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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.