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Isolation and characterization of heparan sulfate from crude porcine intestinal mucosal peptidoglycan heparin

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Abstract

A method for the preparation of heparan sulfate from peptidoglycan heparin is described. The objective of this research was to provide a basis for the development and validation of an industrial process to support the preclinical development of heparan sulfate and/or heparan sulfate derivatives. In the preparation of heparan sulfate, heparin was recovered by alcohol fractionation and dermatan sulfate was isolated by selective precipitation. The remaining crude heparan sulfate was fractionated by anion-exchange chromatography into five subfractions. The biological activities of these subfractions were examined by anticoagulant and amidolytic assays. Molecular weight and molecular size were determined using capillary viscometry and polyacrylamide gel electrophoresis. Charge density and degree of sulfation were determined by cellulose acetate electrophoresis and elemental analysis. Oligosaccharide and disaccharide analysis relied on enzymatic depolymerization using heparin lyases followed by polyacrylamide gel and capillary electrophoresis. ^{1}H NMR analysis provided detailed structural information on each subfraction.

Abbreviations: GAG, Glycosaminoglycan; GlcN*p*, 2-Amino-2-deoxy-D-glucopyranose; Ac, Acetate; GlcA *p*, Glucopyranosyluronic acid; S, Sulfate; IdoA *p*, Idopyranosyluronic acid; UA *p*, Hexopyranosyluronic acid; ΔUA *p*, 4-Deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; t-PA, Tissue plasminogen activator; CE, Capillary electrophoresis; PAGE, Polyacrylamide gel electrophoresis.

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Crude heparan sulfate and its subfractions showed significant differences in physical, structural and biological properties.

Keywords: Heparan sulfate; Peptidoglycan; Heparin

1. Introduction

Natural heparin-like polyanions found in heparin sidestreams have been credited with numerous therapeutic properties, including anti-hypolipemic [1], anti-inflammatory, anti-angiogenesis [2], anti-dementia [3], anti-coagulant and anti-arteriosclerotic effects [4,5]. It is common knowledge that the early heparinoids [6] consisted of dermatan sulfate and heparan sulfate-rich fractions often in association with residual chondroitin sulfate, heparin and deoxyribonucleinates. The pharmaceutical use of modified or degraded heparin [7] may have been triggered by the limited availability of heparan sulfate in the past. Following the development of the low molecular weight heparins [8] and other heparin derivatives [9], better characterized heparinoids containing as much as 80% heparan sulfate-like material were introduced [10]. The high concentration of heparan sulfate in all of these latter heparinoids also suggests a need for the large-scale isolation and characterization of pure heparan sulfate.

Heparan sulfate is a generic term describing polysaccharides that are linear and consist of approximately equal numbers of *N*-acetylated [→ 4-α-D-GlcN*p*Ac-(1 → 4)-β-D-GlcA *p*-(1 →] and *N*-sulfated disaccharides [→ 4-α-D-GlcN*p*S-(1 → 4)-β-D-GlcA *p* or α-L-IdoA *p*-(1 →] that are arranged mainly in segregated domains. Approximately 25% of the total polymer is formed by alternating arrangements of the two disaccharide units, → 4)-α-D-GlcN*p*S-(1 → 4)-UA *p*-(1 → 4)-α-D-GlcN*p*Ac-(1 → 4)-UA *p*-(1 → 4)-α-D-GlcN*p*S-(1 → . [11]. The polymer is initially synthesized as a repeating → 4)-α-D-GlcN*p*Ac-(1 → 4)-β-D-GlcA *p*-(1 → disaccharide sequence that is attached to a serine residue of a core protein through a tetrasaccharide, glucuronosyl → galactosyl → galactosyl → xylosyl, linkage region. It then undergoes partial *N*-deacetylation followed by *N*-sulfation of the newly exposed amino groups, partial C-5 epimerization of D-GlcA *p* to L-IdoA *p* and *O*-sulfation.

Heparan sulfate can be distinguished from most other glycosaminoglycans (GAGs) by their saccharide compositions and positive optical rotations. In contrast to heparin, heparan sulfate is characterized by a ratio of GlcN*p*Ac to GlcN*p*S of > 3.0, a sulfate content of $< 20\%$, a D-glucuronic (D-GlcA *p*) to L-iduronic (L-IdoA *p*) acid ratio of more than 2, and a carbazole-to-orcinol ratio of less than 2 [12]. Heparan sulfate shows similarity to heparin in distribution of *N*-sulfate and *N*-acetyl groups, but differs in the disposition of *O*-sulfates and uronic acid epimers [11]. *O*-Sulfates are always found in proximity to *N*-sulfates, which enhances the clustering of the sulfate residues and the heterogeneity in chemical composition and charge density of heparan sulfate [13]. Heparan sulfate, obtained as a byproduct from the production of heparin, is very similar in structure to heparan sulfate that is extracted from many other different tissues [14]. Heparan sulfate can be analyzed enzymatically [15,16]. The enzymatic cleavage of heparan sulfate by heparin lyases I, II and III is stoichiometric, and the cleavage sites are

known [15,16]. These enzymes are eliminases that yield the same nonreducing terminal unsaturated uronic acid residues, ΔUA*p*, from either D-glucuronic acid or L-iduronic acid.

