Chromatographic methods for product-profile analysis and isolation of oligosaccharides produced by heparinase-catalyzed depolymerization of heparin

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Abstract

A two-dimensional chromatography method for monitoring the formation of oligosaccharides produced by heparinase-catalyzed depolymerization of heparin is reported. In the first step of the two-dimensional method, the depolymerized heparin is size-fractionated by high-performance gel permeation chromatography (HPGPC). The size-uniform fractions are then separated on the basis of charge by strong anion-exchange (SAX) HPLC on a high resolution CarboPac PA1 column. To demonstrate application of the two-dimensional product-profile analysis method, data are presented for the heparinase I-catalyzed depolymerization of heparin in the absence and presence of histamine, a ligand that binds site-specifically to heparin. Results from the two-dimensional analysis indicate that histamine alters the extent of depolymerization and the product-profiles for the tetrasaccharide and hexasaccharide fractions. The use of CarboPac PA1 columns for the semi-preparative scale separation of oligosaccharides in size-uniform fractions isolated from depolymerized heparin by low-pressure (gravity flow) GPC is also reported. The semi-preparative scale CarboPac PA1 column gives high resolution and excellent reproducibility after repeated use over an extended period of time, making it possible to reliably combine fractions from multiple separations. The oligosaccharides are eluted from the CarboPac PA1 column with a NaCl gradient at relatively low pH (3 or 7) where they are stable. An efficient two-step procedure is described for desalting oligosaccharides separated by SAX-HPLC.   © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Heparin is a highly sulfated, linear polysaccharide largely made up of $1\rightarrow4$ linked repeating sequences of the trisulfated disaccharide [(α-L-IdoA(2S))-$(1\rightarrow4)$-(α-D-GlcNS(6S))], where α-L-IdoA(2S) represents 2-O-sulfated iduronic acid and α-D-GlcNS(6S) represents N- and 6-O-sulfated glucosamine (Fig. 1A). Minor constituents, including β-D-glucuronic acid, N-acetyl-glucosamine and disaccharide repeat units with different degrees of O-sulfation (Fig. 1B), are present in varying amounts, depending on the origin of the heparin [1–4].

Heparin is widely used as a blood anticoagulant. It also has antilipaemic, antihaemostatic and antithrombotic activities. The anticoagulant activity of heparin is a result of its binding to antithrombin III (AT-III), which accelerates the rate at which AT-III inactivates

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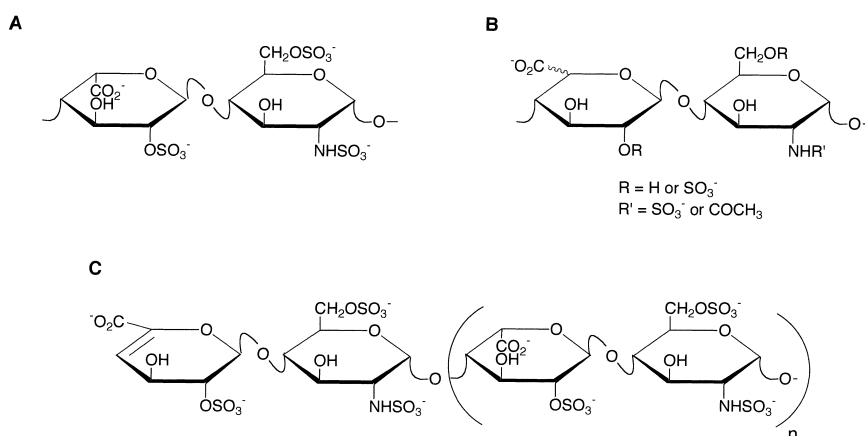*E-mail address:* dlrab@ucrac1.ucr.edu (D.L. Rabenstein).

Fig. 1. Structures of (A) the major heparin disaccharide repeating unit, (B) the minor heparin disaccharide repeating unit and (C) a representative heparin-derived oligosaccharide produced by heparinase I-catalyzed depolymerization of heparin. The heparin-derived oligosaccharide in (C) is comprised of the major repeating disaccharide (A) and contains the $\Delta^{4,5}$ double bond introduced into the uronic acid at the nonreducing terminus by the depolymerization reaction.

proteolytic enzymes of the coagulation cascade [5–7]. With the discovery that the anticoagulant activity resides in a unique pentasaccharide sequence with AT-III binding properties, and that this sequence is present in only about one-third of heparin polymer chains [7–12], there has been increased interest in the primary structure of heparin and the contribution of unique oligosaccharide sequences to its biological activity [13–15].

