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Size-exclusion chromatography of heparin oligosaccharides at high and low pressure

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Abstract

Recent findings on specific and non-specific interactions of glycosaminoglycans (GAGs) accentuate their pivotal role in biology and the call for improved sequencing tools. The present study evaluates size-exclusion chromatography (SEC) of heparin oligosaccharides at high and low pressure, requiring amounts as low as 0.2 microgram, using conventional UV detection after depolymerization with heparin lyases. Because of their high charge at physiological pH, SEC elution volumes of heparin oligosaccharides depend on both molecular size and charge repulsion from the matrix. As a consequence, SEC elution volumes of GAGs are smaller than those of globular proteins of similar molecular weight, and this might be exploited. Accordingly, larger heparin oligosaccharides are best separated according to their size at high ionic strength of the mobile phase (>30 mM); in contrast, disaccharides are best separated according to their charge at low ionic strength, compatible with on-line coupling to mass spectrometry. Optimized SEC affords separation of characteristic heparin trisaccharides that contain uronic acid at the reducing end and suggest cellular storage of heparin as a free glycan.

Keywords: Bio-Gel; Heparan sulfate proteoglycans; Heparin-binding proteins; Heparinase; Heparin oligosaccharides; Mass spectrometry; Size-exclusion chromatography; Superdex; van Deemter plot

1. Introduction

Heparin is one of the top-selling drugs worldwide with yearly sales reaching nearly 2.5 billion dollars [2]. Besides its medical importance as an anticoagulant, it is the glycosaminoglycan (GAG) with the highest charge and structural variability. GAGs are unbranched, sulfated polysaccharides containing between 20 and 1500 repeating units of disaccharides, and are located primarily on the surface of cells and in the extracellular matrix (for a review, see [3,4]). Their high anionic charge at physiological pH provides on one hand a non-specific water-retaining capacity essential for proper functioning of the extracellular matrix, and on the other hand, the capacity to interact *specifically* with cationic molecules, such as basic proteins, through non-covalent electrostatic forces. In contrast to the water-retaining capacity, this protein binding may require a specific primary [3] and secondary [5] structure of the GAG where a well-defined structure ("binding epitope") of GAGs seems compulsory to make a binding specific. Such interactions of proteins with GAGs mediate various biological functions such as growth-factor signaling, cell-adhesion, cell proliferation, host–pathogen interaction, endocytosis, protein folding and misfolding, or blood coagulation. Therefore, the interest in such interactions has been considerably increased as part of glycomics research [4].

In contrast to DNA and proteins, however, the sequencing of such complex polysaccharides is technically very demanding and requires multiple chromatographic steps [4,6]. In addition,

Abbreviations: AT-III, antithrombin-III; dp *n*, degree of polymerization (equivalent to *n* pyranosyl rings); ESI, electrospray ionization; GAG, glycosaminoglycan; Gal, galactose; GlcN, 2-*N*-glucosamine; HexN, 2-*N*-hexosamine; HL-I, heparin lyase type-I; HL-II, heparin lyase type-II; HL-III, heparin lyase type-III; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; I-H, ΔUA(2S)-GlcN(6S); I-S, ΔUA(2S)-GlcNS(6S); IV-H, ΔUA-GlcN; IV-S, ΔUA-GlcNS; MS, mass spectrometry; SEC, size-exclusion chromatography; TRIS, tris(hydroxymethyl) aminomethane; UroA, 6-uronic acid; stereochemistry at C-5 not explicitly specified; ΔUA, Δ4,5-UroA; double bound resulting from heparin lyases; V_0, volume of excluded molecules (e.g. SEC fraction of dextran blue); V_t, total bed volume (e.g. SEC fraction of acetone); Xyl, xylose (pentose as pyranosyl ring structure)

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GAGs are not synthesized as homologous copies of templates as is the case for DNA, RNA or proteins. Instead, GAGs are poly-disperse with regard to chain length, substitution and isomerization. This dispersion is the result of various enzymes for GAG modification as part of cell adaptations, where the balance of regulation and random modification is not yet fully understood. Much of current understanding of the structure-activity relationship of GAGs is therefore not based on the structural analysis of homologue copies of native GAG chains. Instead, heterogeneous GAG pools are commonly extracted from biological tissue, subsequently depolymerized into to smaller oligosaccharides, and then compared in order to analyze their structure ("sequencing") and to understand the correlation between structure and biological activity [3].

In this process, a rapid and sensitive size-exclusion chromatography (SEC) method would facilitate multiple components in the structural characterization of GAGs such as measurement of size dispersions of crude GAG extracts (varying from 10 to 750 kDa), isolation of disaccharide fractions essential for "disaccharide analysis" [7], knowledge of the enzyme-resistant fraction in *full* digests, and finding the best instant to stop the depolymerization in *partial* digests where short fragments (<12 pyranosyl rings) are technically mandatory for analysis with NMR and MS, though complete cleavage destroys the biologically active binding epitopes on the other side [8].

Here, classical low-pressure SEC has been often used to separate oligosaccharides from *bulk* quantities of GAGs such as heparin from slaughterhouse-derived porcine intestine. For rapid comparison of *small* amounts of biological samples (e.g. heparan sulfate from cell culture, *Drosophila* or *C. elegans*), however, such preparative techniques are too low in sensitivity and too lengthy (1–4 days) mainly through dilution effects in preparative columns and poor pressure-resistance of classical SEC matrices, respectively. Reported chromatographic conditions [9–15] and resulting resolution vary widely among published protocols and baseline separation of disaccharides has rarely been obtained.

Therefore, the present study evaluates the optimization of a faster and more sensitive SEC protocol at high pressure—appropriate to compare small amounts (>0.2 μg) of GAGs with baseline resolution for disaccharides. Possible applications are discussed, such as rapid disaccharide analysis with mass spectrometry, and results are compared with an optimized SEC at low pressure. Heparin was chosen for these experiments, because it has the highest charge and, together with heparan sulfate, the highest structural variability of the GAGs.

2. Experimental

2.1. Chemicals and reagents

Porcine intestinal mucosa heparin (sodium salt, 166 anti-(Factor Xa) U/mg, average MW: 13.0 kDa, ≈dp46)[1] was from Celsus Laboratories (Cincinnati, OH). Heparin Lyase-I (EC 4.2.2.7, MW: 42.5 kDa), II (EC number not yet assigned, MW: 85.8 kDa), and III (EC 4.2.2.8, MW: 73.2 kDa) from *Flavobacterium heparinum* with a manufacturer-specified activity of 109, 17 and 57 IU/mg, respectively, were from Ibex (Montreal, Canada). Tris(hydroxymethyl) aminomethane (TRIS) was from Mallinckrodt (Phillipsburg, NJ) and all other chemicals of HPLC grade were from Sigma-Aldrich (St. Louis, MO).

2.2. Enzymatic digestion

Five hundred milligrams of heparin in 5 mL of digestion buffer (50 mM TRIS, 50 mM NaCl, 2 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin, pH 7.10) was 0.22 μm filter sterilized (cellulose acetate, Corning; Corning, NY) into a sterile 5 mL glass vial (Infochroma; Zug, Switzerland), placed in an incubator at 35 °C and gently rotated (Labquake, Barnstead Thermolyne; Dubuque, IA) at all times during the enzymatic digestion (72 h). At these conditions, the activity of heparin lyase-I (HL-I) was 20.2% higher than specified by the manufacturer. HL-I was added initially and every 6 h (in order to compensate for progressive activity losses [16]) in a small volume (1–5 μL) at an operative activity of 0.6 mIU enzyme/mg of heparin. Initial salt content and pH were at optimum conditions [16,17], and the pH dropped only faintly to pH 6.9 at the end of the 72 h digestion.

