10 Analysis of the Structure of Heparin and Heparan Sulfate by High-Resolution Separation of Oligosaccharides

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10.1 Introduction

Heparin and heparan sulfate are mixtures of highly sulfated linear polysaccharides having a molecular weight (MW) ranging from 5000 to 40,000 with an MW avg of 10,000 to 25,000 (1). They are members of a family of molecules called glycosaminoglycans (GAGs). Both heparin and heparan sulfate are biosynthesized as a repeating →4)α-D-GlcNpAc(1→4)-β-D-GlcAp(1→ disaccharide sequence that undergoes partial *N*-deacetylation followed by *N*-sulfation of the newly exposed amino groups, partial C-5 epimerization of D-GlcAp to L-IdoAp and *O*-sulfation. A single disaccharide sequence, [→4)α-D-GlcNpS6S-(1→4)-α-L-IdoAp2S (1→], accounts for 80 to 90% of the heparin polymer. Minor sequences contain β-D-GlcAp residues, reduced sulfation as well as *N*-acetylation. Heparan sulfate is structurally similar to heparin but has substantially lower sulfation. Heparan sulfate consists primarily of unsulfated disaccharide [→4)α-D-GlcNpAc(1→4)β-D-GlcAp(1→] and monosulfated disaccharides [→4)α-D-GlcNpS-(1→4)-β-D-GlcAp(1→] and [→4)α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→].

Heparin is stored in mast cells found in tissues such as intestinal mucosa and lung, while heparan sulfate is ubiquitous, occurring in a wide variety of tissues (1). Heparin is commonly used as a clinical anticoagulant with over 33 metric tons manufactured worldwide each year, representing over 500 million doses (2). Heparin has a number of other biological activities (1) that are currently under investigation, including effect on lipoprotein lipase, effect on smooth muscle proliferation, inhibition of complement activation, anti-inflammatory activity, angiogenic and antiangiogenic activities, anticancer activity, antiviral activity, potential use in treating Alzheimer's disease, regulation of nuclear events and an ability to interact with hundreds of different proteins. Heparan sulfate may be the endogenous molecule responsible for many of the biological roles often ascribed to heparin.

Intact heparin and heparan sulfate can be analysed by a variety of spectral, chromatographic and electrophoretic methods. Electrophoresis is useful in determining GAG identity, MW avg and polydispersity (3, 4). The structural information obtained from such analyses is limited primarily to the result of the structural complexity of these polysaccharides. Thus, the major approach to analysing glycosaminoglycans begins by their fragmentation to a mixture of smaller oligosaccharides having simpler structures more amenable to determination.

Heparin lyases eliminatively break the glycosidic linkages between hexosamine and uronic acid in heparin and heparan sulfate, giving a new hexosamine reducing end and an unsaturated uronic acid (ΔUAp) at the nonreducing end (5, 6). There are three commercially available heparin lyases: heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC number) and heparin lyase III (EC 4.2.2.8). The specificity of these enzymes has been extensively studied (7–10). Heparin and heparan sulfate can also be broken down chemically (5).

Heparin and heparan sulfate can be analysed following enzymatic (or chemical) depolymerization. Treatment with a single heparin lyase affords a mixture of oligosaccharides often ranging in size from disaccharides to dodecasaccharides. The enzymatic cleavage of heparin or heparan sulfate using a mixture of heparin lyases I, II and III results primarily in disaccharide products, although some enzyme-resistant tetrasaccharides are also formed (refs 6, 11; also see Chapter 4). Since these enzymes are eliminases, they yield identical nonreducing terminal ΔUAp residues, derived from either D-glucuronic acid (D-GlcAp) or L-iduronic acid (L-IdoAp). Thus, the identity of the uronic acid residue is lost in enzyme-based disaccharide analysis. Lyase specificity, oligosaccharide mapping and nuclear magnetic resonance (NMR) spectroscopy are useful in determining the content of D-GlcAp and L-IdoAp.