The biological activities of heparan sulfate have recently come under greater scrutiny. Heparan sulfate does not delay the activation of prothrombin but expresses its anticoagulant activity by catalyzing the inhibition of thrombin as it is formed in plasma [17]. Like heparin, heparan sulfate has components that bind antithrombin III [18] and exhibit enhanced specific thrombin-inhibitory and factor-Xa inhibitory activities. The ability of heparan sulfate to activate heparin cofactor II or antithrombin III depends at least in part on the sulfate content of the polymer [19]. More recent studies suggest that only heparan sulfate, which has an antithrombotic effect similar to heparin [20] and may function as the endogenous inhibitor of smooth muscle cell proliferation [21], is involved in high affinity interaction with thrombin [22], increases fibrinolytic activity [23], increases the rate of both t-PA and urokinase-mediated plasmin formation [24,25], and may play a protective role in platelet formation from megakaryocytes [26]. Heparan sulfate also inhibits protease-resistant prion protein accumulation by interfering with the interaction of prion protein with an endogenous GAG or proteoglycan [27] and may inhibit cell metastasis [28,29] by modulation of cell-surface heparan sulfate content.

2. Experimental

Materials.—Raw heparin (stage 14, porcine intestinal mucosal peptidoglycan heparin) [30] and the chromogenic substrates Chromogenic TH and XA (ethylmalonyl-thioprolyl-arginyl-paranitroanilide hydrochloride and 4-methyl-(2*R*)-amino[methoxy(ethoxycarbamate)]pentanoyl-glycyl-arginyl-paranitranilide hydrochloride), human heparin cofactor II (HCII), and human antithrombin III (ATIII) were supplied by Celsus Laboratories (Cincinnati, OH). Human thrombin (Factor IIa) and bovine Factor Xa were supplied by Haemachem (St. Louis, MO). Sheep plasma was supplied by Irvine Scientific (Santa Ana, CA). Purified Water, USP [31] prepared by deionization to a maximum resistivity of 18 megohm/cm, followed by a "depth" prefiltration, reverse osmosis and Pyrogard™ ultrafiltration was used throughout this study. The Milli-RO™ Reverse Osmosis 350, the Pyrogard™ Ultrafilter, the PUF™ Process Ultrafiltration System and membranes were supplied by Millipore Corporation (Bedford, MA). The Virtis 100 SRC Sublimators were from the Virtis Co. (Gardiner, NY). The ZIP Zone Chamber, Super Z Application System and the auxiliaries were supplied by Helena Laboratories (Beaumont, TX). Heparin lyase I (heparinase I, EC 4.2.2.7) was from IBEX Technologies (Montreal, Canada). Heparin lyase II (no EC assigned) and heparin lyase III (EC 4.2.2.8) were prepared from *Flavobacterium heparinum* and purified to homogeneity [32]. The disaccharide standard ΔUA*p*2S-(1 → 4)-D-GlcN*p*S6S was prepared from heparin and characterized [33]. Disaccharides ΔUA*p*2S-(1 → 4)-D-GlcN*p*S and ΔUA*p*-(1 → 4)-D-GlcN*p*Ac6S were from Sigma Chemical Co. (St. Louis, MO). Disaccharides ΔUA*p*-(1 → 4)-D-GlcN*p*S, ΔUA*p*2S-(1 → 4)-D-GlcN*p*Ac6S, ΔUA*p*-(1 → 4)-D-GlcN*p*Ac6S, ΔUA*p*2S-(1 → 4)-D-GlcN*p*Ac and ΔUA*p*-(1 → 4)-D-GlcN*p*Ac were from Grampian Enzymes (Aberdeen, UK). Acryl-

amide (ultrapure), Tris(hydroxymethyl)aminomethane, Alcian blue, bromophenol blue and ammonium persulfate were obtained from Boehringer-Mannheim (Indianapolis, IN). EDTA, sucrose, *N,N,N′,N′*-tetramethylethylenediamine and sodium borate (decahydrate, 99%) were from Fisher Chemical Co. (Fairlawn, NJ). Sodium dodecylsulfate (99%) was from BDH Chemicals (Poole, England). All other chemicals were of the highest commercially available purity.

This paper describes the preparation of a crude heparan sulfate and various subfractions obtained by charge fractionation. Their physical and structural characteristics are examined and their in vitro anti-coagulant properties determined.

General methods.—Preparation of crude heparan sulfate. Crude heparan sulfate, (batch HI-10094) was prepared from porcine intestinal mucosal peptidoglycan heparin [34]. Briefly, peptidoglycan heparin was manufactured from porcine intestinal mucosa, preserved with 1.5–2.5% sodium bisulfite. The pH of the porcine intestinal mucosa slurry was adjusted to 8.5, and the slurry was heated to 90–100°C for 30 min. The temperature was reduced to 50–60°C, 1 g of a proteolytic enzyme from *Bacillus subtilis* (Alcalase 2.4 L, NovoNordisk, Bagsvaerd, Denmark) per liter of slurry was added, and the mucosa was hydrolyzed for 8–16 h. The digested slurry was filtered and added to macroporous anion-exchange resin (Lewatit MP500A, Bayer, Pittsburgh, PA), washed with a 5% (w/v) sodium chloride, the peptidoglycan heparin was then eluted with 20% (w/v) sodium chloride and precipitated from 50% (v/v) ethanol. Residual amino acid components of the peptidoglycan heparin and bacterial endotoxins were removed by degradation with alkaline hydrogen peroxide, followed by chemical inactivation of any vegetative microorganisms, spores and viruses. The relatively mild conditions of reacting peptidoglycan heparin in 0.2% (v/v) hydrogen peroxide at pH 10 for 16 h at 55°C causes the degradation of both bacterial endotoxins and the linkage region in the peptidoglycan heparin, resulting in a loss of amino acid residues and a reduction of molecular weight of about 705 Da without loss of USP heparin activity. Heparin was recovered by alcohol fractionation, and dermatan sulfate was selectively precipitated with copper sulfate under alkaline conditions as previously described [35]. The resulting crude heparan sulfate was diafiltered against Purified Water USP using a 1000 MWCO membrane and freeze dried. From 0.5 kg of peptidoglycan heparin, 5 g of dermatan sulfate and 20 g of crude heparan sulfate (HI-10094) were obtained.

