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Notes & Tips

Microscale separation of heparosan, heparan sulfate, and heparin

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ABSTRACT

The separation and quantification of glycosaminoglycan (GAG) chains with different levels of sulfation from cells and media, and prepared through chemoenzymatic synthesis or metabolic engineering, pose 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 various concentrations of sodium chloride. Disaccharide analysis by LC–MS was used to monitor the chemical structure of the various GAG chains that were recovered.

The heparan family (uronic acid α- or β-(1 → 4)-*N*-acetylglucosamine-β-(1 → 4)) of glycosaminoglycans (GAGs)[1] consists of polydisperse, structurally complex, polysaccharides. This GAG family has varying 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 or 3 sulfo groups/disaccharide) [1–3]. 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], 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 even certain bacteria are 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 under way to control the fine structure of HS and HP [7]. Previously, our laboratory successfully developed a microscale separation of various GAGs (HS, CS, HN, and HA) in cell and tissue samples accompanied by ultra-high-performance liquid chromatography–mass spectrometry for total disaccharide analysis (17 disaccharides from five different GAGs) [8,9]. In a 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, and 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 *Escherichia coli* K5 [11] were used separately and in a mixture to optimize their recovery and separation using a mini strong anion exchange (SAX) column (Vivapure Mini Q H spin columns; Sartorius Stedim Biotech,

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[1] *Abbreviations used:* AMAC, 2-aminoacridone; CS, chondroitin sulfate; CHO-S, Chinese hamster ovary cells wide-type; EIC, extracted-ion chromatography; GAG, glycosaminoglycan; HA, hyaluronan; 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; RP, reversed phase.

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Bohemia, NY, USA). HP, HS, and HN (80 μg) were individually, and as a mixture (50 μg each), added to 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 a YM 10-kDa 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 preequilibrated with urea-surfactant solution, by centrifugation at 700*g*. After the columns were washed twice with urea-surfactant solution, the HN, HS, and HP were eluted by washing twice with 300 μl of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.6 M aqueous NaCl. The recovered GAG in each wash was subjected to carbazole assay [12] to measure total GAG and for disaccharide analysis to identify each GAG based on its composition [8].

HN, HS, and HP in BSA were first individually recovered from solution using the 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 recovered from the mixture of 50 μg HP, HS, and HN with BSA, using a mini-SAX column, were next analyzed by disaccharide analysis (Fig. 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 nonsulfated disaccharide $0S_{HS}$ from HN eluted from the column in 0.6 M NaCl (Fig. 1B) with only 6% $0S_{HS}$ eluting in 0.8 M NaCl (Fig. 1B). From 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 (Fig. 1C and D). HP, composed primarily (80%) of trisulfated disaccharide $TriS_{HS}$, began to be eluted with 1.2 M NaCl (19%) and was completely eluted with 1.6 M NaCl affording 81% of the HP (Fig. 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, desalted, and further digested to disaccharides by heparinases. Because of 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 0.1 mol/L AMAC (acetic acid/dimethyl sulfoxide 3/17 v/v) in the

