International Journal of Mass Spectrometry 305 (2011) 109–115

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Electrospray ionization Fourier transform mass spectrometric analysis of intact bikunin glycosaminoglycan from normal human plasma

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ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form
10 September 2010
Accepted 20 September 2010
Available online 29 September 2010

Keywords:
Glycosaminoglycan
ESI FTMS
Plasma bikunin

ABSTRACT

A mixture of glycosaminoglycan (GAG) chains from a plasma proteoglycan bikunin was fractionated using native, continuous-elution polyacrylamide gel electrophoresis, and the resulting fractions were analyzed by electrospray ionization Fourier transform mass spectrometry (ESI FTMS). Molecular mass analysis of the intact GAG afforded information about the length and composition of GAG chains in the mixture. Ambiguity in the interpretation of the intact GAG mass spectra was eliminated by conducting an additional experiment in which the GAG chains of known molecular mass were treated with a GAG-degrading enzyme, chondroitinase ABC, and the digestion products were analyzed by ESI FTMS. The plasma bikunin GAG chains consisted predominantly of odd number of saccharides, although few chains consisting of even number of saccharides were also detected. Majority of the analyzed chains were tetrasulfated or pentasulfated and comprised by 29–41 monosaccharides.

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1. Introduction

Protein glycosylation is a common post-translational modification (PTM) which enables participation of the resulting glycoproteins and proteoglycans in many biological processes such as cell differentiation, migration, and recognition, immune response, bacterial and viral infection, inflammation, tumor progression, and metastasis [1–4]. The growing understanding of biological importance of glycans has resulted in increased efforts directed toward their structure elucidation using state-of-the-art technology [5–8]. Proteoglycans (PGs) comprise a subset of species' proteome in which core proteins are modified with one or more glycosaminoglycan (GAG) chains in addition to other PTMs. GAG components of PGs are linear, sulfated polysaccharides varying in the number, structure, and sequence of their disaccharide building blocks. Based on the structure of the GAG disaccharide building blocks, PGs are divided into three major families: heparin/heparan sulfate PGs, chondroitin sulfate/dermatan sulfate PGs, and keratan sulfate PGs [9].

Bikunin GAG characterized in the present work belongs to the chondroitin sulfate (CS) family. Disaccharide building blocks of the CS GAGs are comprised by D-glucuronic acid (GlcA), which can be 2-*O* sulfated, and *N*-acetylgalactosamine (GalNAc), which can be 4-*O* sulfated and/or 6-*O* sulfated. Bikunin is encoded by AMBP_HUMAN (P02760) and is modified with a single chondroitin 4-sulfate (CS-A) GAG chain (Fig. 1) at the Ser_{10} residue of its 16 kDa protein core [10–13]. In addition to the CS-A GAG, bikunin core protein is *N*-glycosylated with a 2 kDa complex-type biantennary glycan on the Asn_{45} residue [12]. More than 98% of circulating bikunin is present as a part of the so-called multi-chain bikunin proteins [14], in which one or two heavy chain (HC) polypeptides (HC1, P19827; HC2, P19823; HC3, Q06033) are attached to the CS chain of bikunin through an ester linkage between 6-*O* position of a mid-chain GalNAc residue and α-carbon of the C-terminal Asp residue of the HC polypeptides [11,15,16]. The multi-chain bikunin proteins include a 225 kDa inter-α-inhibitor (IαI) containing HC1, HC2, and bikunin, a 125 kDa Pre-α-inhibitor (PαI) containing HC3 and bikunin, and a 125 kDa HC2·bikunin [13,15,16], the most abundant of which is IαI [17]. The concentration of free bikunin in normal plasma is low compared with that of IαI: <2.5 μg/mL and 25–700 μg/mL respectively [14]. In plasma, free bikunin is

Abbreviations: 4S, 4-*O*-sulfo; CS-A, chondroitin 4-sulfate; DEAE, diethylaminoethyl weak-anion exchanger; dp, degree of polymerization; ESI-FTMS, electrospray ionization Fourier transform mass spectrometry; GAG, glycosaminoglycan; Gal, galactopyranose; GalNAc, 2-deoxy-2-acetamidogalactopyranose; GlcA, glucopyranosyluronic acid; LR, linkage region; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NRE, non-reducing end; PAGE, polyacrylamide gel electrophoresis; PG, proteoglycan; PMF, peptide mass fingerprint; PTM, post-translational modification; RE, reducing end, includes linkage region; SAX, strong-anion exchange; Xylol, xylitol; ΔUA, 4-deoxy-α-l-threo-hex-4-enopyranosyluronic acid.