2.3. Progression of the enzymatic digestion

Along the 72 h enzymatic digestion, aliquots of 5 μL were regularly taken (intervals ranging from initially 15 min to finally 6 h) and split for both immediate analytical SEC (0.01–2 μL) and measurement of the progression of the enzymatic reaction with bulk UV spectroscopy (2.5 μL). For the latter purpose, 2.5 μL of the digest mixture (containing 0.25 mg of heparin) were placed into 1497.5 μL of 0.1N hydrochloric acid (pH 1). At these conditions and a wavelength of 232 nm, the molar extinction coefficient of a monounsaturated disaccharide is estimated at 5500 [18]. The increase in UV absorption during the digestion, as caused by enzymatic formation of double bonds, was measured using a Beckman (Fullerton, CA) DU-50 spectrophotometer (1 cm sample length). Considering the specific sample dilution, spectrophotometer cell length, extinction coefficient, average molecular weight (approximating Gaussian distribution) and non-absorbance of 1 disaccharide from the non-reducing terminus per heparin chain, the complete digestion of 0.25 mg of heparin to purely disaccharides I-S (MW: 665.4 Da, sodium salt) or II-S (MW: 563.4 Da, sodium salt) would result in a maximum absorbance of 1.307 or 1.557, respectively. The measured absorbance, however, was below 1.0 (and thus in the linear range of the spectrophotometer) suggesting incomplete digestion to disaccharides (see Section 3.1). Enzymatic digestions were performed in four repetitions, and results were averaged.

2.4. Preparative SEC

For preparative SEC, six aliquots, each containing 800 μL of the enzymatic digest (corresponding to 80 mg of heparin),

[1] In literature, the length of a GAG chain has been commonly described by the term "degree of polymerization" (dp *n*) where *n* is the number of pyranosyl rings. Accordingly, a disaccharide or tetrasaccharide is assigned as dp2 or dp4, respectively.

were sampled at 2, 4, 8, 16, 36 and 72 h. The enzymatic reaction within these samples was stopped by removing them from the incubator and denaturing the enzyme by heating to 100 °C for 3 min in a water bath. The samples were stored at −80 °C for a maximum of 20 days until the preparative SEC was performed. Lyophilization at these conditions was not favorable because the high salt content, required during the digestion, leads to cap formation during lyophilization and prevents further evaporation of the solvent.

For SEC, a glass column (170 cm × 1.5 cm; Bio-Rad, Hercules, CA) was filled with a polyacrylamide gel Bio-Gel P-10f; (Bio-Rad, Hercules, CA) having a finer particle size (hydrated particle size: 45–90 μm) and larger pore diameter than the commonly used Bio-Gel P-6. Ionic content (0.2 M NH_4HCO_3) and constant hydrostatic flow (3 mL/h; 25 °C) were optimized such as to produce a maximum number of plates for the dp2 peak. The effluent was monitored using an UV-spectrophotometer (Waters 493E; Millford, MA) tuned at 232 nm (0.5 cm sample length). Fractions of 1 mL were collected. Removal of NH_4HCO_3 was effected by lyophilizing (RVT4104/SC110-A; Thermo Savant; Holbrook, NY) the fractions three times in the original volume of nanopure water (Milli-Q, Millipore; Billerica, MA).

2.5. Analytical SEC

Unless otherwise stated, SEC conditions were used that were optimized to yield the most narrow and symmetric heparin I-S peak. For this purpose, two commercially available Superdex-75 PC 3.2/30 columns (Amersham Biosciences; Piscataway, NJ) with an average particle size of 13 μm were run in series (=0.32 cm × 60 cm; combined bed volume of 4.8 mL). Superdex is a composite of cross-linked agarose and dextran, stable for a pH range of 1–14 and a temperature range of 4–40 °C. A Beckman System Gold 125 HPLC solvent module (Fullerton, CA) was coupled to a Beckman UV–vis 166 variable wavelength detector equipped with a 4 μL micro flow cell (0.5 cm sample length). The HPLC system was equipped with two μ-flow heads capable of producing a stable flow rate of 10 μL/min and higher. The degassed mobile phase (150 mM NH_4HCO_3, pH ≈ 7.8) was supplied at a flow rate of 20 μL/min via a preparative 20 mL precolumn sample loop (Rheodyne; Rhonert Park, CA) used to keep the pump heads (degassed water only) free of saline. The mobile phase passed sequentially through a 17.2 bar (250 psi) hardware pressure limitation valve (Upchurch Scientific; Oak Harbor, WA), a sample injector equipped with a 5 μL sample loop (7725i, Rheodyne; Rhonert Park, CA), and a 0.22 μm precolumn filter into the columns followed by the UV detector (232 nm), back pressure regulator (Rheodyne, 60 psi, to avoid bubble formation in the detector) and fraction collector (Bio-Rad Econo; Hercules, CA). Digital sampling rate was 1 Hz. Exclusion volume (V_0) of large analytes and total bed volume (V_t) of the mobile phase were measured with dextran blue (20 μg; mean MW: 2 MDa) and acetone (10%, v/v; MW: 58.08 Da), respectively, each producing an absorbance of ≈0.2 at 232 nm when injected through the 5 μL solvent loop. The "dead" volume between detector and fraction collector was measured by the interval of the peak maximum of cyanocobalamine (25 μg) between detector and collected 30 s fractions.

The maximum tolerated pressure-over-column was 20 bar (290 psi), 24 bar (348 psi), and 12 bar (174 psi) for the Superdex-"Peptide", "75", and "200" column, respectively, as specified by the manufacturer. As a precaution to protect the column, the pressure was constantly recorded and limited by software (flow rate reduction) and hardware (overpressure valve) to a maximum of 17 bar, except for higher flows used in van Deemter plots. Manufacturer-recommended sample volumes were 2–25 μL and specified separation range for globular proteins was 0.1–7 kDa, 3–70 kDa, and 10–600 kDa for the Superdex-"Peptide", "75", and "200" column, respectively. The effective separation range for sulfated glycans, however, was smaller—as described in the *results*. Along the enzymatic digestion, equivalents of the original heparin digests were regularly sampled, diluted with running buffer in order to not pass the linear (±5%) analytical range of the detector (0–1.5 AU) and injected through the 5 μL sample loop.

2.6. Optimum mobile phase velocity

The number of theoretical plates (N) of the columns was determined from the experimental peak width at half-height ($w_{0.5}$) and retention time (t_R) of the solutes and was

$$N = 5.55\left(\frac{t_R}{w_{0.5}}\right)^2 \quad (1)$$

The height equivalent to a theoretical plate (HETP; the smaller the better the resolution) was derived by dividing the length of the column (L) by N:

$$\mathrm{HETP} = \frac{L}{N} \quad (2)$$

Experimental values for HETP were plotted as a function of the mobile phase velocity (u), and the experimental data were approximated by a least-square fitting to the van Deemter equation

$$\mathrm{HETP}(u) = A + \frac{B}{u} + C\,u \quad (3)$$

where A, B, and C are constants for eddy diffusion (particle and filling homogeneity of matrix), longitudinal diffusion (back-and-forth diffusion) and resistance to mass transfer (non-equilibrium between phases; non-diffusion into particles), respectively [19].

2.7. ESI-MS

The oligosaccharides were analyzed by electrospray ionisation mass spectrometry (ESI-MS) using a Bruker Daltonics (Billerica, MA) Esquire 3000 quadrupole ion trap instrument equipped with a Picoview 200 source (New Objective, Woburn, MA). Spectra were averaged during 1 min at lowest available scanning rate (1650 u/s) providing isotopic resolution at all charge states observed. Source conditions were negative mode, off-line nanospray (≈40 nL/min flow rate), custom-pulled (P-97

micropipet puller, Sutter; Novato, CA) borosilicate tips (1 μm inner tip diameter). Gentle desolvation conditions (8 V skimmer, −15 V capillary exit offset) and solvents (30% methanol, 0.1% ammonium hydroxide, pH 8) were used to minimize in-source losses of sulfate groups [20]. The capillary was only moderately heated (200 °C) in order to favour higher charge states. The concentration of an oligosaccharide fraction was typically ≈10 μM. Fragmentation for MS/MS and MS^n spectra was induced via collision-induced dissociation (Helium gas) of isolated ion species. Structures were assigned by closest match (typically <0.2 u) between experimental *m/z* values and a database containing all heparin oligosaccharides structures up to dp10, and by mandatory correspondence of the fragmentation pattern in MS/MS and MS^n spectra.