A variety of methods can be applied to the analysis of intact GAG chains and enzymatically prepared oligosaccharides and disaccharides. Discontinuous gradient polyacrylamide gel electrophoresis (PAGE) has been used for high-resolution analysis of GAGs and oligosaccharides (12). MW and polydispersity determination require oligosaccharide standards (3, 4). Preparative gradient PAGE is also useful for obtaining small quantities of sulfated oligosaccharides (13). Visualization in PAGE analysis relies on cationic dyes such as Alcian blue, Azure A or toluidine blue to detect microgram quantities of GAG or oligosaccharide. Enhancement of dye-stained gels with silver improves detection sensitivity by 1000-fold and is even useful for the detection of nanogram quantities present in biological fluids (14, 15).

Strong-anion-exchange high pressure liquid chromatography (SAX-HPLC), with salt gradient elution, is valuable in the quantitative analysis of oligosaccharides and disaccharides obtained through enzymatic depolymerization of GAGs. SAX-HPLC is both a robust method of analysis and useful as a preparative method (16).

Capillary electrophoresis (CE) is a technique, using a fused silica capillary, capable of rapid, high-resolution separation, requiring extremely small amounts of sample (17, 18). CE is performed at high voltage in normal polarity (positive mode) or reverse polarity (negative mode) (19). In normal polarity the sample is applied at the anode and detected at the cathode. Negative analytes are prevented from moving under electrophoresis towards the anode by the bulk flow of solvent towards cathode. This flow results from electroosmosis caused by the charged silanol residues of the capillary column surface. In reverse polarity, the silanol residues on the capillary column are uncharged because of the low pH. Since the polarity of the electrode is reversed, the sample is applied at the cathode and migrates under electrophoresis towards the anode. The resolution achieved by CE under a given set of conditions is mainly dependent on the charge, mass and molecular mobility of the analytes present. Detection is typically by ultraviolet absorbance or fluorescence emission.

10.2 Technical Procedures

Materials-Methods-Equipment

- Heparin, sodium salt, from porcine mucosa and bovine lung are available from Sigma Chemical Company, St. Louis, MO, and in larger quantities from Celsus, Cincinnati, OH. Heparan sulfate, sodium salt, from bovine kidney and porcine intestine are available from Seikagaku, Tokyo, and in larger quantities from Celsus. Heparin disaccharide standards were from Seikagaku, Sigma, Dextra Laboratories, Reading, UK, or Grampian Enzymes, Aberdeen, Scotland. Oligosaccharide standards are available from Celsus. Heparin lyase I, heparin lyase II and heparin lyase III from *Flavobacterium heparinum* can be obtained

from Seikagaku, Grampian Enzymes or Sigma. Sulfopropyl Sephadex and Dowex macroporous resin are from Sigma. Spectrapore dialysis membranes of 1000 MWCO were from Spectrum Medical, Los Angeles, CA. SAX-HPLC used a dual-face programmable LC-7A titanium pump from Shimadzu, Kyoto, Japan, and 5 mm Spherisorb columns from Phase Separation, Norwalk, CT, of dimensions 0.46 cm × 25 cm (analytical). Gradient PAGE used a Bio-Rad Protean II vertical slab gel equipped with Model 1420B power source from Bio-Rad, Richmond, CA. Acrylamide (ultrapure), Tris, Alcian blue dye, bromophenol blue dye and ammonium persulfate were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Glycine hydrochloride, disodium EDTA, Azure A dye, boric acid, sucrose, *N,N*-methylene bisacrylamide and *N,N,N',N'*-tetramethylenediamine (TEMED) were from Fisher Chemical Company, Fair Lawn, NJ. CE was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, Model I, equipped with high voltage power supply capable of constant or gradient voltage control using a fused silica capillary, 75 µm i.d. × 375 µm o.d., 70 cm long from Dionex Corporation, Sunnyvale, CA. Freeze drying was performed on a Virtis lyophilizer. Centrifugation used a Beckman (Palo Alto, CA) microfuge. Conductimetric and pH measurements used a 4503A Solution Analyzer from Amber Science, San Diego, CA.