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doi:10.1016/j.ijms.2010.09.020

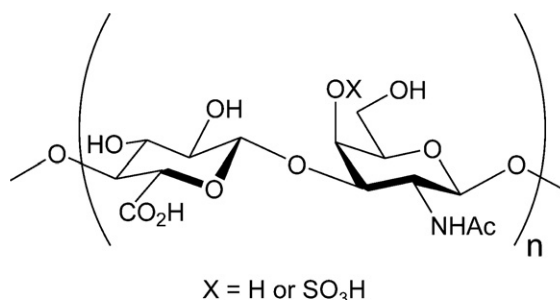


Fig. 1. The disaccharide repeating unit of bikunin GAG.

released from the multi-chain proteins as a result of increased serine protease activity in response to inflammation [14,17] and quickly passes into urine. An average half-life of free bikunin in plasma is approximately 10 min [14,18]. Elevated concentrations of bikunin in plasma and urine are associated with the acute-phase inflammatory response [14,17]; and the increased chain length and decreased sulfation of its GAG component have been observed in patients with different inflammatory syndromes [19,20]. A number of reports describe characterization of bikunin GAG by chromatography and mass spectrometric (MS) techniques such as ESI MS and MALDI MS [19,21,22], but due to their inherent limitations in resolution, these methods yield ambiguous results.

High-resolution technique, Fourier transform ion cyclotron resonance MS (FTICR-MS) has been used in our laboratory to determine the chain length and composition of intact urinary bikunin GAG [23]. The direct MS analysis of a complex polysaccharide mixture, such as bikunin GAG, was complicated by the presence of multiple charge states and Na/H heterogeneity products, which diminish ion signal of any single channel. To achieve sufficient signal-to-noise for the accurate mass measurement, a quadrupole mass filter was used to isolate ions over a narrow m/z window [23]. The urinary bikunin GAG chains were found to consist of odd number of saccharides terminating with a GalNAc residue at the non-reducing end (NRE). This raised the question whether the odd number of saccharides is a unique feature of the urinary bikunin GAG, incurred during the process of renal elimination, or is it a characteristic feature of bikunin GAG from both urine and plasma.

The goal of the present work was to measure the molecular mass of the intact plasma bikunin GAG to determine its composition: the number of saccharides comprising the chains and the degree of sulfation. The described here approach relies on a high-resolution preparative separation of the GAG mixture by polyacrylamide gel electrophoresis (PAGE) followed by ESI FTMS analysis of the intact chains. The advantage of separating a mixture prior to the FTMS analysis is that it permits MS detection of the minor components, giving a more realistic idea about the mixture composition. In addition, preparative-scale separation used in the present study made it possible to further characterize the chains of known molecular mass using an enzyme treatment followed by ESI FTMS and MS^2 analyses of the products, which facilitated the interpretation of MS data obtained for the intact GAG.

2. Materials and methods

2.1. Chemicals

Pooled normal human plasma in sodium citrate (Innovative Research) was received on dry ice and stored at −20 °C. Electrophoresis grade acrylamide, *N*,*N*′-methylene-bis-acrylamide, sucrose, glycine, ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) were from Bio-Rad (Hercules, CA). Alcian blue, sodium hydroxide, and sodium borohydride were from Fisher (Pittsburg, PA). Chondroitin sulfate lyase ABC from *Proteus vulgaris* (chondroitinase ABC, EC 4.2.2.4) was from Associates of Cape Cod (Seikagaku America, East Falmouth, MA). Actinase E (pronase E, EC 3.4.24.4) was from Kaken Pharmaceuticals (Tokyo, Japan). All solvents were HPLC grade and all other chemicals were molecular biology grade or electrophoresis grade.

2.2. Isolation of bikunin-containing proteins from human plasma

Plasma, 50 mL, was thawed at 4 °C overnight, centrifuged at 4000 × *g* for 30 min, and the resulting supernatant was diluted with 200 mL of a 15 mM Tris–HCl buffer, pH 7.4, containing 75 mM NaCl (loading buffer). Plasma in the loading buffer was added to 75 mL of weak-anion-exchange resin (DEAE Sepahrose, GE Healthcare) pre-conditioned in the loading buffer and allowed to equilibrate at room temperature for 1 h with gentle shaking. The resin was allowed to settle, the supernatant was discarded (unbound crude), 75 mL fresh loading buffer was added to the resin, the resin was packed into a 5 cm × 100 cm glass column (Bio-Rad), and the column packing flow-through was discarded. The resin was washed with 300 mL (4 CV) of loading buffer, followed by elution with 150-mL volumes of 15 mM Tris–HCl buffer, pH 7.4 containing 0.2 M, 0.3 M, 0.4 M, and 0.5 M NaCl. During the elution, 50-mL fractions were collected, their absorbance at 280 nm was measured, and each fraction was analyzed by SDS-PAGE using 4–15% gradient mini-gels (Bio-Rad). The gels were stained with Gel Code Blue solution (Bio-Rad), protein bands migrating with or above 250 kDa MW marker (IαI) were excised and subjected to the in-gel trypsinolysis followed by MALDI-TOF-MS analysis of the resulting peptides to confirm the presence of IαI (Supplementary Fig. 1). Two fractions, in which IαI was detected, were desalted against the loading buffer and reduced in volume to a total of 5 mL using Amicon Ultra 30,000 MWCO centrifugal filter (Millipore).