Anti-coagulant activity. The anti-coagulant activity of the heparan sulfate and its fractions was determined according to a modified USP method as the amount that will cause 1 mL of sheep plasma to half-clot when kept for 1 h at 20°C [36].

Anti-Xa and anti-IIa activities. Normal human plasma was diluted 4-fold in water. Anti-Xa activity was determined by incubating 50 μL of heparan sulfate in diluted normal human plasma with 50 μL of bovine Factor Xa (60 PRP ICTH/2.5 mL) at 37°C for 120 s. Then, 50 μL of Chromogenic XA (2 μmol/mL) was added, and residual Factor Xa was measured at 405 nm, as previously described. [36] Anti-IIa activity was determined by incubating 50 μL of heparan sulfate in diluted normal human plasma with 50 μL of human thrombin (12 NIH units/mL) at 37°C for 30 s. Then, 50 μL of Chromogenic TH (2.5 μmol/mL) was added, and the amidolytic thrombin activity was measured at 405 nm, as previously described [37]. Measurements were performed on an ACL 300 Plus instrument from Instrumentation Laboratory (Lexington, MA) and

calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention, Inc. (Rockville, MD).

Nitrogen and sulfur analysis. Nitrogen and sulfur content were determined by elemental analysis by Galbraith Laboratories (Knoxville, TN).

Optical rotation. Optical rotations were measured at 589 nm. The polarimeter employed, Polysciences Model SR-6, was obtained from Fischer Scientific (Cincinnati, OH).

Estimation of molecular weights. Samples were dissolved to 0.8% (w/v) in 0.5 M aq sodium chloride. The outflow time in seconds was measured with an Ostwald capillary viscometer (Fischer Scientific, Cincinnati, OH) in a water bath equilibrated to 25°C. The molecular weight was calculated as previously described [38].

Cellulose acetate electrophoresis. Cellulose acetate electrophoresis was performed, as previously described [39], using either 0.2 M calcium acetate or 0.1 M barium acetate. Both solutions were adjusted to pH 7.25 with acetic acid. Titan III Zip Zone cellulose acetate plates (6.0 × 7.5 cm) were equilibrated with electrolyte, blotted, and samples [1% (w/v)] were applied using the Super Z Application System. The loaded plates were then subjected to electrophoresis. Calcium acetate runs proceeded at constant current of 7.5 mA (per plate) for 120 min; barium acetate runs were at 9 mA constant current (per plate) for 90 min. Following electrophoresis, the plates were air dried and stained with 0.5% (w/v) Toluidine blue in 3% (v/v) acetic acid for 5 min. Destaining proceeded in 1% (v/v) acetic acid for 1 min, and then the plates were submerged in tap water for an additional 10 min. Finally, the plates were dried between filter papers. Electropherograms were quantitated by scanning densitometry using software from pdi (protein + dna) imageWage systems (Huntington Station, NY).

^{1}H NMR analysis. For ^{1}H NMR spectroscopy, approximately 1 mg of each sample was treated repeatedly three times with 0.5 mL portions of ^2H_2O (99.96%, Sigma, St. Louis, MO), followed by desiccation over P_2O_5 in vacuo to exchange the labile protons with deuterium. The thoroughly dried sample was redissolved in 0.7 mL of ^2H_2O (99.96%), and transferred to the NMR tube (5.0 mm o.d. × 25 cm, PP-528; Wilmad Glass Co., Buena, NJ). All spectra were determined on a Varian UNITY-500 spectrometer at an operating frequency of 500 MHz. The instrument was equipped with a VXR 5000 computer system having a process controller and an array processor. The operation conditions for one-dimensional (1D) spectra were as follows: frequency, 500 MHz; sweep width, 6 kHz; flip angle, 90 (6.6 μs); sampling point, 48k; accumulation, 256 pulses; temperature, 323, 313, 303 and 298 K. Quantitative analysis was performed at each temperature with a pulse delay five times the longest T_1 (5.5 s), estimated from an inversion-recovery experiment. Chemical shifts were indicated by ppm from the signal of 3-trimethylsilyl[$^2H_4$]propionic acid, sodium salt, as an internal standard. A small amount of acetone was used as an internal standard (2.225 ppm at 298 K). The water resonance was suppressed by selective irradiation during the relaxation delay.

Two-dimensional (2D) double-quantum-filtered (DQF)-, triple-quantum-filtered (TQF)-COSY spectra, and homonuclear Hartman-Hahn (HOHAHA) spectra were recorded using the phase-sensitive mode. All 2D spectra were recorded with 512 × 2048 data points and a spectral width of 3200 Hz. HOHAHA (MLEV-17) spectra were recorded with a mixing time of 100 ms. The water resonance was suppressed by

selective irradiation during the relaxation delay. A total of from 128 to 256 scans were accumulated for each t_1, with a relaxation delay of 1.4 s. The digital resolution was 3.2 Hz/point in both dimensions with zero-filling in the t_1 dimension. A phase-shifted sine function was applied for both t_1 and t_2 dimensions in the case of DQF-COSY, a Lorentz-Gauss function was applied in all other cases.