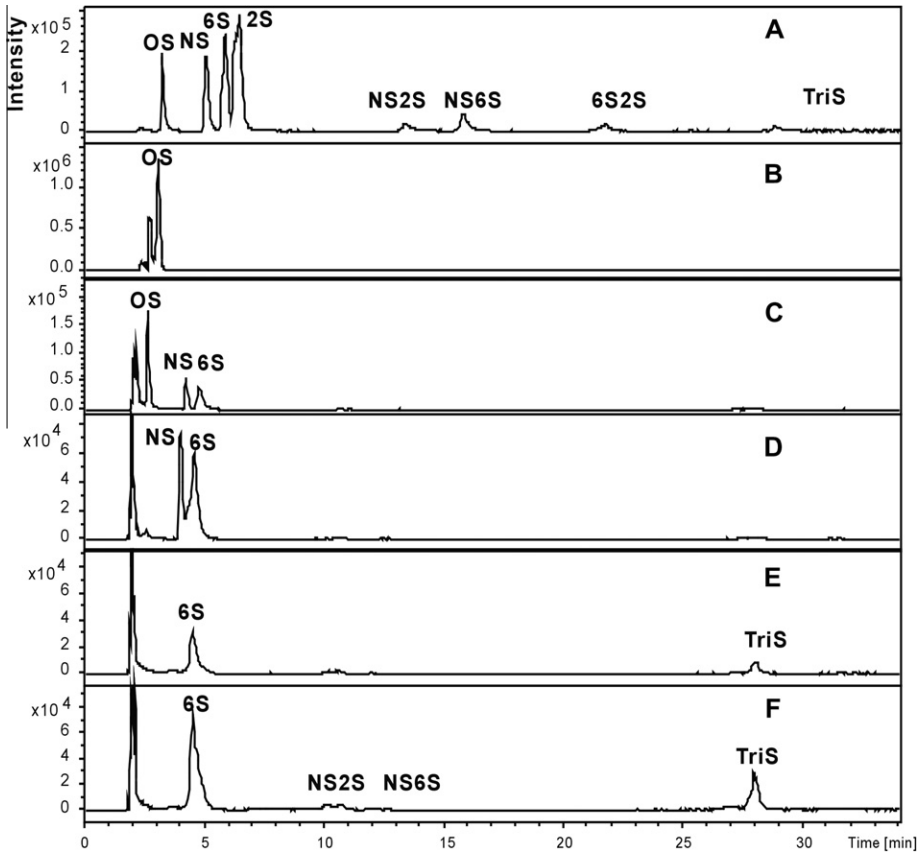

Fig.1. GAGs recovered from a mixture of heparosan, heparan sulfate, and heparin (50 μg of each) and albumin were identified by disaccharide analysis using RP–IP–HPLC–MS with EIC detection. (A) Eight standard disaccharides from HS/HP (disaccharides elute from low to high sulfation). (B) GAGs recovered 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.

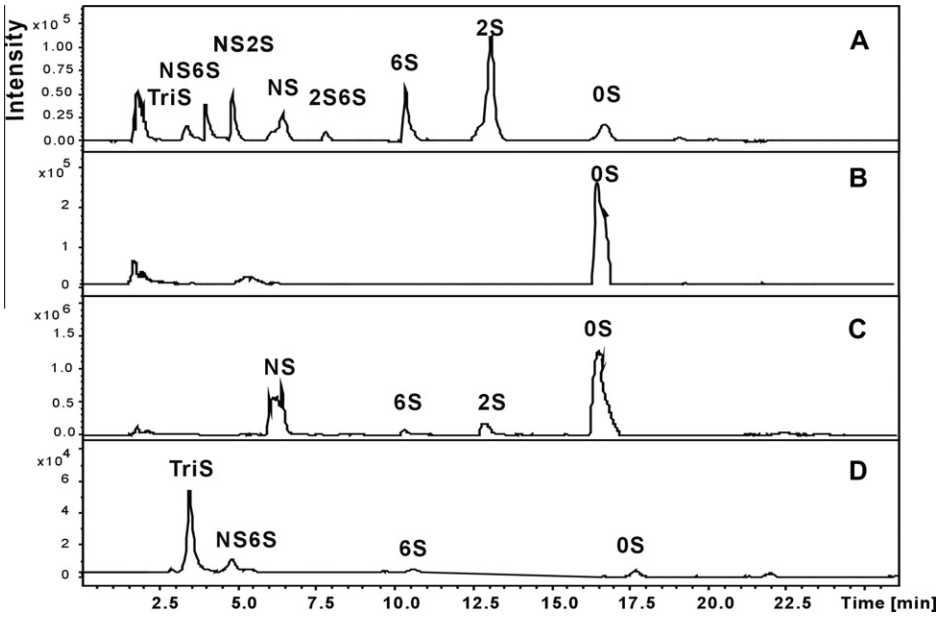

Fig.2. GAGs recovered from CHO-S and MST cells were identified by disaccharide analysis, following AMAC tagging, using RP–HPLC–MS with EIC detection. Buffers A and B were 80 mM NH_4HCO_3 and methanol. (A) Eight standard AMAC–disaccharides from HS/HP (disaccharides elute from high to low sulfation). (B) 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.

presence of 1 mol/L $NaBH_3CN$. The extracted-ion chromatograms (EICs) of the AMAC–disaccharides obtained from the CHO-S and MST cell GAGs showed that the disaccharide separation and quantification are very much successful, with high sensitivity (Fig. 2). AMAC labeling with reversed-phase separation is useful for analyzing nanogram amounts of low-purity GAG samples, such as those 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 in no more than one 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 were 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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