- Buffers used in electrophoresis include: 1, PAGE resolving buffer, 0.1 M boric acid, 0.1 M Tris and 0.01 M disodium EDTA at pH 8.3; 2, PAGE stacking gel buffer, identical to resolving gel buffer except adjusted to pH 6.3 with hydrochloric acid; 3, PAGE upper chamber buffer, 1.24 M glycine and 0.2 M Tris; 4, CE normal polarity buffer, 10 mM sodium borate and 50 mM SDS, pH 8.80; and 5, CE reverse polarity buffer, 20 mM phosphoric acid adjusted to pH 3.48 with 1 M dibasic sodium phosphate.

Protocol 10.1

Preparation of samples

Pure samples of heparin or heparan sulfate that are available in milligram amounts are simply prepared as a 10 mg/ml stock solution in distilled water and used directly for analysis. Samples containing heparin or heparan sulfate at concentrations 1–100 µg/ml, obtained from biological samples (i.e. tissue, cell culture, biological fluids), also contain proteins and buffer salts that must be removed prior to analysis.

1. Dialyse 1 ml sample against distilled water using 1000 MWCO membrane.
2. Adjust sample pH to 3 with 0.1 M hydrochloric acid and load sample onto a 2-ml SP-Sephadex column (equilibrated at pH 3). Wash column with 2 mL of 25 mM hydrochloric acid and collect wash and add 1 mL of 0.1 M sodium phosphate pH 7 buffer.
3. Dialyse against distilled water using 1000 MWCO membrane and freeze-dry. Reconstitute sample in minimum volume of water.

If sample is protein-free, then analyse, but if it still contains residual protein, proceed to step 4.

4. Prepare a 1-ml column of macroporous Dowex resin by washing with 10 column volumes each water, methanol, water, saturated sodium chloride and distilled water.
5. Load sample dissolved in water onto column and wash column with 2 ml each of water, 1% sodium chloride and 16% sodium chloride. Collect the 16% sodium chloride solution containing heparin or heparan sulfate.
6. Recover sample from sodium chloride by (1) dialysis (MWCO 1000 membrane) and freeze drying, or (2) precipitation of heparin or heparan sulfate by adding 4 volumes of methanol to 1 volume of sample in 16% sodium chloride and recovering pellets by centrifugation. Reconstitute sample in minimum volume of water and use for analysis.

Protocol 10.2 **Enzymatic preparation of oligosaccharides**

1. Prepare stock solutions of heparin lyase, I, II and III at an activity of 1 mU/µl in 50 mM sodium phosphate buffer a pH 7.5 (1 U = 1 µmol product formed/min). Freeze 10 µl aliquots of each enzyme in 500 µl polypropylene centrifugation tubes and store at -60°C prior to use.
2. Add 100 µl of sample (≤1 mg heparin or heparan sulfate) to a polypropylene tube containing 10 µl of heparin lyase I, II or III. Add 10 µl of 500 mM sodium phosphate buffer at pH 7.5 and incubate for 8–12 h at 30°C. Boil 5 min to inactive enzyme.
3. Analyse samples immediately or store frozen at -60°C prior to analysis.

Protocol 10.3 **Oligosaccharide and polysaccharide analysis by gradient PAGE**

1. Prepare gradient polyacrylamide resolving gel from two resolving gel buffer solutions (see "Methods" section), one containing 11.52% (w/v) acrylamide with 0.48% (w/v) *N,N*-bisacrylaminide and 1% sucrose and the second containing 20.02% (w/v) acrylamide with 2% (w/v) *N,N*-bisacrylamide and 15% (w/v) sucrose.
2. Pour gel vertically between two glass plates (16 cm × 20 cm gel size) separated by 1.0-mm spacers. Pour a gradient gel by adding 17.5 ml of 12% solution to the reservoir (back) and 17.5 ml of 22% solution to the mixing chamber (front) of the linear gradient maker.
3. Add ammonium persulfate [75 µl of 10% (w/v) in water], followed by 17.5 µl of TEMED to both the reservoir and mixing chambers.
4. Stir the solution in the mixing chamber continuously using a magnetic stirrer.
5. Open the valve between the reservoir and the mixing chamber and pass the polyacrylamide solution from the mixing chamber by gravity to the top of the glass plates to form a linear gradient from the bottom up to the top.
6. Overlay the unpolymerized gel with water.