Linear baseline correction was applied to all 1D spectra prior to integral calculations. The relative concentrations of the monasaccharides in each fraction were determined by measuring the integral at their characteristic chemical shifts.

Gradient PAGE analysis. Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 32 cm vertical slab gel unit SE620, from Hoefer Scientific Instruments (San Francisco, CA), equipped with Model 1420B power source from Bio-Rad (Richmond, CA). Polyacrylamide linear gradient resolving gels (14 × 28 cm, 12–22% total acrylamide) were prepared and run as previously described [40]. The molecular sizes of the oligosaccharide samples were determined by comparing with a banding ladder of heparin oligosaccharide standards prepared from bovine lung heparin [40]. Oligosaccharides were visualized by Alcian blue staining. [40]

Disaccharide analysis. Each sample (HS-100-1, -2, -3, -4 and -5) was prepared at a concentration of 50 mg/mL with distilled water. One mg (20 μL) of each fraction was then diluted to 100 μL with lyase buffer (50 mM sodium phosphate buffer, pH 7.1 and 100 mM NaCl) resulting in a 10 mg/mL solution. The fractions were treated with heparin lyase I, II and III, heparin lyase III only, heparin lyase I only, and lyase omitted as follows. A small volume addition (1–2 μL) of the appropriate lyase (5.6 mU) was added to each vial, and the initial absorbance was measured at 232 nm. All of the samples were sealed and incubated at 37°C. After 12 h, and additional 2 mU of each appropriate lyase was added, followed by an additional 1 mU each at 24 h and another 2 mU at 36 h for a total of 10.6 mU of each lyase per sample. The digestion was considered to be complete after 72 h, and the vials were frozen at −20°C until analyzed by capillary electrophoresis and gradient polyacrylamide gel electrophoresis.

Capillary electrophoresis. The experiments were performed on a Dionex capillary electrophoresis system (Sunnyvale, CA) equipped with a variable wavelength ultraviolet detector. System operation and data handling were fully controlled using version 3.1 A1-450 chromatography software on a IBM-compatible PC. The CE system was operated in the reverse-polarity mode by applying the sample at the cathode and running using 20 mM phosphoric acid adjusted to pH 3.5 with 1 M dibasic sodium phosphate as previously described [41]. The capillary (75 μm i.d., 375 μm o.d, 77 cm long) was manually washed before use with 0.5 mL of 0.5 M sodium hydroxide, followed by 0.5 mL of distilled water, then 0.5 mL of running buffer. Samples were applied using gravity injection (20 s) by hydrostatic pressure (45 mm), resulting in a sample volume of 9.2 nL. Each experiment was conducted at a constant 15 000 V. Data collection was at 232 nm. Peaks were identified by co-injection with disaccharide standards.

3. Results and Discussion

Preparation of heparan sulfate subfractions.—Crude heparan sulfate (HI-10094) was fractionated by anion-exchange chromatography on a column of Lewatit S-6238-A

Table 1
Characteristics of heparan sulfate (HI-10094) and its subfractions (HS-100-1-5)

	HI-10094	HS-100-1	HS-100-2	HS-100-3	HS-100-4	HS-100-5
Recovery (from 15 g)		2.1 g	1.9 g	2.4 g	5.8 g	2.4 g
Average molecular weight	10000	15500	16500	8500	7000	8000
Optical rotation, °	+66	+75	+69	+56	+55	+53
Nitrogen, %	2.2	2.7	2.4	2.1	2.0	2.0
Total sulfur, %	8.8	4.8	6.2	7.9	9.0	10.3
Anticoagulant activity, U/mg	60	< 4	< 4	10	38	107
Anti-Xa, U/mg	N.D. [a]	< 3	4	13	42	138
Anti-IIa, U/mg	N.D.	< 3	7	15	48	122

[a] N.D. is not determined.

supplied by Bayer (Pittsburgh, PA). Fifteen grams of crude heparan sulfate in 750 mL of 0.5 molal NaCl was applied to a 4.4 × 43 cm column equilibrated with 0.5 molal NaCl at a flow rate of 100 mL/h, and the column was washed with one column volume of 0.5 molal NaCl. Heparan sulfate subfractions were subsequently eluted with two column-volumes of 1.0, 1.2, 1.4, 1.6 and 3.0 molal NaCl to yield fractions HS-100-1 through HS-100-5. Carbohydrate-containing fractions were located by a qualitative Molisch test [42] and combined. GAGs were precipitated with ethanol and dehydrated with acetone. Solvents were removed in vacuo. From 15 g of crude heparan sulfate, 14.6 g of five subfractions were obtained (Table 1).

Characterization of heparan sulfate subfractions.—The physical, chemical and biological characteristics of the crude heparan sulfate and its subfractions are given in Table 1. The average molecular weight of the crude heparan sulfate starting material of 10 000 was reflected in the average molecular weights of the subfractions ranging from 16 500 to 7 000. Fractions HS-100-1 and -2 had substantially higher molecular weights than did HS-100-3, -4 and -5. The percentage of nitrogen decreased slightly through the subfractions, while the percentage of sulfur dramatically increased. Bioassays showed HS-100-1 and -2 to be nearly devoid of anti-coagulant activity, while HS-100-5 was comparable to heparin (~ 150 U/mg). These analyses are consistent with HS-100–1 and -2 being heparan sulfate and HS-100-5 being heparin.

