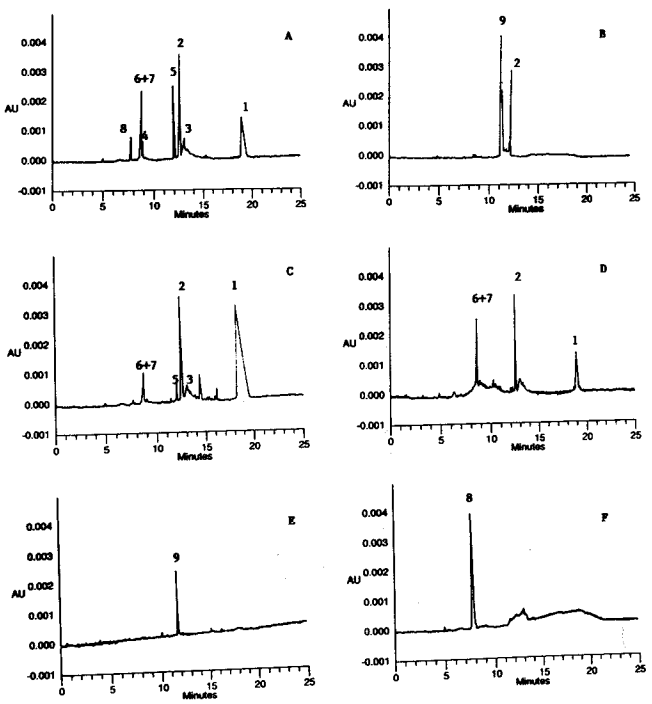

FIGURE 4: Capillary electropherograms of enzymatic digestions of chemically modified heparins. The electrophoresis was performed using 10 mM sodium borate containing 50 mM boric acid at pH 8.8 under a constant voltage of 20 130 V. (A) shows the electropherogram of a disaccharide mixture of **1**–**8**. (B) shows heparin lyase II digested GalAHep; (C) shows heparin lyase II digested heparin. Note the intensity of peak **1** due to the most prominent repeating disaccharide unit in heparin. (D) shows heparin lyase II digeted heparan sulfate. The small peaks around 12–17 min are due to a mixture of higher oligosaccharides. (E) shows disaccharide **9** isolated by SAX-HPLC. (F) shows heparin lyase III cleaved CDSNAc. The broad humps between 10 and 15 min are a result of poorly resolved higher oligosaccharides.

the unusual disaccharide **9** was isolated for structural characterization. The migration position on CE (Figure 4E) suggested that the disaccharide was disulfated. The ^{1}H NMR spectrum (data not shown) showed a peak at δ 5.8 (d), assignable to 4-H of the unsulfated uronic residue, in addition to anomeric protons at δ 5.5 and 5.25 (Table II). The complete assignment of saccharide protons was carried out using *J*-correlated spectroscopy (contour plot not shown). The upfield resonance of 2-H at δ 3.85 suggests that the uronic acid residue is unsulfated. The coupling pattern resulting in a 4-H doublet, in contrast to the doublet of doublet nature of 4-H in unmodified uronic acid residues, suggests stereochemical differences in the uronic acid residue of this unusual disaccharide **9**. The stereochemical assignment was performed using a NOESY experiment. The NOESY contour plot of disaccharide **9** shows a very intense cross-peak between the 2-H and 3-H of the uronic acid residue. The coupling constants of 1-H, 2-H, and 3-H indicate that the uronic acid ring is in a distorted chair conformation, with 2-H and 3-H being pseudoequatorial and *synclinal*. In contrast, in disaccharide **3**, the 2-H and 3-H of the uronic acid residue are *antiperiplanar*, leading to weak dipolar coupling. This suggests reversal of stereochemistry at the 2-position and 3-position, confirming the unusual peak in CE is structure **9** ((1*R*,-2*S*,3*S*)ΔUAp(1→4)-D-GlcNp2S6S). The isolation of disaccharide **9** in the heparin lyase II digestion of GalAHep establishes the unexpected activity of heparin lyase II on this unnatural linkage.

Heparan sulfate is most susceptible to heparin lyase III (Figure 2D, lane E). UV absorbance measurements (Table I) show that heparin lyase III degrades the polymeric chain of heparan sulfate to a greater extent (approximately 95%) than heparin lyase I degrades heparin (approximately 55%).