Identification of oligosaccharide sequences in heparin with specific biological activities is difficult due to the polydispersity and micro-heterogeneity of heparin. One approach to this problem is to enzymatically or chemically depolymerize heparin and then isolate and characterize pure oligosaccharide fragments. These well-defined oligosaccharides can then be used to characterize features of the primary structure of heparin and to study structure–activity relationships with other biomolecules. An extension of this approach is to carry out the depolymerization in the presence of heparin-binding biological molecules. The expectation is that, if the biological molecule binds to a unique oligosaccharide sequence, binding will alter the depolymerization reaction so that oligosaccharides containing this sequence will be enriched in the product mixture. For example, basic fibroblast growth factor (bFGF) prevents heparinase I (EC 4.2.2.7) from degrading the related glycosaminoglycan heparan sulfate from

Chinese hamster ovary cells, presumably by binding at the same sites as cleaved by the enzyme [16].

The progress of heparinase-catalyzed depolymerization of heparin can be monitored by measuring the absorbance of the $\Delta^{4,5}$ double bond formed by the depolymerization reaction (Fig. 1C). However, because heparin–biomolecule complexes are likely to dissociate rapidly [17], it is possible that heparin sequences that bind biomolecules will also be degraded at longer depolymerization times. Thus, it is desirable to monitor the progress of the depolymerization at the product oligosaccharide level to optimize conditions for formation of biomolecule-binding oligosaccharide sequences.

In this paper, we report a two-dimensional chromatography method for monitoring the product-profile distribution during heparinase I-catalyzed depolymerization of heparin. With this method, the formation of individual oligosaccharides can be monitored as the depolymerization reaction is carried out in the absence or presence of heparin-binding ligands to determine if the product-profile distribution is altered by the presence of the heparin-binding ligand, and if so, the method can be used to optimize conditions for the formation of specific oligosaccharides. Application of the method is demonstrated by results presented for depolymerization of heparin in the presence of histamine. The diprotonated form of histamine binds site-specifically to heparin, with

the imidazolium ring located in a cleft formed by the [(α-L-IdoA(2S))-(1→4)-(α-D-GlcNS(6S))-(1→4)-(α-L-IdoA(2S))] trisaccharide sequence. The carboxylate group of the IdoA(2S) at the reducing end of the trisaccharide sequence and the sulfamide group of the GlcNS(6S) residue are essential for site-specific binding [18,19].

We also report improvements in the semi-preparative scale procedures used for isolating sufficient quantities of heparin-derived oligosaccharides for characterization and further study.

2. Material and methods

2.1. Materials

Flavobacterium heparinase I (EC 4.2.2.7), bovine lung heparin and porcine intestinal mucosal heparin were obtained from Sigma (St Louis, MO, USA). Low-pressure (gravity flow) gel permeation chromatography (GPC) was done on a 200×3 cm column packed with 45-90 μm Bio-Gel P6 (Bio-Rad Labs., Richmond, CA, USA). Strong anion-exchange (SAX) separations were performed on CarboPac PA1 columns (250×9 mm and 250×4 mm) obtained from Dionex (Sunnyvale, CA, USA). The Sephadex G10 55–166 μm resin used in the desalting column (500×10 mm) and the Superdex Peptide HR GPC column (30 cm ×10 mm) were obtained from Pharmacia Biotech (Piscataway, NJ, USA). SAX-HPLC with the CarboPac columns and gel permeation chromatography with the Superdex Peptide column were performed on a Dionex 500 ion chromatography system, equipped with a GP40 gradient pump and an AD20 UV–Vis detector. A column pressure of less than 200 p.s.i. was used with the Superdex Peptide column to avoid damage to the column (1 p.s.i.=6894.76 Pa). Water was purified with a Millipore (Bedford, MA, USA) water purification system. All other reagents were of the highest quality available.

2.2. Enzymatic depolymerization of heparin

Bovine lung and porcine intestinal mucosal heparin were depolymerized using the enzyme heparinase I. Both small and large-scale depolymeri-

zations were carried out, the former for detailed depolymerization product-profile analysis studies and the latter for preparative purposes. In the small-scale depolymerizations, 100 or 200 mg of heparin were dissolved together with 50 U of heparinase I (Sigma units; 1 U is defined as the quantity of enzyme that will form 0.1 μmol of unsaturated uronic acid product per h at pH 7.5 and 25°C) in 2 or 4 ml of pH 7.5 solution that contained 25 mM sodium acetate and 10 mM calcium acetate [20]. Depolymerization reactions were carried out at 25 or 37°C in a water bath. The progress of the reaction was monitored by product-profile analysis as described below. Similar reaction conditions were used in the large-scale depolymerizations, except that 1 g of heparin was dissolved together with 250 U of heparinase I in 50 ml of depolymerization solution. The progress of the large-scale depolymerization was monitored by diluting 20-μl aliquots of the reaction solution with 1.0 ml of 0.03 M HCl and measuring the absorbance at 232 nm. When the absorbance reached a constant value, the reaction mixture was lyophilized.