7. After polymerization is complete, remove the water layer and add 5 ml of solution of stacking gel made of 4.75% (w/v) acrylamide and 0.25% (w/v) *N,N*-bisacrylamide in stacking gel buffer (see "Methods") containing 5 µl of TEMED and 75 µl 10% (w/v) of ammonium persulfate to the top of the resolving gel.
8. Insert a comb (well formers). After polymerization, remove the comb and rinse each well with upper chamber buffer (see"Methods").
9. Combine samples in distilled water (1–10 µg/25 µl and 1–10 ng/25 µl of intact polysaccharides or heparin lyase depolymerized oligosaccharide sample for detection with Alcian blue and silver staining, respectively) with an equal volume of 50% (w/v) sucrose containing trace quantities of phenol red and bromophenol blue and load into the bottom of each well. Standards, analysed on the same gel, should include: disaccharide (Structure 8 in Fig. 10.3) (MW 665), tetrasaccharide (MW 1330), hexasaccharide (MW 1995), octasaccharide (MW 2660), decasaccharide (MW 3325) and dodecasaccharide (MW 3990) mixtures [pure oligosaccharides can also be prepared for use as standards (12)].
10. Perform electrophoresis at 400 V with circulating cooling water (between 0 and 5°C) for 5 h.
11. Remove the gel from the glass plates and stain with Alcian blue 0.5% (w/v) and 2% (v/v) acetic acid solution in water for 30 min.
12. Destain with several 200-ml volumes of 5% (v/v) acetic acid solution.

If sample banding can be detected, go to step 18; if not, proceed to step 13.

13. After Alcian blue staining, soak the gel in 50% (v/v) aqueous methanol for 4 h, followed with distilled water for 2 h.
14. Prepare silver staining solution (200 mL), immediately prior to use, by slowly adding 2 ml of 4.7 M silver nitrate with mixing to 2 ml of 7.5% (w/v) sodium hydroxide and 3.3 ml of concentrated ammonium hydroxide in 192.7 ml of distilled water.

15. Stain the gel with gentle shaking for 1 h, followed by three washes (200 ml each) with distilled water over 30 min.
16. Develop the gel in freshly prepared developing buffer made by adding 25.2 mg of citric acid and 250 µl of 37% (w/v) formaldehyde to 500 ml of distilled water. Bands start to develop within 1 to 15 min.
17. Stop the reaction by placing the gel in 10% (v/v) aqueous acetic acid solution containing 30% (v/v) methanol.
18. Determine molecular weight using a standard curve prepared by plotting the migration distance of the major band in each oligosaccharide mixture as a function of log molecular weight.
19. Make a photographic record of the gel (Fig. 10.1).

While gradient PAGE analysis affords sharp bands for oligosaccharides, intact heparin and heparan sulfates tend to run as broad smears because of their high inherent polydispersity and structural heterogeneity.

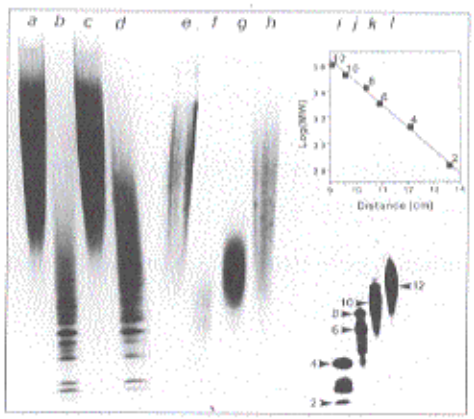
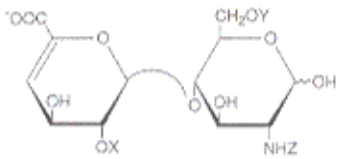


Figure 10.1 Gradient PAGE analysis of heparin, heparan sulfate and oligosaccharide standards *Lanes a and e correspond to intact heparin and heparan sulfate, respectively. Lanes b and f correspond to heparin and heparan sulfate, respectively, treated with an equi-unit mixture of heparin lyase I, II and III. Lanes c and g correspond to heparin and heparan sulfate, respectively, digested to completion with heparin lyase III only. Lanes d and h correspond to heparin and heparan sulfate, respectively, digested to completion with heparin lyase I only. Lanes i, j, k and l correspond to commercially available tetra-, hexa-, octa- and decasaccharide standards, respectively, with the degree of polymerization shown. Inset: Plot of log MW versus migration distance of the standard oligosaccharides. The best-fit line was generated using linear regression analysis.*