3. Results and discussion

3.1. Enzymatic digestion

For SEC separation of GAG oligosaccharides, the sensitivity and resolution are considerably influenced by the specific combination of original GAG and enzyme(s) used. Specifically, the depolymerization with heparin lyases (EC 4.2.2) creates UV absorbent chemical bonds [21–23] affecting the sensitivity for UV detection. Concerning the chromatographic resolution, oligosaccharides have typically a *paired* number of pyranosyl rings (dp2, 4, etc.) due to the repeating disaccharide motif $(UroA\text{-}GlcN)_n$ of heparin and the specificity of the enzyme (Fig. 1). However, within this coarse size-fraction based on the number of pyranosyl rings (e.g. dp2), the molecular weight may scatter considerably from unsubstituted (MW: 359.3 Da, sodium salt) to fully sulfated disaccharides (MW 767.4 Da, sodium salt) depending on the enzyme used for depolymerization. For example, HL-I generates mainly the disaccharide I-S (MW: 665.4 Da, sodium salt); other heparin lyases are less specific and generate, in function of available GAGs, disaccharides with a much wider range of substitutions [24].

By exploiting the UV absorbance of continuously formed oligosaccharides, the progression and extent of the enzymatic cleavage was appropriately monitored (Fig. 2A and B). As measured from the increase of the UV absorbance towards its plateau value (0.662), the reaction was completed to 11, 30, 67 and 100% after 2, 6, 12 and 36 h, respectively. Knowledge of this reaction completion is essential, especially, for a reproducible preparation of intact binding epitopes, where a *maximum* of specific size fractions is observed at a *certain* reaction completion; thereafter,

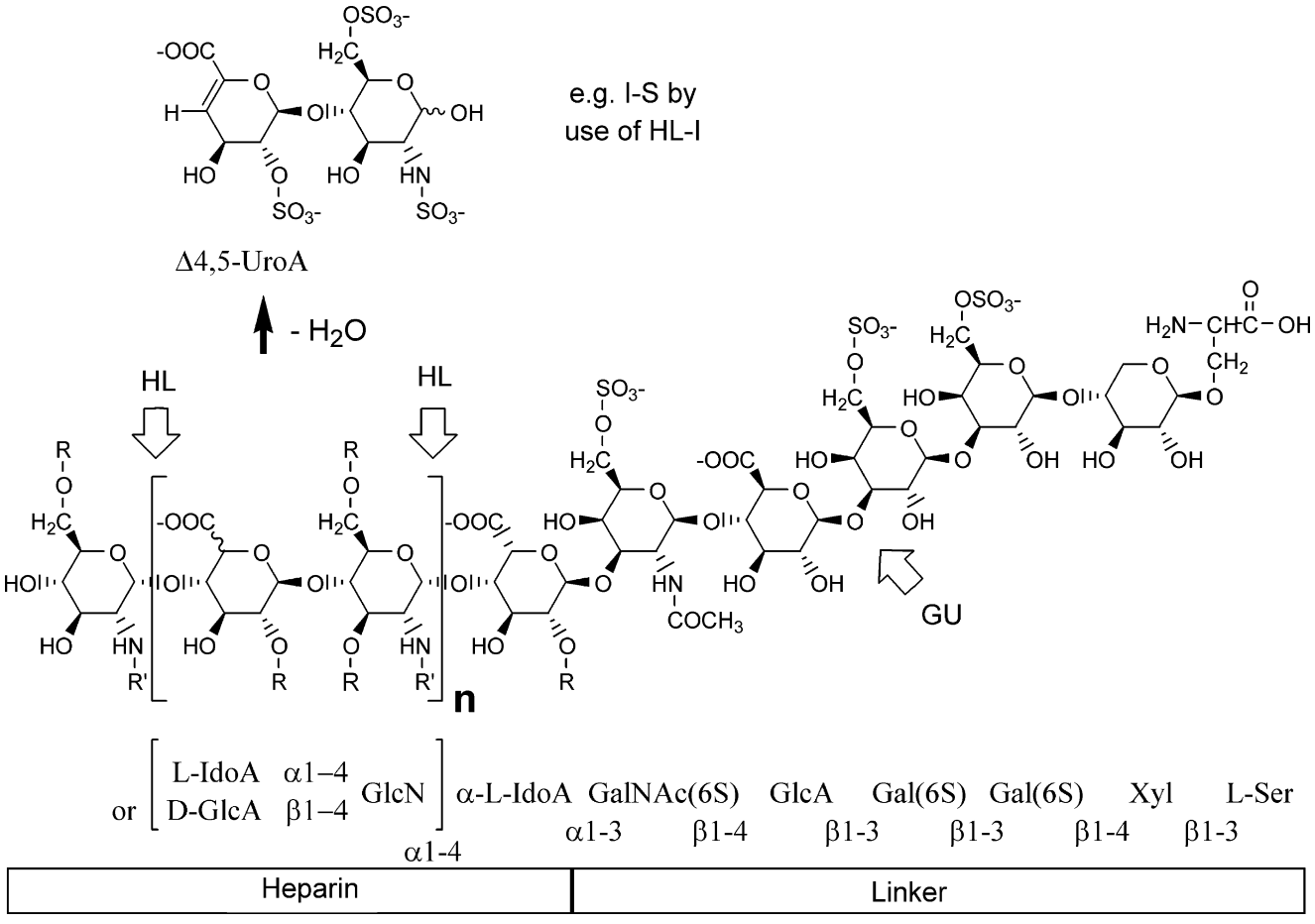

Fig. 1. Heparin's basic structure and consequences for its enzymatic depolymerization. Heparin lyases (HL) cleave between glucosamine and uronic acid producing thus smaller oligosaccharides with a *paired* number of pyranosyl rings leaving glucosamine at the reducing end. A uronic acid at the reducing end may only result under influence of a β-endo-glucuronidase (GU). Upper insert: HLs depolymerize heparin by a H_2O elimination reaction leaving a UV absorbent Δ4,5 double bond at the uronic acid.

