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Microscale separation of heparosan, heparan sulfate and heparin

Xue Zhao[a,b,c], **Bo Yang**[b,c], **Kathryn Linkens**[d], **Payel Datta**[e], **Akihiro Onishi**[b], **Fuming Zhang**[b,f,*], and **Robert J. Linhardt**[b,c,d,f,*]

[a]College of Food Science and Technology, Ocean University of China, Qingdao 266003, PRC

[b]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, 12180, USA

[c]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[d]Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[e]Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

[f]Departments of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

Abstract

The separation and quantification of glycosaminoglycan (GAG) chains, with different levels of sulfation, from cells, media and prepared through chemoenzymatic synthesis or metabolic engineering, poses a major challenge in glycomics analysis. A method for microscale separation and quantification of heparin, heparan sulfate and heparosan from cells is reported. This separation relies on a mini-strong anion exchange spin column eluted stepwise with different concentrations of sodium chloride. Disaccharide analysis by LC-MS was used to monitor the chemical structure of the different GAG chains that were recovered.

Keywords

heparosan; heparan sulfate; heparin; glycosaminoglycan; anion exchange chromatography; disaccharide analysis

The heparan family (uronic acid α- or β-(1→4)-*N*-acetylglucosamine-β- (1→4)) of glycosaminoglycans (GAGs) is polydisperse, structurally complex, polysaccharides. This GAG family has different levels of sulfation, ranging from no sulfo groups (*i.e.*, heparosan (HN)), to low or moderate sulfation (heparan sulfate (HS) 0–1.5 sulfo groups/disaccharide repeating unit), to high sulfation (heparin (HP) 2–3 sulfo groups/disaccharide) [1–3]. Heparin (HP) is a major anticoagulant drug that is essential for the practice of modern medicine. HS and HP GAG chains are biosynthesized within the Golgi organelle [4],

*Corresponding authors: Address: 4005, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic, Institute, Troy, New York, 12180, Fax: (518) 276-3404, linhar@rpi.edu or zhangf2@rpi.edu.

starting with the building of an intermediate, the linear HN polysaccharide backbone. HN is biosynthesized through the synthase-catalyzed alternating addition of two UDP-sugars and certain bacteria are even capable of this step [5]. HN is then enzymatically *N*-deacetylated, *N*-sulfonated, *O*-sulfonated and epimerized, at selected locations and to different extents, affording HS and HP [4]. The enzymes involved in HS/HP biosynthesis are known and many have been cloned in the past decade [2]. Currently these enzymes have been used for the chemoenzymatic synthesis of HS, HP and their derivatives [6]. Metabolic engineering of Chinese hamster ovary (CHO) and mastocytoma (MST) cells is also underway to control the fine structure of HS and HP [7]. Previously, our laboratory had successfully developed a microscale separation of different GAGs (HS, CS, HN and HA) in cell and tissue samples accompanying with ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) for total disaccharide analysis (17 disaccharides from 5 different GAGs) [8, 9]. In previous study, we also showed that HN was present in CHO-S cells, suggesting that no chain modification took place in nearly a third of the CHO-S HS-GAG chains [10]. These results suggested that a fast microscale method for the separation and quantification of GAGs with the same backbone chains and three levels of sulfation (found in HN, HS and HP) from cells, media, chemoenzymatic synthesis reactions and in metabolic engineering studies would greatly facilitate glycomics research on this critical family of glycans.

Porcine intestinal HP and HS were from Celsus Laboratories (Cincinnati, OH, USA) and HN purified from *E. coli* K5 [11] were used separately and in a mixture to optimize their recovery and separation using a Mini-strong anion ion exchange (SAX) column (Vivapure Mini Q H spin columns, Satoriou Stedim Biotech, Bohemia, NY, USA). HP, HS and HN (80 μg) were individually, and as a mixture (50 μg each), added bovine serum albumin (BSA, 50 μg) to simulate proteins present in biological samples. Each sample, dissolved in 1 mL water, was proteolyzed for 20 h at 55°C with 5-mg/mL actinase E (Kaken Biochemicals, Tokyo, Japan). The enzymatic products from each sample were filtered using an YM-10kDa MWCO centrifugal filter (Millipore, Billerica, MA, USA) and washed three-times to remove peptide products. Each GAG sample was recovered from the top layer of the filtration membrane, lyophilized and dissolved in urea-surfactant solution (8 M urea containing 2% CHAPS at pH 8.3). The GAG sample was then bound on the Mini-SAX columns, which had been pre-equilibrated with urea-surfactant solution, by centrifugation at 700 × g. After washing the columns twice with urea-surfactant solution, the HN, HS and HP were eluted by washing twice with 300-μL of 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M and 1.6 M aqueous NaCl. The recovered GAG in each wash was subjected to carbazole assay [12] to measure total GAG and disaccharide analysis to identify each GAG based on its composition [8].