Protocol 10.4

Oligosaccharide analysis by SAX-HPLC

1. Equilibrate the analytical SAX-HPLC column with water adjusted to pH 3.5 with dilute hydrochloric acid.
   Each oligosaccharide sample (50 µg in 5 µl) is analysed using a 120-min linear gradient from 0.2–2.0 M (for depolymerized heparin) and 0–1.0 M (for depolymerized heparan sulfate) sodium chloride at pH 3.5 at a flow rate of 1 ml/min. The elution profile is monitored at 232 nm at 0.02 absorbance units full scale (AUFS) (Fig. 10.2).
2. Major components in the oligosaccharide maps are disaccharides corresponding to *8* (heparin) and *1*, *2* and *3* (heparan sulfate) as described in Figure 10.3. These can be identified by separately adding 25 µg of each disaccharide standard to 50 µg of each oligosaccharide map and observing the peak having an enhanced area.



1. X=Y=H, Z=CH_3CO
2. X=H, Y=SO_3^-, Z=CH_3CO
3. X=SO_3^-, Y=H, Z=CH_3CO
4. X=Y=H, Z=SO_3^-
5. X=SO_3^-, Y=SO_3^-, Z=CH_3CO
6. X=H, Y=SO_3^-, Z=SO_3^-
7. X=SO_3^-, Y=H, Z=SO_3^-
8. X=Y=Z=SO_3^-