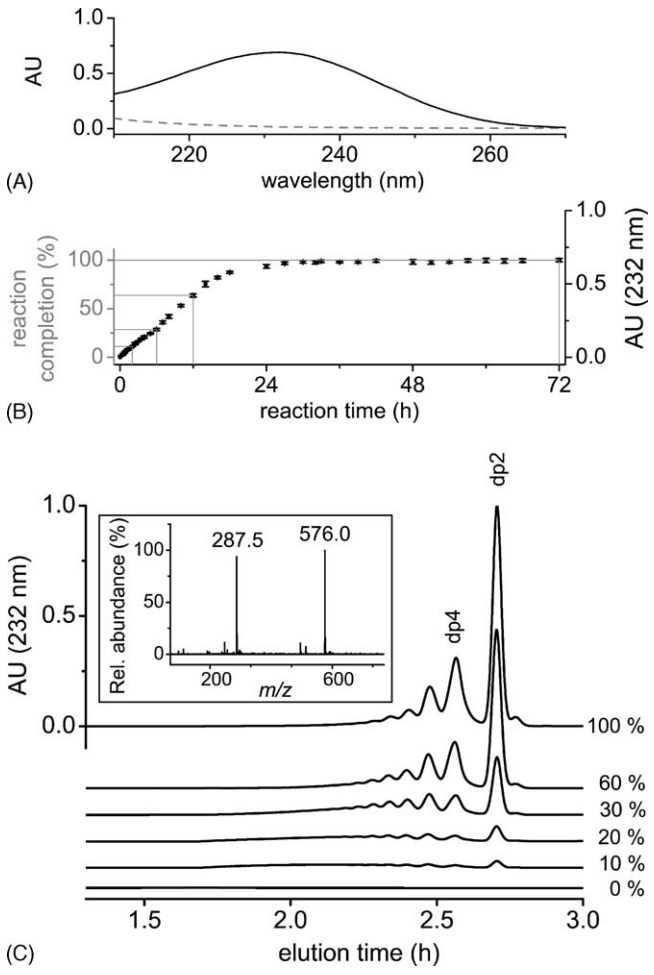

Fig. 2. Enzymatic generation of heparin oligosaccharides. (A) Bulk UV spectrophotometry. The UV absorbance of heparin (0.25 mg heparin/1.5 mL; pH 1) before (dashed line) and after (solid line) exhaustive digestion with heparin lyase-I produced a maximum UV absorbance at 232 nm. (B) Progression of the enzymatic digestion. 500 mg of porcine intestinal heparin ($n = 4$, mean ± STDV) were digested in 5 mL of buffer at 35 °C with repeated additions (every 6 h) of heparin lyase-I (0.6 mIU/mg heparin). At indicated time points (dots ± STD), aliquots of heparin were sampled, diluted in 0.1N HCl (0.25 mg heparin/1.5 mL) and measured at 232 nm (right scale) using a 1 cm sample length. The complete digestion of 0.25 mg of heparin into merely disaccharides would have resulted in an absorbance of (A) = 1.307 (see Section 2.3). Consequently, an enzyme-resistant fraction of larger oligosaccharides remained after 100% of reaction completion with HL-I. (C) Analytical SEC of heparin oligosaccharides at various time points during digestion with heparin lyase-I. Five hundred milligrams of porcine intestinal heparin was digested with repeated (6 h) additions of enzyme (0.6 mIU/mg heparin). Aliquots were regularly taken during the 72 h digestion at intervals ranging from initially 15 min to finally 6 h. Aliquots containing 50 μg of heparin were separated with analytical SEC (2 Superdex-75, 20 μL/min, 0.1N NH_4HCO_3), and some chromatograms are displayed for 0, 10, 20, 30, 60 and 100% of reaction completion (equal *y*-scale for all chromatograms). Insert: ESI-MS (and unpublished MS^n) of the corresponding dp2 fraction reveals essentially the *trisulfated* disaccharide I-S, detected in its $[M - 1H]^{1-}$ and $[M - 2H]^{2-}$ charge state with corresponding *m/z* (monoisotopic) of 575.96 and 287.48, respectively.

intermediate chain length oligosaccharides are further digested to smaller molecules (see Section 3.2).

No further enzymatic cleavage occurred after 36 h, suggesting that the digestion had reached its endpoint, however, not meaning that 100% of the heparin was digested to disaccharides. Accordingly, the fraction of uncleaved bonds ("enzyme-resistant fragments") was roughly approximated by comparing experimental UV absorbance and its theoretical maximum at given conditions. For the present digest (Fig. 2B), the measured absorbance (0.662) was only 50.6% of the theoretical maximum of 1.307 (see Section 2.3) indicating that only this percentage of bonds was cleaved by HL-I.

3.2. Size-dispersion during digestion

In order to better characterize the above approximation of the enzyme-resistant fraction (Fig. 2A and B), the size-dispersion of heparin oligosaccharides was measured along the depolymerization by using rapid and sensitive analytical SEC at high pressure (Fig. 2C). For this purpose, aliquots of 50 μg of digested heparin were sampled at 38 different time points during the 72 h digestion (Fig. 2B) and separated on two commercial Superdex-75 columns coupled in series. Fig. 2C displays the progression of the oligosaccharide production at six representative time points during the digestion of the initially non-UV absorbent heparin. The figure clearly illustrates that the endpoint of the digestion results in a mixture of di- and oligosaccharides. Furthermore, it is obvious that the sensitivity for UV detection depends on the progression of the enzymatic reaction. Moreover, the amount of intermediate chain length oligosaccharides ($V_e = 2$–2.4 h) first increases, then decreases during digestion. Specifically, a maximum amount of dp12 and dp8 were observed at 30 and 67% of reaction completion, respectively.

At 100% reaction completion with HL-I (for other enzymes, see Section 3.4), the contribution of undigested larger oligosaccharides was not negligible, and this finding is important for "disaccharide analysis" [7] of GAGs. In this technique, GAGs of different tissues and diseases are compared on the base of released disaccharides only [7]. Therefore, it is likely that important information on existing binding epitopes is located in enzyme-resistant fractions that are not analyzed on the base of a disaccharide analysis. Present results suggest, however, that such an analysis could be readily performed within 3 h or less from a very small amount of material sampled without further purification from the enzymatic digest. Information on sequence coverage is also essential, because binding epitopes in general represent usually only a very small part of the GAG (e.g. 3.6% of the total heparin structure is a binding epitope for AT-III).[2] In addition, such enzyme-resistant fractions may result from unusual structural variations that prevent the enzymatic digestion, but are likely to define important binding epitopes [3].

3.3. Sensitivity in UV detection

GAGs originating from cell cultures and small laboratory animals are often available in microgram quantities (e.g. 2.8 μg

[2] It has been shown that a binding epitope for AT-III is contained in only every third heparin molecule [1]. The fraction of this binding epitope (a dp5) with respect to the average heparin molecule (here ≈dp46; on the basis of two sulfate groups per disaccharide; sodium salt) and occurrence in only every third heparin molecule represents thus less than 4% of the total structural information.

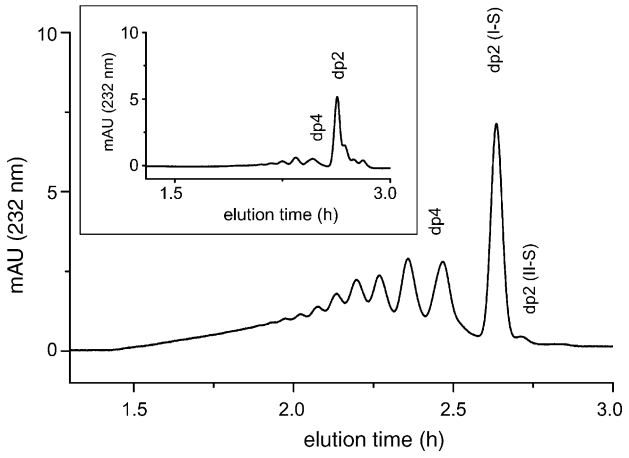

Fig. 3. (Sub-)microgram SEC using UV detection. One microgram of heparin, previously digested with HL-I to 30% of reaction completion, was separated with analytical SEC. Insert: 0.2 μg of heparin, digested with a mixture of all three heparin lyases to 100% reaction completion, was separated with analytical SEC. Chromatographic conditions: two serially connected Superdex 75-columns (V_0 was 1.45 h × 1.2 mL/h; V_t was 3.31 h × 1.2 mL/h), each at a dimension of 3.2 mm × 300 mm and a particle size of 13 μm.

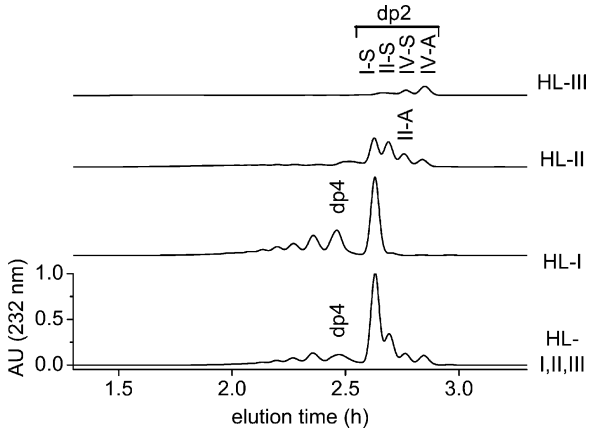

Fig. 4. Heterogeneity and peak width of the dp2 fraction in function of enzymes used. 500 mg of porcine intestinal heparin were digested in 5 mL of buffer at 35 °C with repeated additions (every 6 h) of heparin lyase-I (HL-I), II (HL-II), III (HL-III), or a mixture of all three lyases (HL-I,II,III) each at 0.6 mIU/mg heparin. After 100% of reaction completion, as determined by a plateau value in UV spectroscopy (similar to Fig. 2B), aliquots of 50 μg of depolymerized heparin were separated with analytical SEC (2 Superdex-75, 20 μL/min, 0.1N NH_4HCO_3, equal *y*-scale for all runs).

of HSPG/g wet tissue [25]), necessitating a sensitive detection method for depolymerized GAGs in order to enable study of their patterns of expression under (patho-) physiological conditions. For this purpose, modern HPLC equipment provides a stable UV detector baseline and constant pump rates over several hours, so that measurements in the 1–10 mAU range should be reliably reproduced at flows of a few microliters per minute. This stability was tested for the SEC separation of 1 μg of heparin using a conventional UV detector with a 0.5 cm sample length (Fig. 3). In this experiment, heparin digested to 30% reaction completion with HL-I was chosen, because at this reaction completion, the UV intensities, and thus *molar* contribution, for dp4-14 are approximately similar (Fig. 3). The same signal-to-noise ratio for the disaccharide fraction was obtained with as little as 0.25 and 0.2 μg of heparin (Fig. 3, insert), when digested to 100% reaction completion with HL-I or a mixture of all three heparin lyases, respectively (see Section 3.4). These results clearly illustrate, that (sub-)microgram quantities of GAGs obtained from cell cultures and small laboratory animals can be adequately separated and detected by this technique. GAG quantities of >0.2 μg are thus sufficient for reliable routine separations, i.e. displaying a UV absorbance of >5 mU for the dp2 fractions (Fig. 3) that are required in common disaccharide analysis.