2.3. Preparation of bikunin peptidoglycan

Fractions containing IαI were treated with actinase E, 1 mg/mL at 45 °C overnight, and bikunin peptidoglycan (pG) was isolated from the digestion mixture using strong-anion exchange (SAX) spin column chromatography (Vivapure Q Mini H, Sartorius Stedim North America, Bohemia, NY). The anion exchange spin column was pre-equilibrated with 50 mM NaCl, the digestion mixture was brought up to 50 mM NaCl with a 4 M NaCl stock solution and loaded on the column at 500 × *g*. The bound sample was washed twice with 50 mM NaCl, eluted with 1.5 M NaCl, and desalted using an Amicon Ultra 30,000 MWCO centrifugal filter (Millipore).

2.4. Preparation of bikunin GAG

Bikunin GAG was released from pG by base-catalyzed β-elimination under reducing conditions. Under basic conditions used for the β-elimination reaction, the ester linkage between heavy-chain peptides and the 6-*O* position of GalNAc undergoes hydrolysis. A sample containing bikunin pG was incubated with equal volume of 1 M NaOH containing 1 M $NaBH_4$ at 4 °C overnight (16 h), neutralized with glacial acetic acid, and desalted against water using an Amicon Ultra 30,000 MWCO centrifugal filter (Millipore). The GAG concentration in the resulting solution was estimated by the native PAGE analysis with Alcian blue staining.

2.5. Preparative PAGE separation of bikunin GAG

Bikunin GAG was fractionated by the continuous-elution, native PAGE using a Mini Prep electrophoresis cell (Bio-Rad) [24]. A 10-cm resolving gel was cast in a 7 mm i.d. glass tube from 4 mL of 12% total acrylamide monomer solution (12% T) containing 10 μL

TEMED and 100 μL 10% APS. Stacking gel was cast from 300 μL of 5% T monomer solution, pH 6 (HCl) containing 0.3 μL TEMED and 9 μL 10% APS. The sample for electrophoresis was prepared by mixing 100 μL of the bikunin sample with 100 μL of 50% sucrose solution containing 10 μg/mL phenol red, a tracking dye. Electrophoresis was performed at 1 W constant power, and the elution buffer flow rate was 0.08 mL/min. Once the tracking dye reached the bottom of the gel column (approximately 1 h), 240-μL fractions were collected using a fraction collector (Model 2110, Bio-Rad). Fractions were analyzed using 12% T mini-gels, and those selected for MS analysis were purified using SAX spin-columns (Vivapure Q Mini M, Sartorius Stedim North America, Bohemia, NY) as described above, desalted using the 30,000 MWCO centrifugal filters (Millipore), and reduced in volume to 30 μL.

2.6. ESI FTMS analysis

Size-similar fractions of bikunin GAG were analyzed by ESI FTMS in the negative ionization mode using an LTQ XL Orbitrap mass spectrometer with a standard, factory-installed ion source (Thermo Fisher Scientific, San-Jose, CA). Mobile phase consisting of 0.1% formic acid in 50% aqueous methanol [25] was delivered by an Agilent 1200 nano-LC pump at a flow rate of 50 μL/min. The purified GAG fractions, 5–8 μL, were introduced by direct infusion through an Agilent 1200 autosampler. Mass spectra were acquired at a resolution of 30,000, and the charge deconvolution of each spectrum was performed manually using Excel spreadsheet. The mass spectrometer was tuned to obtain stable signal in the 600–900 m/z region, and the typical parameters were as follows: spray voltage 3–3.5 kV, capillary temperature 270 °C, capillary voltage −10 to −15 V, tube lens voltage −100 V, the sheath and auxiliary gas flow rates were set to 25 and 5 units respectively.

3. Results and discussion

The objective of this work was to measure molecular mass of intact bikunin GAG from human plasma to determine whether the GAG chains are comprised by odd or even number of saccharides, which may provide an insight into the GAG biotransformation. Bikunin GAG is perhaps the simplest analytical target for the intact GAG analysis, yet its characterization is far from trivial. CS chain of bikunin PG is a polydisperse mixture of polysaccharides differing in MW by 1–5%, and the isolation of a size-homogeneous sample is one of the technical challenges in its structural analysis yet to be overcome. In our earlier report, we described an approach for FTICR MS analysis of a urinary bikunin GAG mixture that employed a quadrupole mass filter to select ions over a 20 m/z window and achieve a sufficient S/N for the accurate mass measurement [23]. The approach afforded unambiguous information about the chain length and composition from 10 to 20 μg of the urinary bikunin GAG mixture. In the present work, approximately 200–400 μg of the plasma bikunin GAG mixture was first separated using preparative native PAGE with continuous elution. Fractions eluting after the ion front were analyzed by PAGE in a mini-gel format. Seven fractions, named A through G, eluting between 2 h 40 min and 3 h 50 min from the start of electrophoretic run, and representing various chain sizes, were then selected for further characterization (Fig. 2).