HN, HS and HP in BSA were first individually recovered from solution using Mini-SAX column and GAG content was assessed by carbazole assay. HN was completely eluted by washing the column three-times with 300 μL of 0.6 M NaCl. HS and HP were completely eluted by washing the column three-times with 0.8–1.0 M and three times with 1.2–1.6 M NaCl, respectively (data not shown), thus, establishing the salt concentration required to release HN, HS, and HP from the Mini-SAX column. GAGs were next recovered from the mixture of 50 μg HP, HS, HN with BSA, using a Mini-SAX column, were next analyzed by disaccharide analysis (Figure 1). In disaccharide analysis, HN/HS/HP are converted to disaccharides by exhaustive enzymatic treatment with a mixture of recombinant *Flavobacterial* heparin lyase I, II, and III (10 mU each) for 10 h at 37°C [8]. After boiling at 100°C for 2 min, disaccharide products were recovered in the supernatant by centrifugation at 10,000 × g for 10 min and were determined by reversed-phase (RP) ion-paring (IP) high performance liquid chromatography (HPLC)-mass spectrometry (MS) [8]. Disaccharide analysis by RP-IP-HPLC-MS is useful for analyzing microgram amounts of relatively pure samples of GAGs and has a limit of detection (LOD) of 5–10 ng of GAG. Disaccharide

analysis showed that 94% of non-sulfated disaccharide $0S_{HS}$ from HN eluted from the column in 0.6 M NaCl (Figure 1B) with only 6% $0S_{HS}$ eluting in 0.8 M NaCl (Figure 1B). HS, mainly composed of monosulfated disaccharides NS_{HS} and $6S_{HS}$, 43% eluted in 0.8 M NaCl and 57% in 1.0 M NaCl (Figure 1C and D). HP, composed primarily (80%) of trisulfated disaccharide $TriS_{HS}$, began to be eluted with 1.2 M NaCl (19%) and completely eluted with 1.6 M NaCl affording 81% of the HP (Figure 1E and F). Thus, the complete separation of HN, HS and HP was achieved using a Mini-SAX column under these conditions.

Based on the optimized separation of HP, HS and HN in BSA, CHO and MST cells were similarly treated. After actinase hydrolysis of CHO-S and MST cells (1×10^7) cultured in our laboratory, the endogenous HN, HS and HP recovered from these authentic biological samples were separated on the Mini-SAX column, desalt and further digested by to disaccharides by heparinases. Because the presence of impurities (i.e., salts, peptides) and in an effort to use a minimum number of cells in this analysis, the disaccharides were further freeze-dried and labeled with AMAC by 0.1 mol/L AMAC (acetic acid/dimethyl sulfoxide 3/17 v/v) in the presence of 1 mol/L $NaBH_3CN$. The extracted ion chromatograms (EICs) of the AMAC-disaccharides obtained from the CHO-S and MST cells GAGs showed that the disaccharides separation and quantification are very such successful with high sensitivity (Fig. 2). AMAC-labeling with reverse phase separation is useful for analyzing nanogram amounts of low purity GAG samples, such as ones obtained from cultured cells, and has an LOD of 0.1 ng of GAG. The only GAG recovered from CHO-S cells in 0.6 M NaCl was heparosan (consisting solely of $0S_{HS}$), which was 30% of total GAG content in CHO-S cells (as determined by carbazole assay) (Fig. 2B). Sulfated GAG chains corresponding to HS, composed of $0S_{HS}$, $2S_{HS,}$ $6S_{HS}$ and NS_{HS} disaccharides, eluted in the 1.0 M NaCl wash (Fig. 2C) and, as expected, no HP was observed in the 1.6 M NaCl wash. These results confirmed our previous observation [10] that no sulfate modification takes place on nearly a third of the CHO cell GAG chains. In CHO cells sulfate modification occurred only at C-2, C-6 and N, resulting no more than 1 sulfo group/disaccharide residue, consistent with the structure of HS. In MST cells as shown in Fig. 2D, the GAGs recovered in 1.6 M NaCl wash was composed of 93.2% $TriS_{HS}$, 3.2% $NS6S_{HS}$, 2.4% $6S_{HS}$ and 1.2% $2S_{HS}$, consistent with HP. No GAGs were observed in washes of <1.2 M NaCl, demonstrating that modification was complete and no significant HN or HS could be isolated from cultured MST cells.

In summary, a microscale method has been developed for the isolation of HN, HS and HP from as few as 10^4 cultured CHO cells, containing as little as 0.1 ng of GAG. The effectiveness of this separation was confirmed by AMAC-labeling followed by disaccharide analysis. This approach is general and can also be used to separate chondroitin from chondroitin sulfate or hyaluronan from sulfated GAGs and offers a fast and simple isolation and purification method that should help facilitate glycomics analyses of cultured cells. This approach should also be useful in efforts aimed at the chemoenzymatic synthesis and metabolic engineering of GAGs.