Different GAGs have characteristic migration patterns under electrophoresis. High-purity GAGs usually migrate as single bands. In acidic medium the carboxyl ionization is suppressed, and migration becomes a function of the degree of sulfation. As heparin is the most highly sulfated GAG, it is the fastest moving component. In buffers, such as barium acetate, the electrophoretic mobility is usually an inverse function of shielding of the anionic charges of GAGs by the divalent cation, and GAGs are thus separated into a "slow-moving" component of highly sulfated and higher molecular weight species and "fast-moving" component of less sulfated and low molecular weight species [43]. Cellulose acetate electrophoresis in calcium acetate clearly demonstrates the increased mobility associated with increased charge in the heparan sulfate fractions with an R_H (mobility relative to heparin) of 0.64, 0.69, 0.81, 0.86, 1.0 for HS-100-1 through HS-100-5, respectively. Electrophoresis using barium acetate electrolyte demonstrated the absence of dermatan sulfate in all the samples.

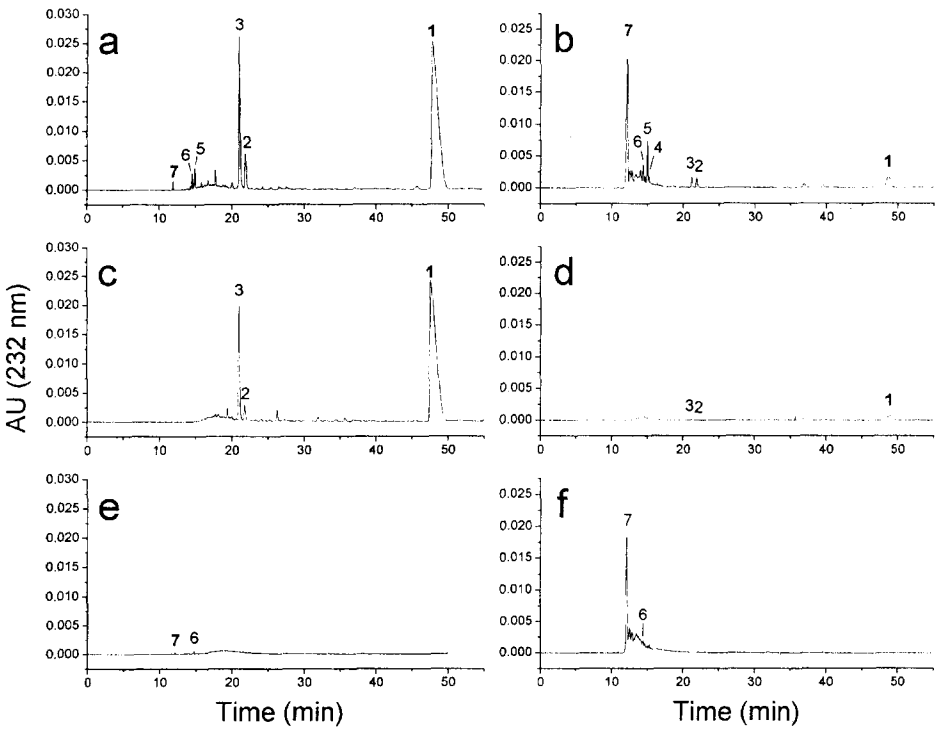

Fig. 1. Selected electropherograms of the disaccharide analysis of the heparan sulfate subfractions. Electropherograms a, c and e correspond to HS-100-1 and b, d and f to HS-100-5. The samples analyzed in a and b were treated with an equi-unit mixture of heparin lyase I, II and III. The samples analyzed in c and d were treated with heparin lyase III. The samples analyzed in e and f were treated with heparin lyase I. The labeled peaks correspond to 1, ΔUA *p*-(1 → 4)-D-GlcN*p*Ac; 2, ΔUA *p*-(1 → 4)-D-GlcN*p*Ac6S; 3, ΔUA *p*-(1 → 4)-D-GlcN*p*S; 4, ΔUA *p*2S-(1 → 4)-D-GlcN*p*Ac6S; 5, ΔUA *p*-(1 → 4)-D-GlcN*p*S6S; 6, ΔUA *p*2S-(1 → 4)-D-GlcN*p*S; 7, ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S. For conditions used in these analyses, see the Experimental section.

Determination of the primary sequence of disaccharides in heparan sulfate, a non-homogeneous structure, is not possible at present. A common approach for structural analysis of heparan sulfate has been to characterize the disaccharide structures following enzymatic degradation [44]. The digestion of heparan sulfate or heparin with an equi-unit mixture of heparin lyase I, II and III leads to nearly complete conversion of these GAGs to disaccharides. Certain sequences can result in the formation of resistant tetrasaccharides. [15,16,45] Heparin lyase III acts only on heparan sulfate with a specificity [15,16] affording the undersulfated disaccharides, ΔUA *p*-(1 → 4)-D-GlcN*p*Ac, ΔUA *p*-(1 → 4)-D-GlcN*p*Ac6S and ΔUA *p*-(1 → 4)-D-GlcN*p*S. In routine analyses, this enzyme is used to confirm the presence of heparan sulfate [44]. Heparin lyase I acts on both heparin and heparan sulfate to produce the more highly sulfated disaccharides, ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S and ΔUA *p*2S-(1 → 4)-D-GlcN*p*S. These disaccharides have a low frequency in heparan sulfate and high frequency in