FT mass spectra of fractions A through G were acquired in the negative ion mode and deconvoluted manually using the expression (m/z + 1.00782) × z. The resulting set of experimental neutral masses (M_{exp}) was used to set the limits for a master list of theoretical masses (M_{theor}) corresponding to all possible GAG compositions within the observed mass range. For example, taking into account that bikunin GAG is comprised by GlcAGalNAc (residue

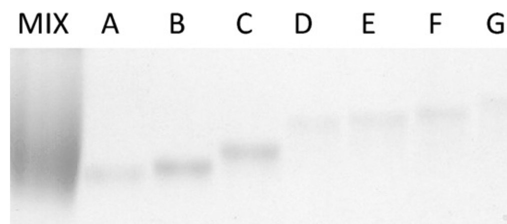
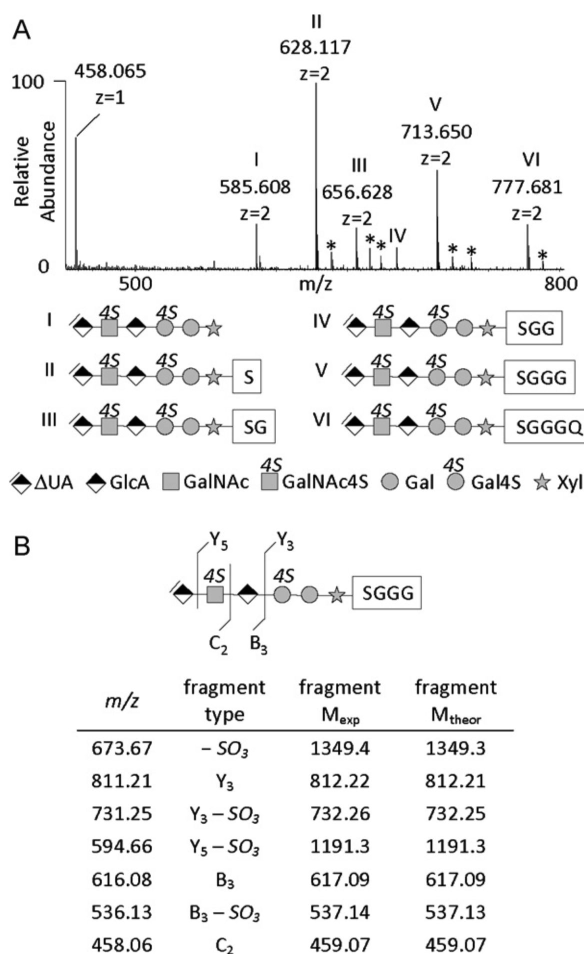


Fig. 2. PAGE analysis of the plasma bikunin GAG mixture (lane MX) and gel-eluted fractions A through G (lanes A–G). Approximately 3% of the GAG mixture isolated from 50 mL of plasma was used for analysis in lane MX. For lanes A–G, 5 μL of the samples prepared for the FTMS analysis was loaded in each well. Fractions B and C represent more abundant constituents of the GAG mixture.

mass 379.111 u) and GlcAGalNAc4S (residue mass 459.068 u) disaccharide repeating units, and its linkage region (LR) tetrasaccharide has sequence GlcAGal4SGalXylol (residue mass 714.152 u) [23,26], an experimental mass of 5725 can represent a minimum of (5725 − 714)/459 + 2 = 13 disaccharides (degree of polymerization 26, dp26) and a maximum of (5725 − 714)/379 + 2 = 15 disaccharides (dp30). The theoretical mass lists were constructed for the H-form of GAG chains (M_{H}) consisting of an odd number or an even number of saccharides. The Na/H exchange products were not considered, because using the 0.1% formic acid solution in 50% aqueous methanol as a spray solvent promotes formation of protonated GAG ions, $[M_{\mathrm{H}}-n\mathrm{H}]^{n-}$ [24,25]. Starting with the base peaks, the theoretical mass lists were searched for matches to the experimental data, but no matches were found with the expected mass accuracy ($\Delta \leq 10$ ppm). At a lower mass accuracy ($\Delta \geq 30$ ppm), experimental masses could be assigned to the even-number chains, each having 3 Na/H exchange sites (Δ 21.982 u × 3) and one dehydration site (Δ −18.011 u). To investigate the validity of this interpretation, especially the location of unsaturated residue, a volume of fraction G remained after the intact chain analysis was divided into two aliquots: one aliquot was treated with 35 mM mercuric acetate at pH 5, which selectively removes the non-reducing end (NRE) ΔUA [27,28]. A CS octasaccharide (dp8), ΔUAGalNAc4S $(\mathrm{GlcAGalNAc4S})_3$, was used as a positive control for the mercuric acetate reaction (Supplementary Fig. 2). Another aliquot was exhaustively digested with chondroitinase ABC to obtain the reducing end (RE) hexasaccharide (dp6) [26]. Products of the two reactions were analyzed by ESI FTMS. An FT mass spectrum of fraction G treated with mercuric acetate exhibited the same m/z values as the one acquired before the treatment, indicating that the NRE is not terminated with ΔUA residue. A mass spectrum of the chondroitinase ABC digest contained peaks at m/z 628.117 (M_{exp} 1258.25 u), 656.628 (M_{exp} 1315.27 u), 685.140 (M_{exp} 1372.30 u), 713.650 (M_{exp} 1429.32 u), and 777.681 (M_{exp} 1557.38 u) in addition to the expected peaks at m/z 585.608 (M_{exp} 1173.23 u, ΔUAGalNAc4SGlcAGal4SGalXylol) and 458.065 (M_{exp} 459.073 u, ΔUAGalNAc4S) (Fig. 3A). The base peak at m/z 628.117 was assigned to the LR dp6 *O*-linked to a Ser (S̲) residue of the core protein, and the remaining peaks corresponded to the LR dp6 *O*-linked to peptide stubs S̲G, S̲GG, S̲GGG, and S̲GGGQ. This interpretation is in agreement with the amino acid sequence of the GAG attachment site in bikunin core protein (Supplementary Fig. 1B) and supported by MS^2 data acquired using collision-induced dissociation (CID) of the doubly charged ion at m/z 713.650 (Fig. 3B). The peak at m/z 777.681 could be interpreted as ΔUAGalNAc4SGlcAGal4SGalXyl-*O*-S̲GGGQ (M_{theor} 1557.36 u) or ΔUAGalNAcGlcAGalNAcGlcAGal4SGalXyl-*O*-S̲ (M_{theor} 1557.39 u). The CID of the m/z 777.681 ion was unsuccessful due to the low amount of available analyte. Although the former assignment appeared to be more reasonable, considering the LR dp6 composition of four other species in the digestion mixture (Fig. 3A), the monosulfated LR dp8 could not be ruled out definitively.