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Abbreviations used

AMAC 2-aminoacridone

CHO-S	Chinese hamster ovary cells wide-type
EIC	extracted ion chromatography
GAG	glycosaminoglycan
HN	heparosan
HP	heparin
HPLC	high-performance liquid chromatography
HS	heparan sulfate
IP	ion-pairing
LOD	limit of detection
MS	mass spectrometry
MST	mastocytoma cells
SAX	strong anion exchange
UPLC	ultra-performance liquid chromatography
RP	reversed-phase

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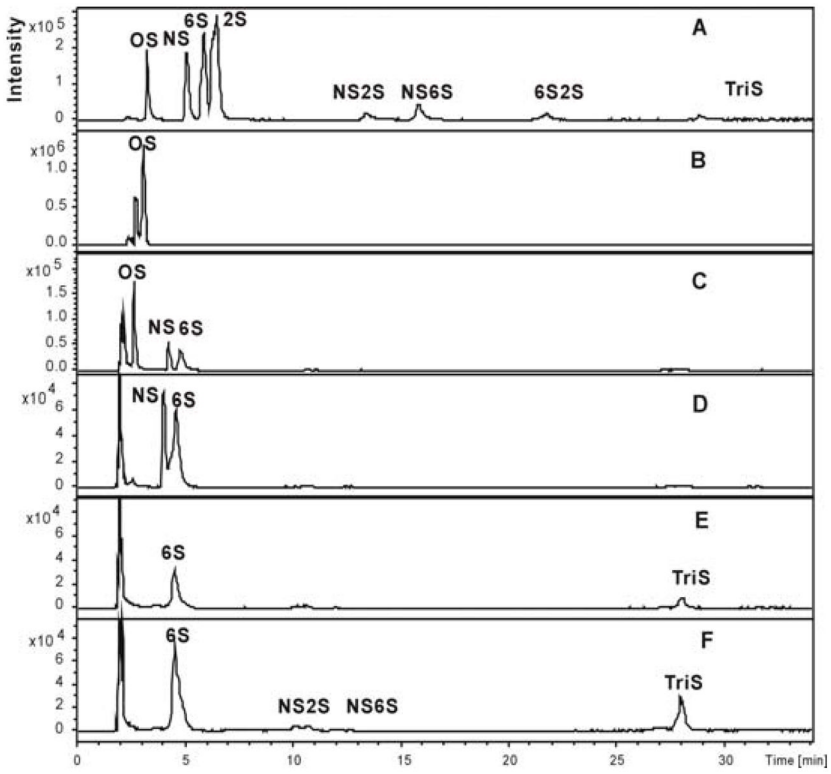

Figure 1.

GAGs recovered from a mixture of heparosan, heparan sulfate, heparin (50 μg of each) and albumin were identified by disaccharide analysis using RP-IP-HPLC-MS with EIC detection. (A) Eight standard disaccharides from HS/HP (disaccharides elute from low to high sulfation) (B) GAGs recovered in 0.6 M NaCl wash. (C) GAGs recovered in 0.8 M NaCl wash. (D) GAGs recovered in 1.0 M NaCl wash. (E) GAGs recovered in 1.2 M NaCl wash. (F) GAGs recovered in 1.6 M NaCl wash.

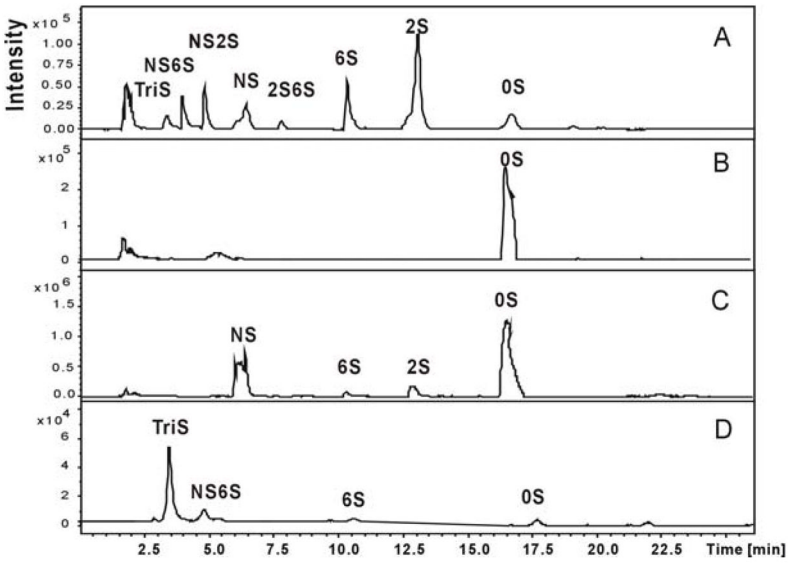

Figure 2.

GAGs, recovered from CHO-S and MST cells, were identified by disaccharide analysis, following AMAC tagging, using RP-HPLC-MS with EIC detection. Buffer A and B were 80 mM NH_4HCO_3 and methanol. (A) Eight standard AMAC-disaccharides from HS/HP (disaccharides elute from high to low sulfation) (B) GAGs recovered from CHO cells in 0.6 M NaCl wash. (C) GAGs recovered from CHO cells in 1.0 M NaCl wash. (D) GAGs recovered from MST cells in 1.6 M NaCl wash.