Table 2
Disaccharide composition (relative percent) [a] subfractions determined by CE analysis

Disaccharide	HS-100-1 [b]			HS-100-2			HS-100-3			HS-100-4			HS-100-5		
	I,II,III	III	I	I,II,III	III	I	I,II,III	III	I	I,II,III	III	I	I,II,III	III	I
ΔUA *p* → GlcN*p*Ac	75.7	84.5	n.d. [c]	58.7	79.3	n.d.	38.2	87.4	n.d.	28.2	74.1	n.d.	11.1	25.3	n.d.
ΔUA *p* → GlcN*p*Ac6S	4.4	2.1	n.d.	10.8	1.2	n.d.	10.7	1.8	n.d.	5.9	7.1	n.d.	4.1	< 0.1	n.d.
ΔUA *p*2S → GlcN*p*Ac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΔUA *p* → GlcN*p*S	14.1	11.1	n.d.	20.9	11.0	n.d.	15.5	9.2	n.d.	5.5	9.1	n.d.	2.9	2.0	n.d.
ΔUA *p*2S → GlcN*p*Ac6S	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	n.d.	2.6	n.d.	n.d.	2.1	n.d.	n.d.
ΔUA *p* → GlcN*p*S6S	1.4	n.d.	n.d.	2.5	n.d.	n.d.	7.2	n.d.	n.d.	11.2	n.d.	n.d.	10.3	n.d.	n.d.
ΔUA *p*2S → GlcN*p*S	1.0	n.d.	65.4	1.4	n.d.	26.6	3.0	n.d.	8.5	4.3	n.d.	4.3	3.9	n.d.	0.7
ΔUA *p*2S → GlcN*p*S6S	0.5	n.d.	34.6	1.6	n.d.	52.6	12.4	n.d.	75.0	29.5	n.d.	74.6	50.3	n.d.	79.2
Total nmoles [d]	880	629	76	778	244	138	498	109	225	495	120	273	436	47	287

[a] Calculated from the relative percent of the total peak area in a given electropherogram.

[b] Each salt subfraction was digested with either a mixture of heparin lyase I, II and III; heparin lyase III; or heparin lyase I (see Experimental section).

[c] Not detected, n.d.

[d] The total moles were calculated using an $\epsilon_{232\ nm}$ of 5500 $M^{-1}\ cm^{-1}$ for the unsaturated residue [49] produced after lyase digestion of 1 mg of each sample.

heparin [41]. Heparin lyase II has a very broad specificity and, when used in combination with heparin lyases I and III can almost completely convert heparin and heparan sulfate to disaccharides [15,16]. Fig. 1 shows the electropherograms of the low-salt (HS-100-1) and high-salt (HS-100-5) subfractions. Each disaccharide peak was identified by co-injection with standards. The retention times were as follows: ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S, 12.0 min; ΔUA *p*2S-(1 → 4)-D-GlcN*p*S, 14.4 min; ΔUA *p*-(1 → 4)-D-GlcN*p*S6S, 14.9 min; ΔUA *p*2S-(1 → 4)-D-GlcN*p*Ac6S, 15.2 min; ΔUA *p*-(1 → 4)-D-GlcN*p*S, 21.0 min; ΔUA *p*-(1 → 4)-D-GlcN*p*Ac6S, 21.8 min; ΔUA *p*-(1 → 4)-D-GlcN*p*Ac, 48.2 min. The disaccharide ΔUA *p*2S-(1 → 4)-D-GlcN*p*Ac, which had a retention time of 21.4 min, was absent in all of the samples. The presence of unidentifiable peaks confirms that there are lyase-resistant oligosaccharides present in the samples. Electropherograms (Fig. 1a, c and e) clearly demonstrate that HS-100-1 affords primarily ΔUA *p*-(1 → 4)-D-GlcN*p*Ac product and is resistant to degradation by heparin lyase I, consistent with its being heparan sulfate. In contrast, electropherograms (Fig. 1b, d and f) clearly show HS-100-5 affords primarily ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S product and is resistant to heparin lyase III, consistent with its being heparin.

The disaccharide analysis (Table 2) showed ΔUA *p*-(1 → 4)-D-GlcN*p*Ac to be the major disaccharide present in the HS-100-1, -2, and -3 subfractions. As expected when using anion-exchange chromatography, as the concentration of the salt eluent increases, the eluted polysaccharide is enriched in trisulfated disaccharide sequences. Similarly, the unsulfated disaccharide content decreases in the increasing salt fractions. Interestingly, the total nmoles of products formed using a mixture of heparin lyases decreased in the increasing salt fractions, suggesting a higher level of lyase-resistant sequences in these fractions. Approximately 4.4 and 12 nmole/mg (0.5 and 1.6%) ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S were observed in the HS-100-1 and -2 salt fractions, respectively. These percentages increased to 62 and 146 nmole/mg (12.4 and 29.5%) in the HS-100-3 and -4 fractions, indicating a significant amount of heparin contamination in these subfractions. The amount of ΔUA *p*2S-(1 → 4)-D-GlcN*p*S6S present in the higher salt fractions

Table 3
Monosaccharide composition of heparan sulfate subfractions by ^{1}H NMR analysis

Subfraction	Hexosamine Residue, mole %		Uronic Acid Residue, mole %		
	D-GlcN*p*Ac	D-GlcN*p*(S or Ac)6S	L-IdoA *p*2S	L-IdoA *p*	D-GlcA *p*
HS-100-1 [a]	71.8	12.4	20.7	6.9	72.4
HS-100-2	64.3	24.4	23.4	9.9	66.7
HS-100-3	38.1	— [b]	36.5	17.9	45.6
HS-100-4	30.1	—	47.2	28.9	23.9
HS-100-5 [c]	18.2	—	57.7	25.8	16.5

[a] Monosaccharide analysis (mole %) of HS-100-1 calculated from CE disaccharide analysis (Table 2) was determined as follows: D-GlcN*p*Ac (6S or 6OH), 80.1; D-GlcN*p*S(6S or 6OH), 17; D-GlcN*p*S6S and D-GlcN*p*Ac6S, 6.3; UA *p*2S, 1.5; and UA *p*, 95.6.