Figure 10.3 Heparin and heparan sulfate disaccharides

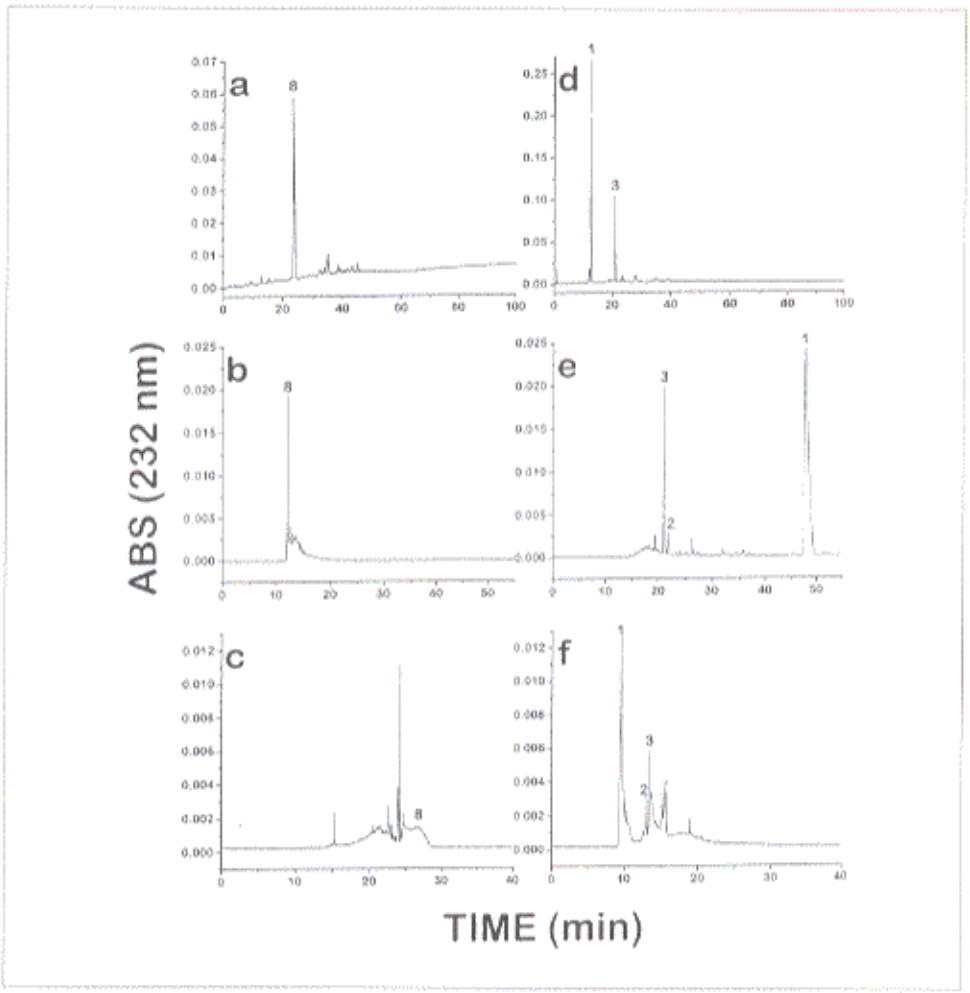


Figure 10.2 Oligosaccharide mapping of heparin and heparan sulfate

Panels a, b and c correspond to heparin treated with heparin lyase I analysed by SAX-HPLC, reverse polarity CE and normal polarity CE, respectively Panels d, e and f correspond to heparan sulfate treated with heparin lyase III analysed by SAX-HPLC, reverse polarity CE and normal polarity CE, respectively. The numbering corresponds to the disaccharides shown in Figure 10.3. In panel a, peak 8 eluted at 0.43 M NaCl. In panel d, peaks 1 and 3 correspond to 0.13 and 0.20 M NaCl, respectively.

Protocol 10.5

Oligosaccharide analysis by CE

1. Separate and analyse the sample using a fused silica (externally coated except where the tube passes through the detector) capillary tube (75 µm i.d., 375 µm o.d., 70 cm long).
2. Insert the capillary into the machine and rinse, using 400 sec pressure injections, of 0.1 M phosphoric acid, 0.5 M sodium hydroxide, distilled water and running buffer (see "Methods").
3. Load oligosaccharide samples (10 mg/ml) with gravity injection, by hydrostatic pressure (45 mm head height); a 15 sec injection period results in an injection of 8 nl.
4. For oligosaccharide mapping of oligosaccharides prepared from pure heparin or heparan sulfate, perform CE using normal polarity (inject analyte into the capillary at the positive electrode and detect in front of the negative electrode) in buffer at 15 kV. Detect at 232 nm at 0.01 AUFS (Fig. 10.2).

Reverse polarity analysis is preferred for oligosaccharides prepared from heparin or heparan sulfate obtained from biological samples, which may be contaminated with protein. Perform CE using reverse polarity (inject analyte at the negative electrode and detect in front of the positive electrode) in buffer at 15 kV. Detect at 232 nm at 0.02 AUFS (Fig. 10.2).

Protocol 10.6

Disaccharide analysis of heparin and heparan sulfate

Use a mixture of heparin lyases I, II and III to completely depolymerize heparin and heparan sulfate.

1. Place the sample (≤1 mg in 100 µl of water) in a 500 µl polypropylene centrifuge tube. Add sodium phosphate buffer (10 µl, 500 mM, pH 7.5) to the sample, then add 5–10 mU each of heparin lyase I, II and III.
2. Digest the sample for 8–12 h at 35°C and analyse immediately or boil 5 min and store frozen at -60°C until analysis.

Protocol 10.7 **Disaccharide analysis by CE**

1. Analyse a completely depolymerized sample (100 μg/10 μl) by CE using reverse polarity as previously described (see "Oligosaccharide analysis by CE").
   Normal polarity can also be used but results in loss of resolution. The disaccharide (Fig. 10.3) composition and migration times are given for both reverse and normal polarity CE analysis in Table 10.1.

Table 10.1 Disaccharide analysis of heparin and heparan sulfate by CE

	Migration Time[a]		Heparin[b]		Heparan Sulfate[b]	
Disaccharide	RP	NP	typical value[c]	range	typical value[c]	range
1	48.2	9.3	<1	0–1	66	45–73
2	21.8	13.9	1	1–3	11	2–12
3	21.4	14.4	<1	0–1	<1	0–1
4	21.0	15.1	1	0–2	10	7–12
5	15.2	20.2	2	1–4	<1	0–3
6	14.9	21.4	7	4–12	4	2–8
7	14.4	22.0	4	2–9	5	4–14
8	12.0	33.5	82	80–90	3	2–21