2.3. Low-pressure GPC

Portions (0.2 g) of the lyophilized product from large-scale depolymerizations were size fractionated by low-pressure GPC on the Bio-Gel P6 column using a 0.5 M NH$_4$HCO$_3$ eluent at a flow-rate of 8–12 ml/h. Fractions (2.0 ml) were collected and the absorbance of each fraction was measured at 232 nm. Fractions containing oligosaccharides of the same size were pooled and desalted by lyophilization.

2.4. High-performance GPC

Aliquots (100 μl) of small-scale reaction mixtures were size fractionated by high-performance GPC with a Superdex Peptide HR column (one column or two columns in series) with a 0.2 M NaCl eluent at a flow-rate of 0.4–0.8 ml/min. The separation was monitored by measuring the absorbance at 232 nm. Peaks corresponding to oligosaccharides of different sizes were collected for further product analysis by SAX-HPLC. Although the Superdex column was designed for GPC separations of peptides, it gave
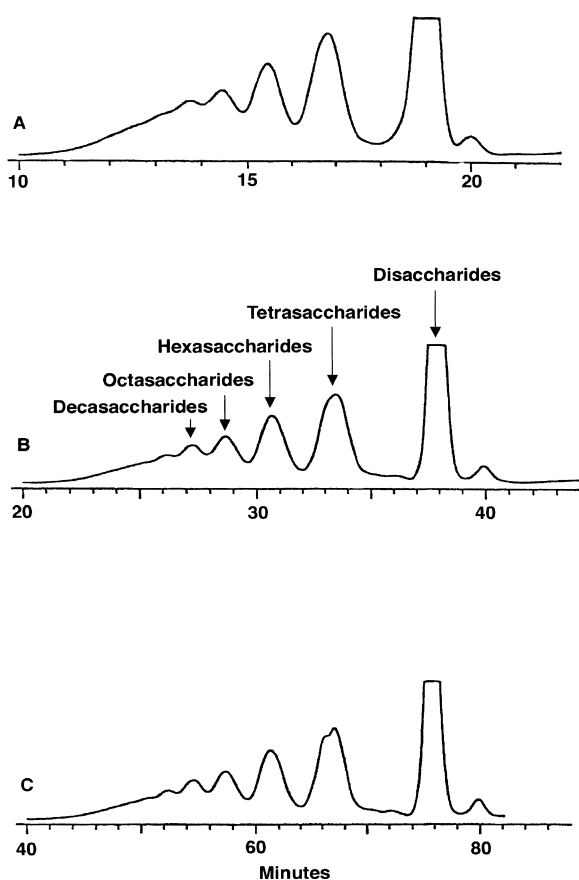
Fig. 2. Size fractionation of heparinase I-depolymerized heparin with the high-performance Superdex GPC column using 0.2 *M* NaCl as the mobile phase. The eluent was monitored by UV absorbance at 232 nm; (A) one column, 0.8 ml/min; (B) two columns in series, 0.8 ml/min, and (C) two columns in series, 0.4 ml/min.

excellent size fractionation of heparin-derived oligosaccharides.

2.5. Strong anion-exchange chromatography

In the procedure used for product-profile analysis, 100-µl samples from the size-uniform fractions collected from the Superdex Peptide column were injected directly on to an analytical-scale CarboPac PA1 column without desalting. The oligosaccharides were eluted with a linear gradient of 70 m*M* pH 3 (or 7) phosphate buffer (solvent A) and 70 m*M* pH 3 (or 7) phosphate buffer plus 2 *M* NaCl (solvent B) at a

flow-rate of 1.2 ml/min. After each run, the column was washed with solvent B for 5 min to elute any oligosaccharides still on the column, followed by solvent A for 5 min to recondition the column. The gradient was optimized for each size-uniform oligosaccharide fraction.

In the procedure used for isolating pure oligosaccharides, the lyophilized size-uniform oligosaccharide fractions obtained by low-pressure GPC were separated according to charge with a semi-preparative scale CarboPac PA1 column. A sample size of 6–8 mg of oligosaccharide was generally used. The chromatographic conditions were similar to those described above, except that a flow-rate of 3.3 ml/min was used.