At the same time, it is evident that the specific combination between enzyme(s) used, number of cleavage sites in the substrate and progression of the reaction finally determine the signal intensity when referring to UV detection.

3.4. SEC peak dispersion in function of enzyme used

The use of different enzymes for heparin depolymerization revealed that the disaccharide fraction of heparin oligosaccharides was, in fact, polydisperse. For 100% reaction completion with HL-I, for example, the intense dp2 peak (Fig. 4, HL-I; 2.63 h elution time) was almost pure in chemical composition and consisted of the unsaturated *trisulfated* heparin disaccharide I-S as evidenced by mass spectrometry (Fig. 2C, insert). The smaller SEC fraction right of it (2.74 h, 3.2% of the I-S peak integral), consisted also of a disaccharide and was identified as *disulfated* disaccharide II-S. When HL-II and HL-III were used instead of HL-I, additional disaccharides were separated (Fig. 4) and were disulfated III-S, monosulfated IV-S and acetylated IV-A as identified by using multistage tandem MS. These disaccharides eluted, according to their smaller molecular size, at higher elution times than I-S. The formation of these disaccharides, as caused by other lyases, was accompanied by a decrease in the abundances of larger oligosaccharides. A similar peak broadening as for the dp2 fraction was observed for the dp4 peak after digestion with all three heparin lyases (Fig. 4).

However, a remarkable enzyme-resistant fraction of oligosaccharides (>dp2) remained even after complete digestion with a mix of all three heparin lyases (Fig. 4) and accounted for 55.3 ± 2.4% (m/m) of the total oligosaccharides as determined by weight after preparative SEC of 80 mg of heparin. On a superficial view, the intense UV integral of the disaccharides (70.3%) may thus lead to underestimation of this fraction, because it reflects *molar* and not *mass* contribution. Integration of the analytical UV signal and multiplication by the dp2 content per size-fraction lead to similar results (enzyme resistant-fraction of 51.4 ± 1.7%, m/m) as compared to the direct determination by weight. The small difference between the two methods is likely caused by fragments originating from the non-reducing end of heparin that do not contain a Δ4,5 chromophore after enzymatic digestion.

3.5. Optimum flow

SEC is a separation technique based purely on the ability of molecules to diffuse into pores of defined size. Because the mobile phase velocity favors or hinders the local diffusion of the

solutes, SEC is much more dependant on an optimum mobile phase velocity as compared to reverse phase chromatography where an optimum gradient of the organic solvent is more critical. Since the diffusion coefficient, in turn, is dependant on the molecular size of the solute (governed by Fick's first law), the optimum flow is not universal, but depends mainly, next to viscosity and temperature of the mobile phase, on the molecular size of the solute. The larger the oligosaccharide, the smaller should be its random diffusion in the column and its contribution to peak broadening at low flow (B-term). As a result, the optimum flow rate for larger molecules was lower (left shift in van Deemter plots).

Accordingly, van Deemter plots (Fig. 5A) illustrate that a minimum HETP for acetone (MW: 58.08 Da) was achieved at a flow of 66 μL/min, corresponding to the manufacturer suggested flow rate of 40–100 μL/min. In contrast, the optimum mobile phase velocity for the heparin disaccharide was much lower (17.5 μL/min). For the even larger dp4 fraction, the B-term could not be determined precisely any longer (lowest available flow rate of the pump = 10 μL/min), a rough approximation of the experimental data suggests that the optimum flow rate is lower than 10 μL/min.

These results notably suggest that optimum SEC resolution of GAGs is achieved at flow rates of 20 μL/min and below for present columns. Another important conclusion can be derived from Fig. 5A. Despite the difference in optimum flow rates, the minimum HETP for acetone (27.43 ± 0.43 μm) and the disaccharide (26.02 ± 0.49 μm) were equal: depolymerization of