m/z	fragment type	fragment M_{exp}	fragment M_{theor}
673.67	$-SO_3$	1349.4	1349.3
811.21	Y_3	812.22	812.21
731.25	Y_3-SO_3	732.26	732.25
594.66	Y_5-SO_3	1191.3	1191.3
616.08	B_3	617.09	617.09
536.13	B_3-SO_3	537.14	537.13
458.06	C_2	459.07	459.07

Fig. 3. (A) FT mass spectrum of fraction G exhaustively treated with chondroitinase ABC. Five compositions of the GAG reducing end are denoted by roman numerals. Peaks marked with an asterisk correspond to the H/Na exchange products. (B) CID data obtained during the fragmentation of the 713.65 *m/z* ion.

The presence of peptidoglycans in a sample that had been subjected to a well-established GAG release protocol, base-catalyzed β-elimination reaction, was unexpected, emphasizing the necessity for validating the MS peak assignment in this type of analyses.

Once the RE compositions were established and the possibility of an unsaturated residue at the NRE was eliminated, theoretical masses in the mass lists were recalculated to include the RE peptide stubs, and the FT mass spectra of the intact chains re-examined. The FTMS peaks with relative abundances at or above 20% were assigned with the expected accuracy ($\Delta \leq 10$ ppm). Table 1 lists the base peak assignments in the FT mass spectra of the 7 gel-eluted fractions; and a complete list of the plasma bikunin GAG compositions determined during this experiment is provided in Supplementary Table 1. GAG chains with the composition abbreviated dpX-Y-S, where X is the number of saccharides, Y is the number of sulfo groups, and S is the GAG attachment site Ser residue, are expected to dominate the spectra, because the RE dp6 attached to the Ser monopeptide is represented by the base peak in the FTMS of the RE mixture (Fig. 3A). The MS signal of chains with the composition dp(X − 2)-(Y + 1)-SGGGQ, which could not be distinguished by mass from the dpX-Y-S chains, is expected to contribute to some extent to the signal of the dpX-Y-S chains. For example, peaks assigned to the composition dp29-4-S should also contain contribution from the signal of the dp27-5-SGGGQ chains, which should be <10% of the total signal, based on the signal intensity of the 777.681 *m/z* ion corresponding to the SGGGQ-terminated LR dp6 in the RE mixture mass spectrum (Fig. 3A). Thus, the SGGGQ-terminated chains were considered a minor component and omitted from Table 1 and Supplementary Table 1.

According to the results of FTMS analyses, each of the PAGE-separated fractions contained GAGs with 4–9 different compositions: 2–3 chain lengths and 2–3 sulfation states, many of which were *O*-linked to peptide stubs, predominantly S or SGGG (Supplementary Table 1 and Figs. 3–5). Mass spectra of fractions A and G, representing the lowest-MW and the highest-MW chains examined in this study, are shown in Figs. 4 and 5 respectively. The full spectra are labeled with the abbreviated GAG compositions (dpX-Y-peptide) and charge states (z). To avoid crowding the figures, not all assigned peaks are labeled. As evident from Figs. 4 and 5, the RE heterogeneity combined with multiple charge states observed for every GAG chain in a fraction generated considerably complex mass spectra, reducing the analytical sensitivity and complicating the MS data interpretation. Simulated isotopic distributions, used for additional validation of peak assignment, were in agreement with the experimental mass spectra (panels B and C in Figs. 4 and 5). The in-source fragmentation resulting in the loss of sulfo groups is often observed during MS analysis of GAGs. To assess the extent of such fragmentation, an FT mass spectrum of a CS dp8 was acquired under the same experimental conditions used for the analysis of bikunin GAG (Supplementary Fig. 2A). Peaks corresponding to the $[M_{\mathrm{H}}-\mathrm{SO}_3-n\mathrm{H}]^{n-}$ ions contributed less than 5% of the total analyte signal. Thus, the number of sulfo groups observed during the FTMS analysis of bikunin GAG reflects the chain composition as opposed to being an artifact of the in-source fragmentation.