The capacity of the CarboPac PA1 columns was not determined, however excellent separations were achieved for hexasaccharide-fraction sample sizes of 0.5 and 6–8 mg with the analytical and semipreparative-scale columns, respectively.

2.6. Desalting procedure

The oligosaccharides obtained by collection of fractions from the semi-preparative CarboPac PA1 column were desalted on a 500×10 mm Sephadex G-10 (superfine) column using Millipore water as eluent and a flow-rate of 9 ml/h or by a two-step procedure described below. Fractions (1.5 ml) were collected from the Sephadex G10 column and monitored both by conductivity and UV absorbance at 232 nm. The desalted oligosaccharides were lyophilized and their purity checked by ^{1}H NMR.

3. Results

Bovine lung and porcine intestinal mucosal heparin were depolymerized with heparinase I, which catalyzes cleavage of glucosamine-(1→4)-uronic acid glycosidic bonds by an eliminative mechanism that results in a $\Delta^{4,5}$ unsaturated uronic acid (ΔUA) at the non-reducing end (Fig. 1C) [21]. Thus, the oligosaccharides formed by the heparinase I-catalyzed depolymerization of heparin differ in size by disaccharide units [22].

3.1. Monitoring the progress of heparinase-catalyzed depolymerization of heparin

The method reported here for product-profile analysis at the oligosaccharide level is a two-step chromatographic procedure. In the first step, the formation of size-uniform oligosaccharide fractions is monitored by GPC with a high resolution/high loading capacity Superdex Peptide column. To illustrate the performance of this column, chromatograms obtained for heparinase I-depolymerized porcine intestinal mucosal heparin are presented in Fig. 2. Chromatogram A was obtained using one column and a flow-rate of 0.8 ml/min, chromatogram B using two columns in series and a flow-rate of 0.8 ml/min, and chromatogram C two columns in series

and a flow-rate of 0.4 ml/min. The chromatograms show that, with two columns in series and a flow-rate of either 0.4 or 0.8 ml/min, di- to decasaccharides can be resolved. The maximum loading capacity was ~10 mg of oligosaccharide. The column was found to be stable and to give excellent reproducibility after more than 150 injections. In the second step of the product-profile analysis, size-homogenous fractions collected from the separation on the Superdex GPC column are separated by SAX-HPLC on an analytical-scale CarboPac PA1 column.

Application of the product-profile analysis method is illustrated by the results in Fig. 3 for the heparinase I-catalyzed depolymerization of bovine lung heparin. Superdex GPC chromatograms for aliquots taken from a depolymerization reaction solution at 3,
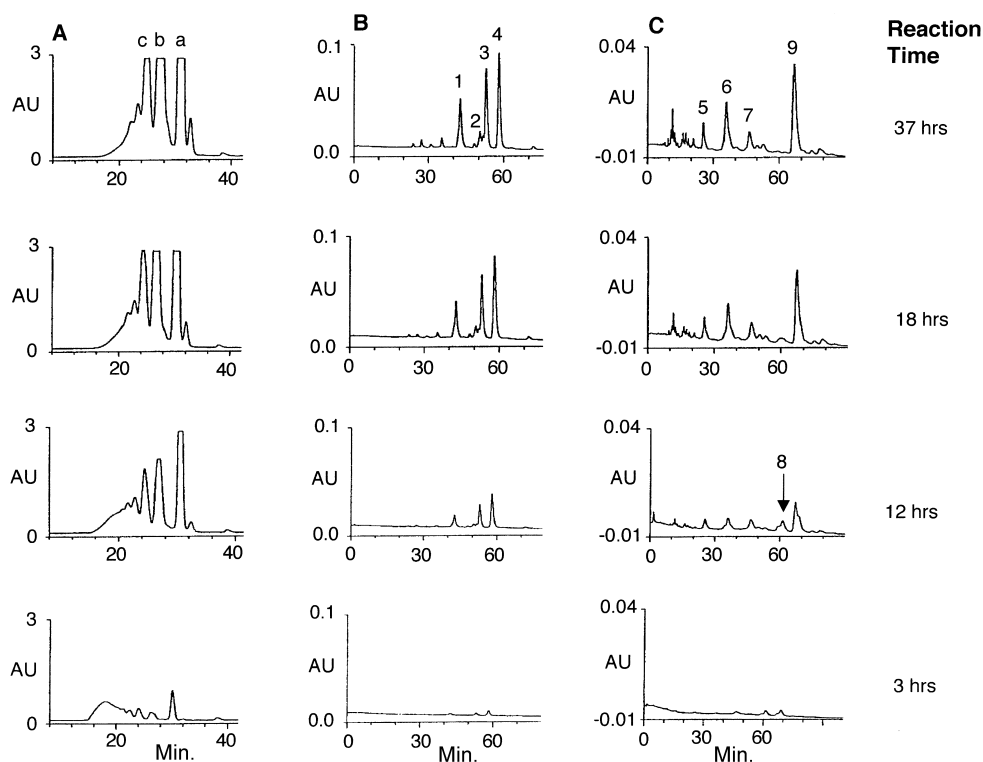