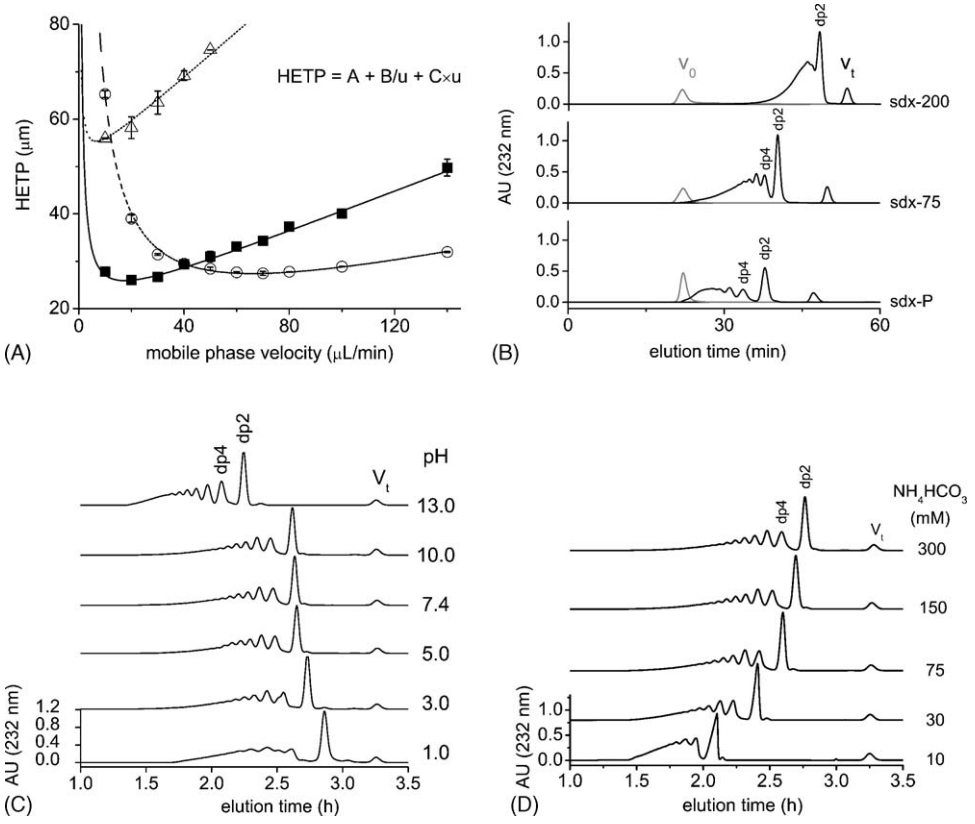

Fig. 5. Optimum SEC parameters. (A) Flow: the height equivalent to a theoretical plate (HETP) was plotted as a function of the mobile phase velocity u over two Superdex-75 columns connected in series (22 °C), and the results were fitted with the van Deemter equation ($HETP = A + B/u + Cu$) where A–C are constants (see Section 2.6). Acetone (- - -, ◯) had a minimum HETP at a flow of 66 μL/min. For heparin disaccharide I-S (—, ■), the optimum flow was 18 μL/min, and for the dp4 fraction (· · ·, Δ), the optimum flow was ≈6.5 μL/min. Conditions: 10% (v/v) acetone or 200 μg aliquots of heparin (digested with HL-I to 30% reaction completion) were applied via a 5 μL sample loop. (B) Matrix pore size. Two hundred micrograms of aliquots of a heparin oligosaccharides (—, MW: 0.5–13 kDa) produced by 30% reaction completion with HL-I were loaded on a single column of equal composition (Superdex, 13 μm particle size), but of different pore sizes: Superdex-200 (upper panel), Superdex-75 (middle) and Superdex-Peptide (lower) with manufacturer specified exclusion ranges of 600, 75 and 7 kDa, respectively, for globular proteins. HPLC conditions: single Superdex 3.2 mm × 300 mm column, flow of 40 μL/min, 0.1N NaCl, 0.1 N Tris, pH 8.0, 200 μg of heparin digested to 30% reaction completion with HL-I. Exclusion volume (V_0) and total bed volume (V_t) of the columns were determined in separate runs (overlay) with 20 μg of dextran blue (- - -, average molecular weight of 2 MDa) and 10% (v/v) acetone (· · ·) in the eluent, respectively. (C) pH. Monovalent acids (or monovalent buffers) were added to the mobile phase at a concentration of 100 mM containing additionally 100 mM NaCl. For this purpose, sodium hydroxide, ammonium buffer, TRIS buffer, acetate buffer, formiate buffer and HCl were used to control the pH to 13.0, 10.0, 7.4, 5.0, 3.0 and 1.0, respectively. Two Superdex 75 columns were serially connected and used at a flow of 20 μL/min and 200 μg aliquots of heparin (digested with HL-I to 30% reaction completion) were applied via a 5 μL sample loop containing 10% (v/v) acetone as a marker for the column's total bed volume (V_t). (D) Ionic strength of the mobile phase. With increasing salt content, the absolute elution volumes and also the separation efficiency for GAGs increased. Two Superdex-75 columns were serially connected and operated at a flow of 20 μL/min (all other conditions as described in Fig. 3) and 200 μg aliquots of heparin (digested with HL-I to 30% reaction completion) were applied via a 5 μL sample loop containing 10% (v/v) acetone as a marker for the column's total bed volume (V_t). As indicated (stacked plots), the ion content of the mobile phase was increased from 10 to 300 mM ammonium bicarbonate.

heparin with HL-I resulted in an isotropic peak for dp2 (trisulfated disaccharide I-S, MW: 645.6 Da, ammonium salt), so that its peak width could be accurately used for determination of the optimal flow with van Deemter approximations. In contrast, the SEC peak for the dp4 fraction was not a single compound, but consisted mainly of pentasulfated (65.4%) and hexasulfated (21.8%) tetrasaccharides as determined by ESI-MS. Accordingly, the mixture of higher sulfated (left SEC peak shoulder) and less sulfated (right peak shoulder) tetrasaccharide contributed to SEC peak broadening compared to acetone and the disaccharide leading to a higher HETP for the tetrasacharides. The substitution heterogeneity was even more pronounced for larger oligosaccharides. This may also explain why SEC peaks of highly sulfated GAGs are generally broader than that of lower sulfated GAGs (such as chondroitin and keratan sulfate; not shown). Also the choice of the enzyme contributes to peak heterogeneity (see Section 3.4).

3.6. Pressure-resistance, flow rate and column coupling

A SEC matrix should ideally be porous and hydrated to allow proper separation of the solutes. The particle homogeneity and relative pore volume, however, are irreversibly reduced, if the pressure in the column exceeds a certain limit. This limit is low for aqueous SEC columns at low-pressure (1–5 bar; e.g. Bio-Gel brandmark) and high-pressure (20–30 bar) compared to common reverse phase columns (200–300 bar). Such damage, however, decreases drastically the chromatographic resolution and must be prevented by software or hardware protections.

In the present experiments (Fig. 5A; two Superdex-75 columns), a flow of 140 μL/min generated a backpressure of 23.5 bar (340 psi) that is very close to the maximum tolerated pressure (24 bar or 370 psi) of the 13-μm particles. At optimum flow of 20 μL/min for dp2, however, the back pressure over these two columns, the detector and back pressure regulator was only 8.3 bar (120 psi), so that four additional columns could be coupled in series without damaging the matrix. Accordingly, the resolution could be increased by a factor of 3, causing tripling of the separation time. The resulting 9 h separation time is still short with respect to that of classical SEC at low-pressure (see Section 3.12). Preferable to 6 columns in series would be a single column of the same length, preventing lateral sample spreading at in- and outlets of the columns which contributes to sample dilution and therefore an irreversible loss of resolution in SEC. Such long columns are not yet commercially available.

3.7. Column range and pore size

Due to the principles of SEC, only the upper limit (V_0) of the exclusion range can be influenced by variation of the matrix' pore size. Because proteomics historically preceded glycomics, the exclusion ranges of SEC matrices are generally specified for globular proteins. In contrast to proteins, however, GAGs have an exceptionally high charge at physiological pH, so that repulsion from the matrix (see also Section 3.9) may cause the exclusion limit, at equal pore size of the matrix, for GAGs to be very much smaller than that for proteins.

This is illustrated in Fig. 5B where heparin oligosaccharides were separated on columns of equal composition (Superdex brandmark, 13 μm particle size), but of different pore sizes. GAG oligosaccharides of 0.5–13 kDa were much better separated on a Superdex-75 matrix (globular protein exclusion: 75 kDa) than on a Superdex Peptide matrix (globular protein exclusion: 7 kDa) of equal chemical composition, particle size and total bed volume. At the same time, the Superdex-75 column offered the highest pressure-resistance allowing thus connection of several columns in series at various flow rates (see Section 3.6). Nevertheless, the Superdex-Peptide provided best efficiency to separate heparin oligosaccharides dp2 from dp4 (Fig. 5B), although the half-width for dp2 and the HETP was larger, decreasing thus the sensitivity. The Superdex-200 matrix (globular protein exclusion: 600 kDa), in turn, was sufficient to quantify the average size of the starting material (13 kDa) and its distribution (chromatogram not shown), but was no longer suited to separate the oligosaccharides (Fig. 5B). The difference between V_0 and V_t, and thus the relative pore volume, was largest for the Superdex-200.

3.8. Optimum pH for SEC

The high content in acid groups of polymeric GAGs requires careful consideration of buffer concentration and pH. Heparin (MW: 13.0 kDa), for example, has an average of 2 sulfate and 1 carboxylic group per disaccharide (MW: 563.4, sodium salt) corresponding to approximately 3 × 23 molar equivalents of monovalent acids. Dilution of 50 μg of heparin in 5 μL (=initial HPLC sample loop) and 49 μL (=final peak half-width of 2.44 min for I-S at 20 μL/min; Fig. 2C) results in 0.77 and 0.08 mM of heparin, respectively. This apparent small polymer concentration, however, corresponds to 53 or 5.4 mM of monovalent acid groups, respectively, so that a much higher buffer concentration (>50 mM) needs to be considered. The consequences of various pH on the separation of GAG oligosaccharides is illustrated in the following.

Present SEC matrix was chosen because of its extraordinary resistance to extreme pH between 1 and 14. Accordingly, the pH stability of the matrix was checked with repeated applications of dextran, cyanocobalamine, and acetone that changed their elution volumes only faintly (<5 min) over the entire pH range from 1 to 13 (Fig. 5C).

In contrast, heparin oligosaccharides drastically changed their elution volume as a function of the pH, depending on whether the GAG was present in its charged (i.e. pH > 1) or uncharged form (pH 1). At pH > 1, the negatively charged GAGs eluted much earlier than at pH 1 (Fig. 5C), most likely due to electrostatic repulsion between the negatively charged GAGs and the matrix [26–29] or through intermolecular repulsion of heparin [30], despite sufficient content of counter ions in the mobile phase (see Section 3.9). Interestingly, this reduction in elution time continued also at pH higher than the p*K*a of the sulfate groups (p*K*a ≈ 0.5–1.5 [31]) suggesting that polarization of the matrix attributes additional repulsive forces to negatively charged GAGs. As a consequence, measured elution volumes of GAGs (0–13 kDa) at pH > 1 differed very much from protein

markers of equal molecular weight and manufacturer suggested column separation range. For that reason, heparin oligosaccharides (0–13 kDa) were best separated on columns specified for much larger proteins (75 kDa exclusion limit). At pH 1 and lower, however, where GAGs are no longer charged, the elution times of GAGs were similar to protein markers of equal size. Concomitantly, the classical view of the oligosaccharide pattern (dp4, dp6, dp8, etc.) disappeared at pH 1, suggesting that the elution of GAGs at higher pH is not only based on molecular size, but also on charge, despite the presence of counterions (see Section 3.9).