[b] Not determined.

[c] Monosaccharide analysis (mole %) of HS-100-5 calculated from CE disaccharide analysis (Table 2) was determined as follows: D-GlcN*p*Ac(6S or 6OH), 17.3; D-GlcN*p*S(6S or 6OH), 67.4; D-GlcN*p*S6S and D-GlcN*p*Ac6S, 66.8; UA *p*2S, 56.3; and UAp, 28.4.

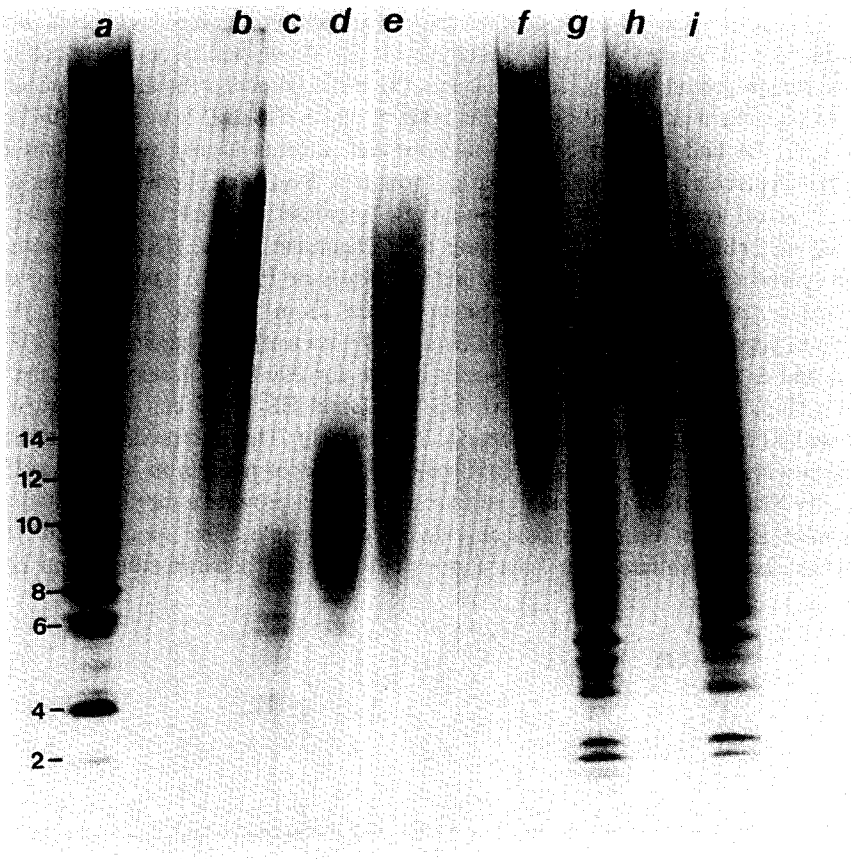

Fig. 2. Analysis of heparan sulfate fractions before and after heparin lyase treatment by gradient polyacrylamide gel electrophoresis. Lane a contains a mixture of heparin oligosaccharides prepared from bovine lung heparin [40]. The degree of polymerization of selected bands are shown to the left of this lane. Lanes b and f correspond to HS-100-1 and -5, respectively. Lanes c and g correspond to heparin lyase I, II, III treated HS-100-1 and -5, respectively. Lanes d and h are heparin lyase III treated HS-100-1 and -5, respectively. Lanes e and i are heparin lyase I treated HS-100-1 and -5, respectively. For conditions used in these analyses, see the Experimental section.

HS-100-4 and -5 suggests that these subfractions are primarily heparin. This disaccharide analysis can be used to calculate the monosaccharide composition (see Table 3, footnotes). Since heparin lyases result in loss of chirality at C-5 of the uronic acid residue, IdoA *p* and GlcA *p* cannot be distinguished and are described simply as UA *p*.

Polyacrylamide gel electrophoresis (PAGE) was also used to analyze the heparan sulfate fractions (Fig. 2). The untreated heparan sulfate fractions stained with increasing intensity as expected with increased sulfation [40]. The level of lyase-catalyzed depolymerization is consistent with the nmoles of product reported in Table 2 [46]. Disaccharides and higher oligosaccharides containing less than two sulfate groups do not stain [46]. This is apparent by viewing lanes c and d. HS-100-1 treated with heparin lyases I,

II, III and heparin lyase III show no disaccharide and little or no tetrasaccharide products in gradient PAGE analysis, while CE analysis clearly shows nearly complete conversion to disaccharide products (Fig. 1a and c and Table 2). Disaccharide and tetrasaccharide products become much more evident in lanes g and i. HS-100-5 treated with heparin lyases I, II, III and heparin lyase I, as expected, affords highly sulfated and easily visualized disaccharide and tetrasaccharide products. Resistant oligosaccharides can be observed in all lyase treated lanes, and these increase with fractions afforded with increased salt elution, as suggested using CE. Treatment of the HS subfractions with heparin lyase III (Fig. 2, lanes d and h) and heparin lyase I (Fig. 2, lanes e and i) can be conveniently used to distinguish heparan sulfate from heparin. HS-100-1 is very sensitive to heparin lyase III and almost completely insensitive to heparin lyase I (Fig. 2, lanes d and e, respectively). The opposite is true for HS-100-5 treated with the same enzymes (Fig. 2, lanes h and i). Thus, HS-100-1 and HS-100-5 can be unequivocally designated as heparan sulfate and heparin, respectively. The intermediate fractions are consistent with increasing levels of cross-contamination of heparin in heparan sulfate.