Fig. 3. Product profile analysis as a function of time for the heparinase I-catalyzed depolymerization of 100 mg of bovine lung heparin in 2 ml of depolymerization buffer. (A) Chromatograms for the size fractionation of depolymerized heparin on the Superdex GPC column; flow-rate 0.6 ml/min. Peaks labelled a, b and c are for the di-, tetra- and hexasaccharide fractions. (B) and (C) Chromatograms for the SAX-HPLC separation of the tetrasaccharide and hexasaccharide fractions, respectively, on an analytical scale CarboPac PA1 column. For B and C, the oligosaccharides were eluted with a linear gradient of 70 mM pH 7 phosphate buffer (Solvent A) and 70 mM pH 7 phosphate buffer plus 2.0 M NaCl (Solvent B). For the tetrasaccharide separations: 2%/min gradient for 10 min and then 0.5%/min for the remainder of the separation; for the hexasaccharide separations: 3%/min gradient for 10 min and then 0.5%/min for the remainder of the separation.

12, 18 and 37 h are presented in Fig. 3A. Fractions corresponding to tetra- and hexasaccharides were collected and further separated by SAX-HPLC using an analytical scale CarboPac PA1 column (Fig. 3B and C, respectively). The time courses for formation of the various oligosaccharides obtained from SAX chromatograms are plotted in Fig. 4 for tetrasaccharides giving peaks labeled 1–4 and hexasaccharides giving peaks labeled 5–9 in Fig. 3B and C. The results indicate conditions for optimizing the formation of certain oligosaccharides. For example, the hexasaccharide giving peak 8 in the hexasac-

charide chromatograms is produced early in the depolymerization reaction but then is consumed later in the time course, while the hexasaccharides giving peaks 6 and 9 reach a maximum relative concentration after ~20 h. The tetrasaccharides giving peaks 3 and 4 in Fig. 3B and the hexasaccharide giving peak 9 in Fig. 3C were isolated from large-scale depolymerizations and their structures were determined by ^{1}H NMR spectroscopy methods [23] to be:

ΔUA(2S)–GlcNS(6S)–GlcA–GlcNS(6S),

ΔUA(2S)–GlcNS(6S)–IdoA(2S)–GlcNS(6S), and

ΔUA(2S)–GlcNS(6S)–IdoA(2S)–GlcNS(6S)–

IdoA(2S)–GlcNS(6S), respectively, where GlcA represents glucuronic acid.

Depolymerization of porcine intestinal mucosal heparin in the presence of histamine was also monitored to demonstrate application of the product-profile analysis method when the depolymerization solution contains a heparin-binding biomolecule. The Superdex GPC chromatograms in Fig. 5 are for aliquots taken from depolymerization solutions that contained no histamine (chromatogram A) and histamine at histamine/heparin ratios of 2:1 and 10:1 (chromatograms B and C, respectively), where the heparin concentration is in terms of disaccharide repeating units. The aliquots were all taken after 43 h of reaction.