3.9. Ionic strength for SEC

Interaction of the analytes with the matrix may occur because most hydrophilic SEC matrices comprise residual negative charges or polarizeable functional groups [26–29]. As a consequence, addition of an electrolyte in the mobile phase may partially compensate this interaction through creation of a diffuse Coulomb layer. Here, the ionic strength of the mobile phase influenced the elution time, peak symmetry and width of the charged oligosaccharides where smallest peak width and best symmetry were obtained at an ionic strength of 75 mM (Fig. 5D). The increase in elution time of GAGs did not proceed linearly with increasing ionic strength, but was only moderate for salt contents higher than 300 mM. This effect was also independent of the nature of the salt, because a combined NaCl/Tris buffer produced similar effects and elution times as displayed for the ammonium bicarbonate buffer (chromatograms not shown). However, an excessive content in ammonium bicarbonate could lead to glycosylamine formation [32] and difficulties in removing ammonium bicarbonate effectively by evaporation. In contrast, the elution time and peak width of the uncharged acetone was not affected by the ionic strength (Fig. 5D) suggesting that only the ionic interaction of the matrix is influenced by the ionic strength, but not the pore size of the matrix. This is also supported by the retardation of cationic peptides tested (MW: 1561 Da; $z = +8$) that eluted much later (3.59 h) than the uncharged, small acetone (3.38 h) even when using 0.5 M NaCl in the elution buffer. Similar retardation and peak broadening of highly cationic peptides (chromatograms not shown) were observed for 4 similar HPLC-SEC matrices tested (Aquagel-OH 30, BioSep S2000, HEMA-BIO 40, TSK gel G3000SW).

3.10. Disaccharide analysis by means of SEC

As illustrated in Fig. 5D (acetone versus dp2), the SEC elution volume depends not only on the molecular size, but also on charge effects, especially at low ionic strength of the mobile phase: when changing from high (150 mM) to low (25 mM) ionic content, the elution time of the trisulfated disaccharide I-S on a single column dropped from 41.6 to 31.1 min (Fig. 6). In contrast, the elution time for the less-charged, non-sulfated disaccharide IV-H, dropped significantly less from 45.1 to 39.4 min (Fig. 6). This different behavior can be exploited for a very useful application leading to rapid disaccharide analysis in combination with mass spectrometry.

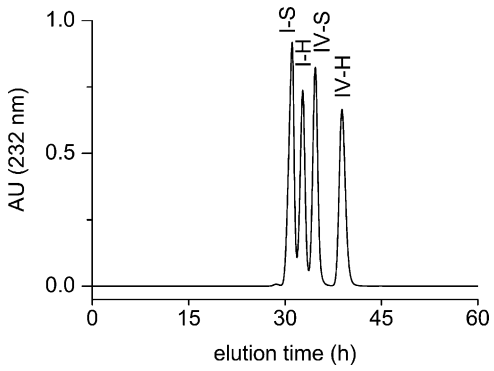

Fig. 6. SEC separation of heparin disaccharides having various number of sulfate groups. A single Superdex 75 column was used (flow of 20 μL/min) to separate a mixture of each 5 nmol of commercial heparin disaccharides heparin I-S, I-H, IV-S, IV-H containing 3, 2, 1, and 0 sulfate groups, respectively. The mobile phase contained 15 mM NH_4OH and 10 mM NH_4HCO_3 resulting in a pH of 9.0. This minimum amount of ammonium bicarbonate was required, since pure ammonium hydroxide resulted again in peak broadening. At pH 9, the peak width was smaller as compared to lower pH, especially for disaccharides without substitution at the amine.

Accordingly, heparin disaccharide standards with different sulfate content (0–3) were separated to near-baseline with a single Superdex-75 column (Fig. 6), whereas the amount of larger oligosaccharides was still separated from these disaccharides as large excluded fraction (Fig. 5D)—an essential parameter ("enzyme resistant fraction") in disaccharide analysis. This separation of heparin disaccharides of different sulfate content was achieved at low ionic strength (25 mM) meeting thus the spraying requirements for on-line coupling to mass spectrometry using heated-capillaries and/or orthogonal source designs. Together with the possibility to distinguish heparin isomers of same charge by collision-induced MS [33,34], this approach may provide a very fast and sensitive contribution to disaccharide analysis avoiding prolonged labeling steps or sample losses. The low flow rate of the optimized SEC (≈1 min half-width of each disaccharide peak using 1 column) additionally provides sufficient time for collision-induced mass spectra.

3.11. Dilution effects

In contrast to affinity chromatography, a pre-column dilution of the sample can no longer be reversed in SEC. Therefore, large injection volumes (sample loops) and lateral spreading of the sample (large column diameter) cause a loss in separation efficiency. On the other hand, too viscous samples (small sample loop) and narrow column diameters reduce the flow homogeneity and packing homogeneity, respectively, and thus, the resolution. For present 3.2 mm diameter columns, a sample loop of 5–20 μL gave the best SEC resolution of the heparin oligosaccharides. Accordingly, application of 200 μg of heparin (digested with HL-1 to 100% of reaction completion) applied with a 5, 10, 20, and 50 μL sample loop resulted in HETPs of 26.0 ± 0.5, 26.8 ± 0.4 μm, 28.0 ± 0.4 μm, and 41.0 ± 0.4 μm, respectively, for the heparin disaccharide I-S ($n = 4$). Although the smallest sample loops (5–10 μL) yielded the best resolution, injection of the same absolute amount of GAGs through smaller

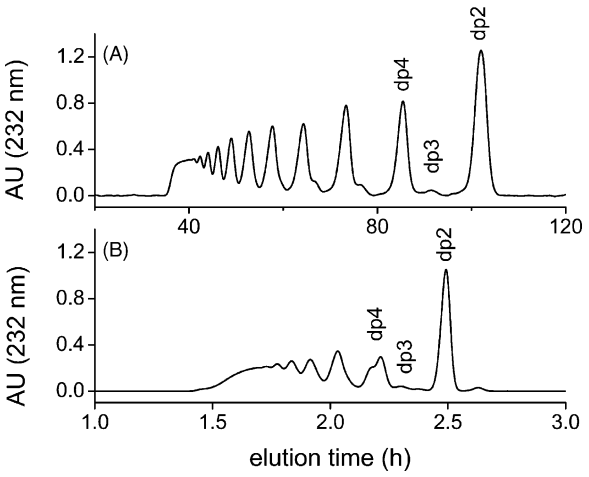

Fig. 7. Isolation of heparin trisaccharides using (A) preparative or (B) analytical SEC. (A) For the preparative SEC, a Bio-Gel P-10f matrix (protein exclusion limit of 20 kDa) of fine particles (hydrated particle size of 45–90 μm) was used. (B) For the analytical SEC, two Superdex-Peptide columns coupled in series (protein exclusion limit of 7 kDa) were used to resolve the trisaccharide fraction (dp3) within a much shorter time.

injection loops may cause problems with lower injector precision, higher sample viscosity, and higher buffer requirements. Accordingly, the same absolute amount of heparin (200 μg) in a 50 and 5 μL-loop corresponds to 212 or 21.2 mM of monovalent acid groups, respectively, with related changes in viscosity (affecting SEC resolution by diffusion) and need for an appropriate buffer capacity. The use of a detector with the double path length also resulted in peak broadening at such low flow rates, and thus not in exact doubling of the sensitivity. Accordingly, the 10 mm detector flow cell (11 μL volume) and 5 mm flow cell (4 μL volume) resulted in an HETP of 28.8 ± 0.4 μm and 26.0 ± 0.5 μm, respectively.

3.12. Comparison with optimized SEC at low pressure

In analogy to the above-described *analytical* SEC at high pressure, the SEC of heparin oligosaccharides was also optimized for a *preparative* matrix (1.5 cm × 170 cm) at low pressure. Fig. 7 illustrates considerable improvements compared to previous reports at low pressure [11,12,14,15]. Essentially, the larger protein exclusion limit of 20 kDa (Bio-Gel P-10) gave a better resolution for heparin oligosaccharides (0.5–13 kDa) than the frequently used Bio-Gel P-6 (exclusion limit: 6 kDa). Also, the very low optimum flow (3 mL/h), as determined for peak width of disaccharide I-S, was much lower than suggested from previous studies, and the generated back-pressure was also below the maximum supported pressure (1 bar). As a consequence, a finer matrix particle size (P-10f; 45–90 μm) was used to further improve the resolution through increased filling homogeneity and relative pore volume. As a result, baseline separation (>95%) was achieved for oligosaccharides up to dp10 (Fig. 7A). Further non-baseline separation was achieved up to dp22, which is excellent compared to previous reports.