Majority of the assigned MS peaks corresponded to the odd-number chains, although several low-abundance peaks corresponding to chains comprised by an even number of saccharides were also detected (panel C in Figs. 4 and 5). The occurrence of large proportion of odd-number chains in plasma bikunin GAG can be rationalized by the normal metabolic processes: the CS chain biosynthesis and/or the action of one or more exolytic glucuronidases. Bikunin is primarily biosynthesized and secreted by hepatocytes [16,29,30], where it is expressed as a 42–50 kDa precursor, containing α_1-microglobulin and bikunin linked by a dibasic peptide [31]. The CS chain is assembled on the precursor core protein in the Golgi through sequential addition of the sugar nucleotides to the GAG attachment site Ser by concerted action of specialized glycosyl transferases [32–34]. The addition of UDP-GalNAc or UDP-GlcA occurs independently in an alternating way, in which the second sugar is added after the concentration of oligosaccharides terminated with the first added sugar is sufficiently high [32]. During the chain polymerization, the sulfation of GalNAc residues is carried out by a GalNAc 4-sulfotransferase [32]. The GalNAc4S and GlcAGalNAc4S NREs have been shown to prevent further addition of GlcA and GalNAc respectively by CS polymerizing enzymes serving as the chain-termination signal [32,35]. Thus, the GalNAc-terminated chains are not uncommon in CS/DS PGs [32,36], and it is possible that GalNAc or GalNAc4S at the NRE of bikunin GAG are the products of its biosynthesis, which would also explain the presence of a small number of GlcA-terminated chains.

It is also possible that GlcA-terminated chains are processed by an exolytic β-glucuronidase to yield GalNAc-terminated or GalNAc4S-terminated chains. β-Glucuronidase is present in urine and serum as well as in most tissues including liver and kidney [37,38]. In this case, a small amount of GlcA-terminated chains could be rationalized by decreased β-glucuronidase activity across the pool or in one or more individuals whose plasma was in the pooled sample used in the present study. It has been shown that β-glucuronidase activity varies greatly between healthy individuals due to the different levels of enzyme expression and environmental factors, such as dietary preferences [39,40]. The reason for the

Table 1
Chain compositions assigned to the base peaks in FT mass spectra of the plasma bikunin GAG in the gel-eluted fractions A through G.

Fraction	Abbreviated composition	M_{exp}	M_{theor}	Δ (ppm)	GAG composition
A	dp29-4-$\underline{S}$	5809.49	5809.47	3	GalNAc(GlcA GalNAc)$_{12}$(SO$_3$)$_4$GlcAGalGalXyl$\underline{\text{Ser}}$
B	dp31-5-$\underline{S}$	6268.59	6268.54	8	GalNAc(GlcA GalNAc)$_{13}$(SO$_3$)$_5$GlcAGalGalXyl$\underline{\text{Ser}}$
C	dp33-4-$\underline{S}$	6567.73	6567.69	6	GalNAc(GlcA GalNAc)$_{14}$(SO$_3$)$_4$GlcAGalGalXyl$\underline{\text{Ser}}$
D	dp39-5-$\underline{S}$	7785.04	7784.98	8	GalNAc(GlcA GalNAc)$_{17}$(SO$_3$)$_5$GlcAGalGalXyl$\underline{\text{Ser}}$
E	dp39-4-$\underline{S}$	7705.09	7705.02	9	GalNAc(GlcA GalNAc)$_{17}$(SO$_3$)$_4$GlcAGalGalXyl$\underline{\text{Ser}}$
F	dp41-5-$\underline{S}$	8164.17	8164.09	10	GalNAc(GlcA GalNAc)$_{18}$(SO$_3$)$_5$GlcAGalGalXyl$\underline{\text{Ser}}$
G	dp41-4-$\underline{S}$	8084.18	8084.14	5	GalNAc(GlcA GalNAc)$_{18}$(SO$_3$)$_4$GlcAGalGalXyl$\underline{\text{Ser}}$

absence of even-number chains in the urinary bikunin GAG could have been an artifact of the analysis: it is virtually impossible to detect a minor component in a mixture of hundred components each of which exhibits multiple charge states and various degrees of Na substitution. Finally, the odd number of saccharides in the plasma bikunin GAG and the urinary bikunin GAG could result from a combination of anabolic and catabolic processes.