The results in Fig. 5 indicate that histamine affects the heparinase-catalyzed depolymerization of heparin, with an increased yield of each size fraction in the disaccharide to tetradecasaccharide range at a histamine/heparin ratio of 2:1. This increased yield was observed in chromatograms taken from the beginning of the reaction until the heparinase lost its activity (data not shown). However, when the histamine/heparin ratio is increased to 10:1, the depolymerization reaction is stopped. SAX-HPLC chromatograms of the tetra- and hexasaccharide fractions for the reactions without histamine and with a histamine/heparin ratio of 2:1 are shown in Fig. 6. A comparison of the chromatograms indicates that histamine modifies the cleavage pattern, with a decrease in the formation of some oligosaccharides and an increase in the formation of others. For example, formation of the tetrasaccharides that elute at ~69 and 70 min in Fig. 6B is greatly enhanced in the presence of histamine, as is the formation of hexasaccharides that elute at ~75, 84 and 102 min.
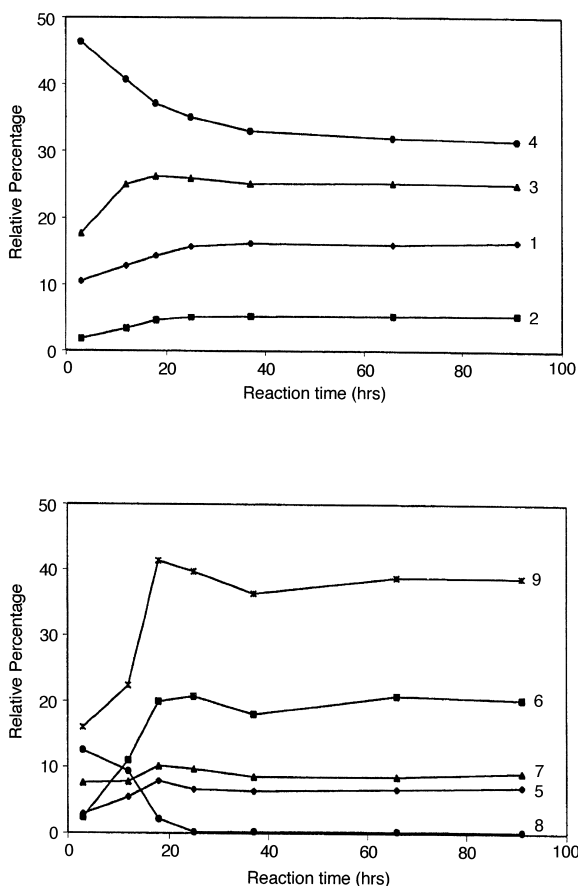

Fig. 4. Time-course plots for the labelled peaks in the tetra- and hexasaccharide SAX-HPLC chromatograms in Fig. 3. The top panel is for tetrasaccharides, the bottom panel is for hexasaccharides. Relative percentage is defined as the percentage each peak is of the total peak area in the tetrasaccharide or hexasaccharide chromatogram.
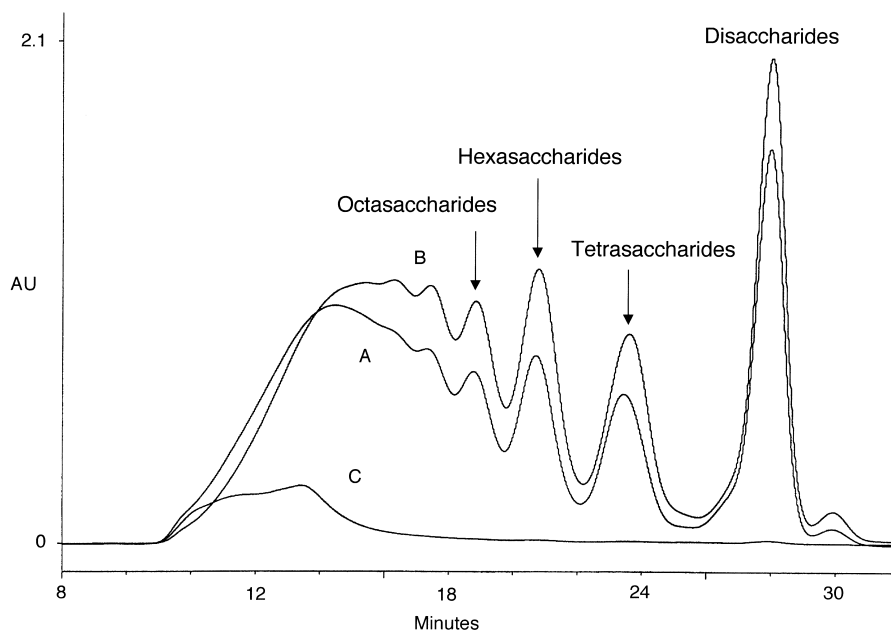
Fig. 5. Size fractionation of depolymerized porcine intestinal mucosal heparin (200 mg of heparin in 4 ml of depolymerization buffer with 50 U heparinase I) with the high-performance Superdex GPC column, (A) without histamine, (B) with histamine at a histamine/heparin disaccharide ratio of 2:1 and (C) with histamine at a histamine/heparin disaccharide ratio of 10:1. The chromatograms are for samples taken after depolymerization for 44 h. One Superdex GPC column was used with a flow-rate of 0.6 ml/min and a mobile phase of 0.2 M NaCl.