[a] *Approximate migration time in a 70-cm capillary at 15 kV using the buffers described in the text for reverse polarity (RP) and normal polarity (NP). Migration times, determined from a disaccharide standard mixture, may vary.*

[b] *Typical percentages of L-IdoAp and D-GlcAp in heparin from bovine lung and porcine intestinal mucosa are 75–90% and 10–25%, respectively; in heparan sulfate from bovine and porcine organs, these ranges are 11–45% and 55–89%, respectively.*

[c] *Relative percentages (peak areas) of disaccharides obtained from porcine intestinal heparin and bovine kidney heparan sulfate, respectively. The peak area of resistant oligosaccharides contained in the enzyme treated sample are not taken into account.*

10.3 Method Selection, Critical Parameters and Troubleshooting

The enzymes used to depolymerize heparin and heparan sulfate are often supplied in units that differ from the international unit. These enzymes should be dissolved in water or buffer (according to manufacturers specifications), divided into convenient aliquots and stored frozen. An aliquot should then be assayed before use (6). A simplified assay involves removing an aliquot of enzyme and adding 1 mg of heparin or heparan sulfate in 100 µl of 50 mM sodium phosphate buffer, pH 7.5, and incubating at 35°C. Samples (10 µl) are removed at various time points and added to 1 mL of 30 mM hydrochloric acid, and absorbance at 232 nm is determined. A plot of A_{232} nm versus time should afford a curve that indicates the length of incubation required for complete reaction. If the enzyme shows no activity: (1) obtain the same enzyme from a second supplier; (2) test the enzyme on a different sample of substrate; or (3) purify the heparin or heparan sulfate substrate (see "Preparation of Samples", 10.1, 1–6). Additional details on the use of these enzymes are given in reference (6).

Discontinuous gradient PAGE analysis is not routinely performed in many laboratories. Discontinuous PAGE at constant acrylamide concentration is effective for separating oligosaccharides with a limited range of sizes; 14, 16 and 18% acrylamide gels separate oligosaccharide mixtures of MW 5000 to 25,000, 2000 to 5000 and 600 to 2000, respectively. Minigels are often satisfactory for such separations.

SAX-HPLC, while best performed using packing material of 5 µm particle size, can also use 10 µm packing with a significant loss of resolution. While any HPLC system can be used, a titanium-based system is preferred, as sodium chloride will corrode stainless steel. When performing SAX-HPLC mapping, if no peaks are observed the oligosaccharides afforded on enzyme treatment might be too large and too highly charged to elute from the column. In this case it is best to use gradient PAGE for mapping. Shorter retention times and peak broadening are common for older SAX-HPLC columns.

Capillary electrophoresis can be performed satisfactorily on virtually any instrument. In analysis, peaks migrating rapidly under normal polarity migrate slowly late under reverse polarity and vice versa. This provides a convenient approach to separations where a contaminant happens to elute very close to a peak corresponding to a component of interest. The observation of broad peaks with short re-

tention times under normal polarity suggest that the GAG sample being analysed is contaminated with protein. Reverse polarity often improves the resolution of such samples, since most protein does not elute from the capillary at low pH. Repetitive analysis using reverse polarity often leads to decreased resolution because of protein adsorption to the capillary walls, requiring the periodic removal and cleaning of the capillary.

Anticipated results

Gradient PAGE should afford the approximate MW and polydispersity of a heparin or heparan sulfate sample (3), the molecular weight of an oligosaccharide and a qualitative oligosaccharide map (7). These oligosaccharide maps easily distinguish heparin from heparan sulfate (Fig. 10.1) as well as other GAGs (7–9). SAX-HPLC affords a quantitative oligosaccharide map (7), and CE using either reverse or normal polarity gives an accurate and reproducible disaccharide analysis (11). As little as 100 μg to 1 mg of heparin or heparan sulfate is sufficient to perform all of these analyses.

Time considerations

The assay of heparin lyases and the depolymerization of multiple samples takes 1–2 days. PAGE analysis can be performed on as many as 30 samples in 1 day. SAX-HPLC requires one-half day to set up and 3 h/analysis. Capillary electrophoresis requires one-half day to set up and 50 min/analysis.

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