All the chemically modified heparins, except GalAHep, were unexpectedly found to be good substrates for heparin lyase III. Gradient PAGE analysis of CNDSNAc, CDSNS, and CDSNAc digested with heparin lyase III (Figure 2D, lanes B–D, respectively) show the formation of tetrasaccharides and intermediate molecular mass oligosaccharides. These results are very similar to product distribution observed when heparan sulfate is digested with heparin lyase III (Figure 2D, lane E). UV absorbance measurements indicate a cleavage of 88% of the susceptible linkages in CDSNAc by heparin lyase III. CDSNS and CNDSNAc are cleaved by heparin lyase III to about 50% and 15%, respectively. These data suggest, for the first time, that heparin lyase III is able to cleave linkages containing unsulfated α-L-idopyranosyluronic acid residues. The cleavage of chemically modified heparins by heparin lyase III indicates that the major requirement for enzyme action is reduced sulfation. The greater susceptibility of CDSNAc and CDSNS toward heparin lyase III than CNDSNAc shows that, as the degree of sulfation increases, the susceptibility toward heparin lyase III decreases. The failure of heparin lyase III to act on GalAHep shows that, while heparin lyase III is capable of cleaving linkages

Table II: ^{1}H Chemical Shifts (ppm) and Coupling Constants (^3J and ^2J, Hz) for Disaccharide **9**[a]

proton[b]	unsatd uronic acid	glucosamine
1-H	5.26 (2.7)[c]	5.48 (3.5)
2-H	3.85 (2.6, 7.5[d])	3.29 (3.6, 10.5)
3-H	4.36 (3.1, 7.5[d])	3.71 (8.9, 10.4)
4-H	5.80 (3.0)	3.84 (m[e])
5-H		4.16 (2.1, 3.5, 10.1)
6-H		4.28 (2.1, 11.0)
6′-H		4.38 (3.9, 11.1)

[a] Data for the α-anomer (major) is presented. The β-anomer accounts for less than 10% of the total population. [b] The assignment of protons was carried out using 2D COSY and 2D NOESY. [c] The numbers in parentheses represent first-order coupling constants. [d] The 7.5-Hz coupling, although slightly high for *synclinal* orientation of 2-H and 3-H, is significantly lower than ~10 Hz that is normally observed for *antiperiplanar* orientation (see text for configurational assignment). [e] Multiplet unanalyzable due to broadening.

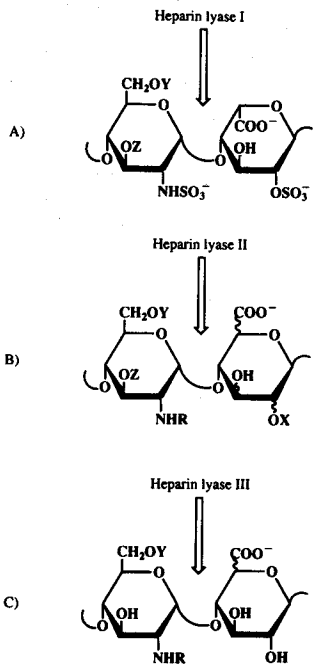

FIGURE 5: Substrate specificity of the heparin lyases. (A) Heparin lyase I cleaves →4)-α-D-GlcNp2S3S(or 3-OH)6S(or 6-OH)(1→4)-α-L-IdoAp2S(1→ linkages. (b) Heparin lyase II cleaves →4)-α-D-GlcNp2S(or 2-Ac)6S(or 6-OH)(1→4)-α-L-IdoAp2S(or 2-OH)(or GalAp)(1→ linkages. Heparin lyase II also cleaves →4)-α-D-GlcNp2S(or 2-Ac)6S(or 6-OH)(1→4)-β-D-GlcAp(1→ linkages. (C) Heparin lyase III cleaves →4)-α-D-GlcNp2Ac(or 2S)6S(or 6-OH)-(1→4)-β-D-GlcAp(or α-L-IdoAp)(1→ linkages. X, Y, and Z represent a S or a H, and R represents a S or an Ac.

containing unsulfated IdoAP and GlcAp residues, it cannot cleave linkages containing unsulfated GalAp residues.

The substrate specificity of heparin lyase III was also analyzed by CE. The capillary electropherogram of the enzymatic digestion mixture of CDSNAc (Figure 4F) shows an intense peak at 8 min. This peak corresponds to disaccharide **8**. Similar experiments on CDSNS (data not shown) show a peak due to disaccharide **4** in a lesser proportion as compared to disaccharide **8**. The observation of these disaccharides in the heparin lyase III digestion of the chemically modified heparins confirms the gradient PAGE and UV absorbance results and unequivocally establishes heparin lyase III can cleave linkages to unsulfated α-L-idopyranosyluronic acid residues.

In conclusion, the structural requirements for cleavage by heparin lyases I–III were examined. Heparin lyase I was found to possess the most stringent structural requirements (Figure 5a), making it potentially the most valuable enzyme for sequence analysis of polysaccharides. Heparin lyases II and III exhibit a broader specificity. Heparin lyase II acts on both natural (IdoAp and GlcAp) as well as unnatural (GalAp) linkages (Figure 5B). Heparin lyase III was unexpectedly found to cleave linkages to either α-L-idopyranosyluronic acid or β-D-glucopyranosyluronic acid residues (Figure 5C). Scientists using these enzymes to study glycosaminoglycan structure should exercise caution in interpreting their results until the full details of the specificity of the heparin lyases have been clarified.

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