3.2. Isolation of pure heparin-derived oligosaccharides

Oligosaccharide mixtures produced by depolymerization of heparin for preparative purposes are generally size-fractionated by low-pressure (gravity flow) GPC. The size-uniform fractions are then separated according to charge by SAX-HPLC [24]. Peak fractions are collected and the same fractions from multiple runs are combined to obtain sufficient quantities of pure oligosaccharides for further study. The purified oligosaccharides are then desalted by GPC. However, because the oligosaccharide concentration is low, a significant fraction of the oligosaccharide can be lost during the desalting step. To minimize this loss, we developed the following two-step procedure. In the first step, the oligosaccharide solution is diluted with Millipore water to reduce the NaCl concentration to below 0.4 M, and then the entire solution is pumped through a CarboPac PA1 column as the mobile phase. The oligosaccharide stays on the column while the NaCl is eluted. The

oligosaccharide is then eluted from the column quickly with a steep NaCl gradient and collected. The oligosaccharide, which now is concentrated in a smaller volume at a smaller NaCl to oligosaccharide ratio, is then desalted by low-pressure GPC on a 500×10 mm Sephadex G-10 column using Millipore water as eluent. Fractions (1.5 ml) were collected from the Sephadex G-10 column and monitored both by conductivity and UV absorbance at 232 nm. The time efficiency of this process can be further increased by doing the concentration/partial desalting process on the CarboPac PA1 column simultaneously for several oligosaccharide fractions that are sufficiently separated in the chromatogram. An additional benefit of this procedure is that the second pass through the CarboPac PA1 column also serves as a second purification step.

4. Discussion

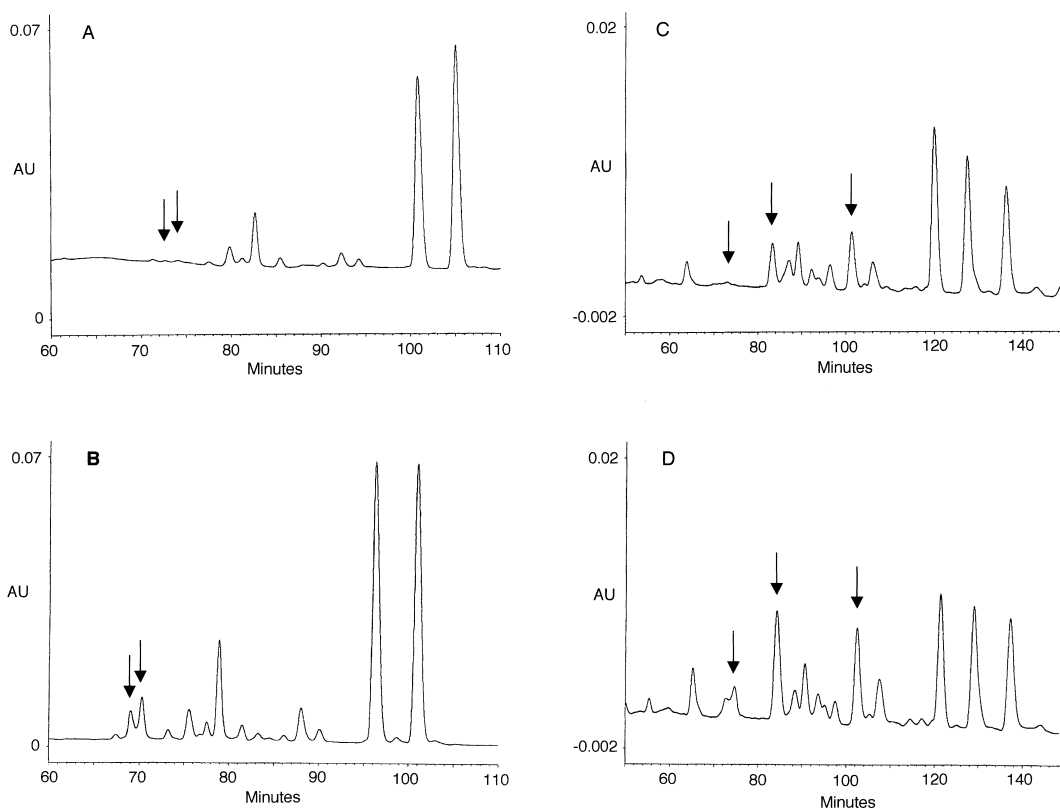The two-step procedure described here for moni-