In contrast to the analytical SEC (Fig. 2C), however, this preparative separation lasted 38 times longer and required 400 times more oligosaccharides to produce the same UV absorbance for the disaccharide fraction. Here, the somewhat lower resolution of the analytical column could be compensated at optimum flow by a similar bed length of 1.8 m (see Section 3.6).

3.13. Trisaccharides

In view of the literature, enzymatic digestion of heparin typically produced oligosaccharides that contained an even number of subunits (dp2, 4, 6, etc.) [13]. Present optimized preparative SEC, however, revealed distinct smaller signals from odd numbered oligosaccharides ($n = 3$, 5 and 7) in the enzymatic digests (Fig. 7A). These trisaccharides were also resolved with the high-pressure SEC, when a Superdex-Peptide matrix was used instead of the Superdex-75 (Fig. 7B). Such signals were previously not reported in preparative size-exclusion chromatography of lower chromatographic resolution [11,12,14,15].

At full enzymatic digestion, these trisaccharides made 1.7% (m/m) of the original amount of digested heparin. Analysis with mass spectrometry revealed that 38% of the trisaccharides had the backbone ΔUroA-HexN-UroA with various sulfate substitutions (spectra not shown). The uronic acid at the reducing end is a somewhat unexpected finding, because it can't originate from specific cleavage with HL-I leaving always glucosamine at the reducing end. This finding thus suggests that trisaccharides with the uronic acid at the reducing end originate from the protein linker region of heparin (reducing end) as proposed in 1975 [35,36]. According to this proposal, heparin is cleaved from its protein core by a tissue-specific endo-glucuronidase between uronic acid and the protein linker Gal-Gal-Xyl-Ser (Fig. 1).

Beside these four trisaccharides from the reducing end of heparin, further four trisaccharides were found containing no ΔUroA suggesting their origin from the non-reducing end of heparin. These molecules were di-, tri-, and tetrasulfated trisaccharides of the general form HexN-UroA-HexN [37].

Present chromatographic results thus extend a recent report where only one heparin trisaccharide from the non-reducing end of heparin was found in similar digests that were separated by reversed-phase ion-pairing chromatography [38].

4. Conclusions

The present results demonstrate that optimized SEC at high pressure offers improved separation of GAG oligosaccharides in a rapid (<3 h) and sensitive way (>0.2 μg) using UV detection and heparin lyases. The method is particularly convenient to analyze and compare GAGs of microgram amounts of biological samples. The size-distribution along their digestion can be readily followed without further purification. This might be especially valuable for the generation of oligosaccharides used in binding studies and to determine the sequence coverage in disaccharide analysis.

Optimized conditions allowed discovery of specific heparin trisaccharides that support heparin storage as a free glycan. The method is versatile, because it allows separation of either oligosaccharides at high ionic strength (>75 mM) or separation

of disaccharides with different sulfate content at low ionic content (25 mM). The latter condition is especially interesting for direct coupling to mass spectrometry where heparin isomers of equal charge can be differentiated as a rapid tool for GAG sequencing on the basis of disaccharides.

Acknowledgments

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References

[1] L.H. Lam, J.E. Silbert, R.D. Rosenberg, Biochem. Biophys. Res. Commun. 69 (1976) 570.
[2] S.A. Mousa, Cardiovasc. Drug Rev. 20 (2002) 199.
[3] U. Lindahl, M. Kusche-Gullberg, L. Kjellen, J. Biol. Chem. 273 (1998) 24979.
[4] H.J. Gabius, S. Andre, H. Kaltner, H.C. Siebert, Biochim. Biophys. Acta 1572 (2002) 165.
[5] D.R. Ferro, A. Provasoli, M. Ragazzi, B. Casu, G. Torri, V. Bossennec, B. Perly, P. Sinay, M. Petitou, J. Choay, Carbohydr. Res. 195 (1990) 157.
[6] R.R. Vives, D.A. Pye, M. Salmivirta, J.J. Hopwood, U. Lindahl, J.T. Gallagher, Biochem. J. 339 (Pt 3) (1999) 767.
[7] A. Linker, P. Hovingh, Fed. Proc. 36 (1977) 43.
[8] G. Yu, L. LeBrun, N.S. Gunay, D. Hoppensteadt, J.M. Walenga, J. Fareed, R.J. Linhardt, Thromb. Res. 100 (2000) 549.
[9] J. Henriksen, L.H. Ringborg, P. Roepstorrf, J. Mass Spectrom. 39 (2004) 1305.
[10] H. Komatsu, T. Takahata, M. Tanaka, S. Ishimitsu, S. Okada, Biol. Pharm. Bull. 16 (1993) 1189.
[11] C. Leteux, W. Chai, K. Nagai, C.G. Herbert, A.M. Lawson, T. Feizi, J. Biol. Chem. 276 (2001) 12539.
[12] G. Pavlov, S. Finet, K. Tatarenko, E. Korneeva, C. Ebel, Eur. Biophys. J. 32 (2003) 437.
[13] K.G. Rice, Y.S. Kim, A.C. Grant, Z.M. Merchant, R.J. Linhardt, Anal. Biochem. 150 (1985) 325.
[14] K. Sugahara, H. Tsuda, K. Yoshida, S. Yamada, T. de Beer, J.F. Vliegenthart, J. Biol. Chem. 270 (1995) 22914.
[15] T. Toida, R.E. Hileman, A.E. Smith, P.I. Vlahova, R.J. Linhardt, J. Biol. Chem. 271 (1996) 32040.
[16] D.L. Lohse, R.J. Linhardt, J. Biol. Chem. 267 (1992) 24347.
[17] L.A. LeBrun, R.J. Linhardt, Methods Mol. Biol. 171 (2001) 353.
[18] K.G. Rice, R.J. Linhardt, Carbohydr. Res. 190 (1989) 219.
[19] J.J. van Deemter, F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sci. 5 (1956) 271.
[20] J. Zaia, Mass Spectrom. Rev. 23 (2004) 161.
[21] A. Linker, P. Hovingh, J. Biol. Chem. 240 (1965) 3724.
[22] C.P. Dietrich, M.E. Silva, Y.M. Michelacci, J. Biol. Chem. 248 (1973) 6408.
[23] Z.M. Merchant, Y.S. Kim, K.G. Rice, R.J. Linhardt, Biochem. J. 229 (1985) 369.
[24] S. Ernst, R. Langer, C.L. Cooney, R. Sasisekharan, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 387.
[25] Y. Park, G. Yu, N.S. Gunay, R.J. Linhardt, Biochem. J. 344 (1999) 723.
[26] N.P. Golovchenko, I.A. Kataeva, V.K. Akimenko, J. Chromatogr. 591 (1992) 121.
[27] X. Guo, M. Condra, K. Kimura, G. Berth, H. Dautzenberg, P.L. Dubin, Anal. Biochem. 312 (2003) 33.
[28] B. Wittgren, A. Welinder, B. Porsch, J. Chromatogr. A 1002 (2003) 101.
[29] Y. Zhu, M. Potschka, P.L. Dubin, C.-H. Cai, Macromol. Chem. Phys. 202 (2001) 61.
[30] J. Seog, D. Dean, A.H.K. Plaas, S. Wong-Palms, A.J. Grodzinsky, C. Ortiz, Macromolecules 35 (2002) 5601.
[31] B. Casu, U. Gennaro, Carbohydr. Res. 39 (1975) 168.
[32] D. Vetter, M.A. Gallop, Bioconjug. Chem. 6 (1995) 316.
[33] J. Zaia, X.Q. Li, S.Y. Chan, C.E. Costello, J. Am. Soc. Mass Spectrom. 14 (2003) 1270.
[34] O.M. Saad, J.A. Leary, Anal. Chem. 75 (2003) 2985.
[35] L. Jansson, S. Ogren, U. Lindahl, Biochem. J. 145 (1975) 53.
[36] H.E. Conrad, Pure Appl. Chem. 65 (1993) 787.
[37] A. Ziegler, J. Zaia, J. Am. Soc. Mass Spectrom. 15 (2004) 62S.
[38] C. Thanawiroon, K.G. Rice, T. Toida, R.J. Linhardt, J. Biol. Chem. 279 (2004) 2608.