The FTMS analysis of bikunin GAG isolated from human plasma demonstrates that a large proportion of this polysaccharide is comprised by chains ranging in size from dp31 to dp41 (fractions B–D, Fig. 2) with 2 sulfo groups present in the LR dp6 and the remaining 2–3 sulfo groups positioned elsewhere along the chain. The 7 gel-eluted fractions analyzed during this study contained GAG chains with at least 20 unique compositions, 4 of which were trisulfated, 8

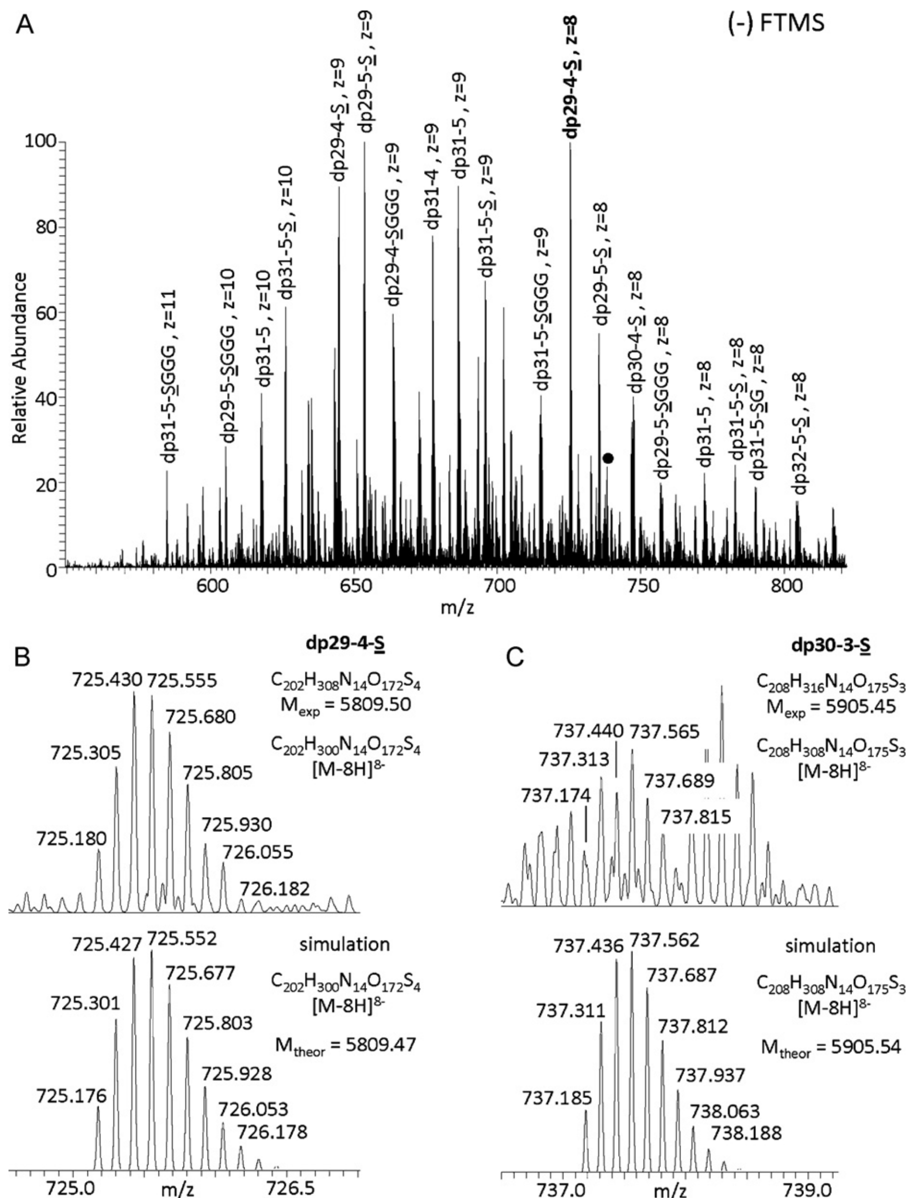


Fig. 4. (A) FT mass spectrum of fraction A, the lowest MW fraction examined in this study. Peaks are labeled with the assigned composition and charge. Experimental and simulated isotopic patterns for two peaks: (B) corresponding to an odd-numbered chain, highlighted in bold in the full spectrum; and (C) corresponding to an even-numbered chain, marked with closed circle in the full spectrum.