Fig. 6. SAX-HPLC chromatograms obtained with an analytical scale CarboPac PA1 column for tetra- and hexasaccharide size-uniform fractions obtained from the high-performance Superdex GPC separation of heparinase I-catalyzed depolymerization of porcine intestinal mucosal heparin without and with histamine present. The samples were taken after 236 h of depolymerization. A and B are for tetrasaccharide fractions from depolymerization solutions (A) without histamine and (B) with histamine present at a histamine/heparin disaccharide ratio of 2:1; C and D are for the corresponding hexasaccharide fractions. The oligosaccharides were eluted with a linear gradient of 70 mM pH 7 phosphate buffer (Solvent A) and 70 mM pH 7 phosphate buffer plus 2.0 M NaCl (Solvent B) at a flow-rate of 1.2 ml/min. For the tetrasaccharide separations: 2%/min gradient for 10 min and then 0.2%/min for the remainder of the separation; for the hexasaccharide separations: 2%/min gradient for 10 min and then 0.25%/min for the remainder of the separation.

toring heparinase I-catalyzed depolymerization of heparin by product-profile analysis at the oligosaccharide level is based on the high resolution that can be achieved for both size fractionation with the Superdex GPC column and SAX-HPLC separation of the size-uniform oligosaccharide fractions with the CarboPac PA1 column. Methods used previously to monitor depolymerization include viscosity measurements to monitor oligosaccharide size and direct analysis of the depolymerization reaction mixture, i.e. without a size-fractionation step, as a function of time by SAX-HPLC [25,26]. Product profile kinetics were reported in the latter studies, however there were some inconsistencies in the interpretation of the

exo- and endolytic activity of heparin lyases and their substrate specificity [27,28]. Because of this ambiguity, an alternative method involving mass spectrometry and capillary electrophoresis was proposed for mechanistic studies [29]. In this method, purified oligosaccharides of known structure are used as substrates to overcome complexities in the analysis due to variations in the primary structure of heparin. Such studies are limited primarily by the lack of pure, homogeneous oligosaccharides larger than tetrasaccharides.

The two-step procedure reported here for monitoring heparinase I-catalyzed depolymerization of heparin is relatively fast and it provides detailed time

course information for multiple oligosaccharides. As demonstrated by results for the depolymerization of heparin in the presence of histamine, it should be of use in studies to determine if the formation of specific oligosaccharide sequences is enriched in the presence of heparin-binding biomolecules and for optimizing conditions for formation of such oligosaccharides. The method should also be of use in studies of the mechanism and substrate specificity of the heparin lyases and other glycosaminoglycan lyases.

Results obtained in this study demonstrate that the CarboPac PA1 column offers excellent performance for the SAX-HPLC separation of size-uniform oligosaccharide mixtures. Standard quaternary-amine-based strong anion-exchange columns have been most widely used for this purpose [21,22]. However, these columns tend to deteriorate under the high salt concentration conditions used to elute heparin-derived oligosaccharides and thus require constant correction of the slope of the NaCl gradient [30,31]. As a consequence, elution times for the different oligosaccharides in a mixture are not very reproducible, which makes it difficult to combine fractions from repeated separations. This is a particular problem for the isolation of pure samples of the larger oligosaccharides, since fractions from many separations usually need to be combined to accumulate adequate amounts for further study [22,32]. Unlike traditional macroporous polymeric ion-exchange materials, the CarboPac PA1 column contains a polymeric nonporous pellicular resin [33] that overcomes the poor mass transfer and peak band broadening problems resulting from slow diffusion of analytes into and out of the pores of resins used in quaternary amine-based SAX columns. In addition, the resin is stable over a wide pH range (pH 0–14).

The CarboPac PA1 column has been used previously to separate unsaturated disaccharides derived from chondroitin sulfate, dermatan sulfate and hyaluronan with high resolution [34,35]. However, the reported procedure requires reduction of the disaccharides to their alditol form to stabilize them at the alkaline pH values of the eluents used. In the procedure described here, the separation of heparin-derived oligosaccharides is done at lower pH, where the oligosaccharides are stable and no further modification is required. Also, the CarboPac PA1 column can be reconditioned after each run in ~5 min, compared to the 25 min we found necessary for standard quaternary amine-based SAX columns.

The chromatograms in Figs. 3 and 6 indicate that excellent resolution is achieved with the CarboPac PA1 column. The resolution remained essentially the same after approximately 300 injections over a 1-year period. With the high resolution and excellent reproducibility of separations on the CarboPac PA1 column, oligosaccharides present in minor abundance can be isolated.

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