The ^{1}H NMR profiles of heparan sulfate and heparin provide a rapid, qualitative and quantitative assay of each monosaccharide in the sample. The spectra also yielded information on the sulfate ester substitutions of the intact heparan sulfate and heparin

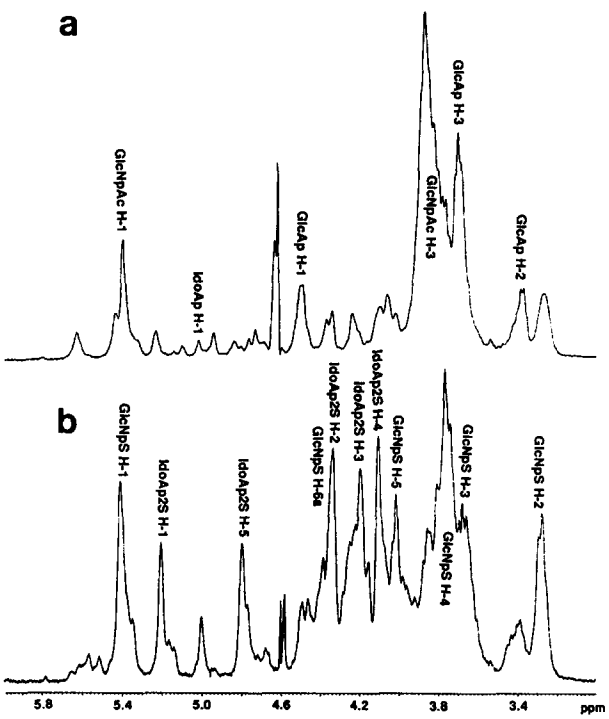

Fig. 3. Analysis of heparan sulfate subfractions by 500 MHz ^{1}H NMR spectroscopy at 313 K. (a) Corresponds to HS-100-1 and (b) to HS 100-5. The peaks that are easily assignable (see Experimental section) are labeled by residue and the position of the resonating proton.

(Fig. 3). 2D ^{1}H NMR spectroscopy has been shown to give a great deal more information about the position and degree of sulfation and about the composition of uronic acids in heparan sulfate and heparin [47,48]. The contents of D-GlcA *p*, L-IdoA *p* and L-IdoA *p*2S residues were calculated from the area of signals at 4.463, 4.997 and 5.203 ppm corresponding to the anomeric protons of each uronic acid. The contents of D-GlcN*p*Ac and D-GlcN*p*S residues in each fraction was obtained from the area of resonance at 5.375 and 5.404 ppm corresponding to the anomeric protons of each glucosamine residue. Additionally, integration of the well-resolved signal area at 2.025 ppm from the *N*-acetyl methyl protons could also reveal the content of *N*-acetylglucosamine residue. The content of D-GlcN*p*(Ac or S)6S residue in each fraction could be revealed from the area of signal at 4.333 ppm. Unfortunately, this signal was frequently overlapped with the signal from H-2 proton of L-IdoA *p*2S.

The monosaccharide compositions determined by CE analysis and ^{1}H NMR analysis were similar (Table 3). The D-GlcN*p*Ac content was nearly identical as measured by both methods. HS-100-1 contained 72–80% of D-GlcN*p*Ac(6S or 6OH), while HS-100-5 contained 17–18% of D-GlcN*p*Ac(6S or 6OH), consistent with their classifications as heparan sulfate and heparin, respectively. The D-GlcN*p*(Ac or S)6S content was easily measured by CE analysis, but extensive signal overlap prevented its accurate measurement by ^{1}H NMR in all but two fractions. The content of D-GlcN*p*(Ac or S)6S went from 12–17% for HS-100-1 to 67% for HS-100-5, again supporting the classification of these fractions as heparan sulfate and heparin, respectively. ^{1}H NMR spectroscopy was most useful in determining the content of D-GlcA *p* and L-IdoA *p* in each fraction, demonstrating that HS-100-1 was heparan sulfate and HS-100-5 was heparin. As discussed earlier, CE analysis relying on heparin lyases could not be used to distinguish L-IdoA *p* from D-GlcA *p* since both were observed as ΔUA *p* in the disaccharide products. It is interesting to note that, while HS 100-5 is classified as heparin by bioassay, electrophoresis and disaccharide analysis, ^{1}H NMR shows twice the level of *N*-acetylation than that typically found in porcine mucosal heparins.

In conclusion, peptidoglycan heparin from porcine intestinal mucosa can be used to prepare both heparin and heparan sulfate on a commercial scale. The heparan sulfate prepared by this method is comparable in all respects to heparan sulfate prepared in very limited quantities by other methods. This new approach should make sufficient quantities of heparan sulfate available for important structural, biological and clinical studies.

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