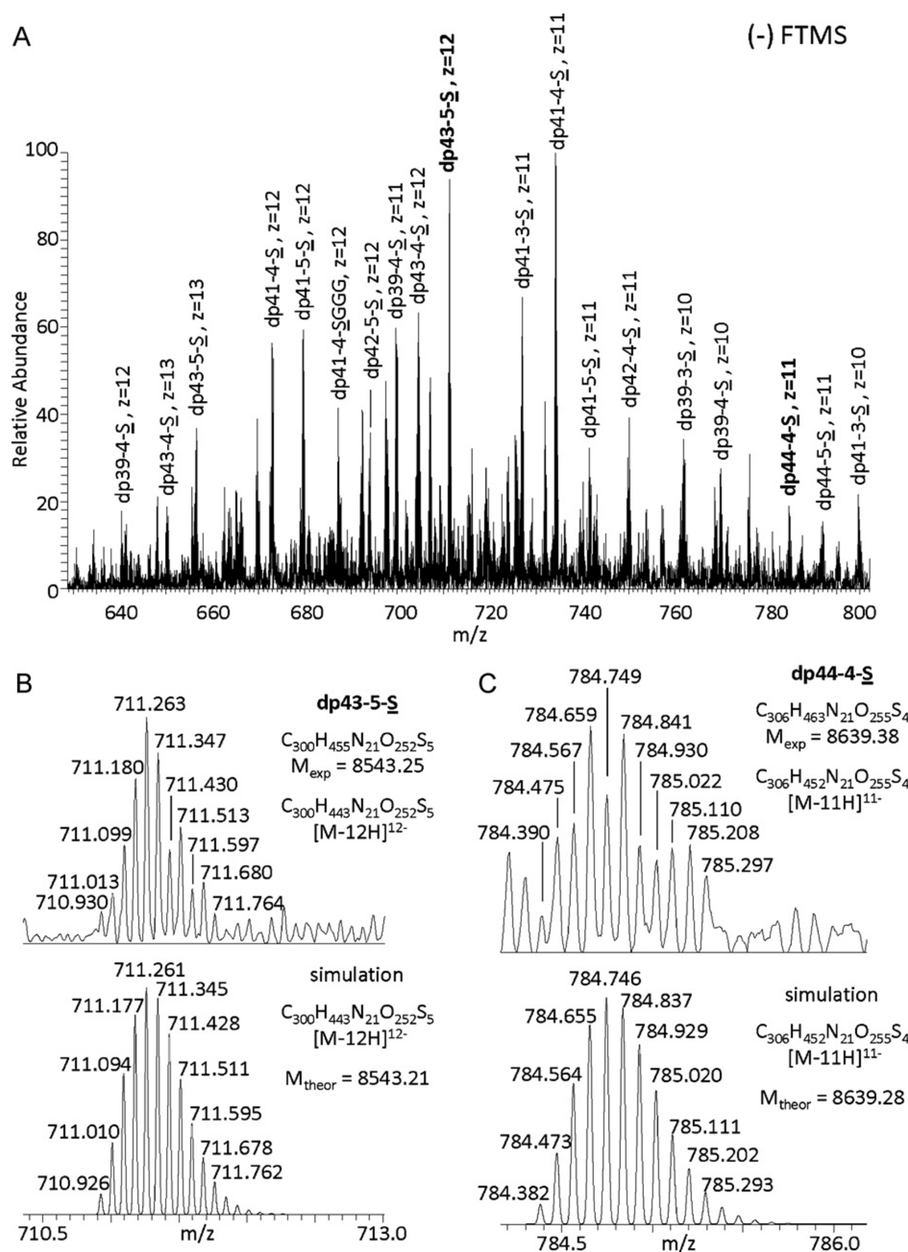


Fig. 5. (A) FT mass spectrum of fraction G, the highest MW fraction examined in this study. Peaks are labeled with the assigned composition and charge. Experimental and simulated isotopic patterns for the two peaks highlighted in bold are shown for an odd-numbered chain (B), and an even-numbered chain (C).

were tetrasulfated, 7 were pentasulfated, and 1 was hexasulfated. The urinary bikunin GAG mixture contained shorter chains (dp27 to dp35) with 6–7 sulfo groups per chain [23]. The differences in chain length and sulfation between the plasma bikunin GAG and the urinary bikunin GAG suggest that these GAGs may have different functions. The modification of the assembled CS chains with the HC polypeptides and the cleavage of bikunin-α_1-microglobulin linkage occur intracellularly, in the trans-Golgi or the secretory vesicles, shortly before the secretion [31]. It has been shown that a significant proportion of bikunin is secreted in a free form, *i.e.* lacking the HC polypeptides [18,30], and rapidly cleared through renal filtration [14,30]. Thus, it is possible that bikunin PG that escapes assembly into multi-chain bikunin proteins and the urinary bikunin PG represent the same biosynthetic product [41]. Bikunin GAG examined in the present study originated predominantly from the multi-chain bikunin proteins: IαI, PαI, and HC2·bikunin, because in plasma, the amount of bikunin in the multi-chain bikunin proteins is 20–30-fold greater than that of free bikunin [14–16] diminishing the likelihood of detecting the GAG from free bikunin. The MS results suggest that the urinary bikunin and bikunin present in the multi-chain plasma proteins, IαI, PαI, and HC2·bikunin, may represent distinct biosynthetic products differing in the composition of their CS chains. Testing this hypothesis will require a systematic study of bikunin GAG isolated from single-donor blood and urine.

In summary, the described here approach for separation, purification, and analysis of GAG components of a PG, alone or in combination with partial enzymatic depolymerization, should be applicable in the characterization of larger, more complex GAGs. Care should be taken to avoid introducing heterogeneity into the sample during its isolation and preparation for MS analysis. Future studies will be directed toward optimizing the separation conditions for isolation of size-homogeneous GAG fractions, a prerequisite for GAG sequencing.

Acknowledgement

This work was supported by NIH Grant GM38060.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.09.020.

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