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Abnormally High Content of Free Glucosamine Residues Identified in a Preparation of Commercially Available Porcine Intestinal Heparan Sulfate

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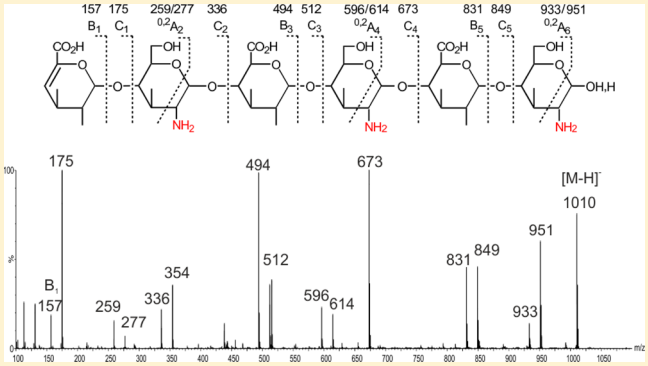
Supporting Information

ABSTRACT: Heparan sulfate (HS) polysaccharides are ubiquitous in animal tissues as components of proteoglycans, and they participate in many important biological processes. HS carbohydrate chains are complex and can contain rare structural components such as *N*-unsubstituted glucosamine (GlcN). Commercially available HS preparations have been invaluable in many types of research activities. In the course of preparing microarrays to include probes derived from HS oligosaccharides, we found an unusually high content of GlcN residue in a recently purchased batch of porcine intestinal mucosal HS. Composition and sequence analysis by mass spectrometry of the oligosaccharides obtained after heparin lyase III digestion of the polysaccharide indicated two and three GlcN in the tetrasaccharide and hexasaccharide fractions, respectively. ^{1}H NMR of the intact polysaccharide showed that this unusual batch differed strikingly from other HS preparations obtained from bovine kidney and porcine intestine. The very high content of GlcN (30%) and low content of GlcNAc (4.2%) determined by disaccharide composition analysis indicated that *N*-deacetylation and/or *N*-desulfation may have taken place. HS is widely used by the scientific community to investigate HS structures and activities. Great care has to be taken in drawing conclusions from investigations of structural features of HS and specificities of HS interaction with proteins when commercial HS is used without further analysis. Pending the availability of a validated commercial HS reference preparation, our data may be useful to members of the scientific community who have used the present preparation in their studies.



Heparan sulfate (HS) is ubiquitous in animal tissues occurring as components of proteoglycans. They participate in many important biological processes interacting with a wide range of proteins, such as cytokines and chemokines,[1] fibroblast growth factors,[2] and the coagulation regulator antithrombin, in addition to pathogenic agents involved in inflammation[3] and amyloid diseases.[4] HS polysaccharides are complex carbohydrate chains. Alternating (1-4)-linked α-*N*-acetylglucosamine (GlcNAc) and β-glucuronic acid (GlcA) comprise most of their primary sequence with heterogeneity arising from different degrees of *N*-deacetylation/*N*-sulfation, isomerization of GlcA to iduronic acid (IdoA), and variation in *O*-sulfation. The most highly sulfated regions of HS contain 3 sulfates per disaccharide unit, 6-*O*- and 2-*N*-disulfoglucosamine and 2-*O*-sulfo-IdoA (GlcNS6S-IdoA2S) whereas the least sulfated regions contain the GlcNAc-GlcA disaccharide unit. There also occur some less common structural elements, such as 3-*O*-sulfo or *N*-unsubstituted glucosamine (GlcN).

In the past, the presence of GlcN in HS chains was generally of little concern. The importance of this rare structural component was recognized only in the 1990s[5−11] and has attracted various interest.[12−15] Although this unusual residue in HS can be formed as an artifactual product introduced during preparation and purification (particularly at low pH), its occurrence in the HS chain is now thought to be formed through regulated, incomplete action of an *N*-deacetylase/*N*-sulfotransferase.[16] GlcN amounts to 0.7 to 4% of total glucosamine in HS[16] or 1 to 2 residues in each HS chain[17] depending on the source. The GlcN residue occurs largely near the polysaccharide-protein linkage region of HS chains, with less frequent peripheral location.[16] The GlcN unit has been shown to be part of HS antigens recognized by monoclonal antibodies 10E4[11,18] and JM403.[5,19] The antigen recognized by

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Table 1. ESI-MS of Oligosaccharide Fractions of Celsus HO-01214 Obtained by Heparin Lyase III Digestion and Bio-Gel P6 Fractionation

	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	Cal'd MW	Compositions	Sequence assignment
F1	416	-	-	417	ΔUA1.GlcNS1	ΔUA-GlcNS
F2a	673	-	-	674	ΔUA1.UA1.GlcN2	ΔUA-GlcN-UA-GlcN
F2b	753	-	-	754	ΔUA1.UA1.GlcN1.GlcNS1	ΔUA-GlcN-UA-GlcNS[b]
	673 (20)[a]	-	-	674	ΔUA1.UA1.GlcN2	ΔUA-GlcN-UA-GlcN
	833 (5)	-	-	834	ΔUA1.UA1.GlcNS2	ΔUA-GlcNS-UA-GlcNS
F3	715	-	-	716	ΔUA1.UA1.GlcN1.GlcNAc1	ΔUA-GlcN-UA-GlcNAc
	1090 (5)	544.6 (98)	-	1091	ΔUA1.UA2.GlcN2.GlcNS1	ΔUA-GlcN-UA-GlcN-UA-GlcNS
	1010 (45)	-	-	1011	ΔUA1.UA2.GlcN3	ΔUA-GlcN-UA-GlcN-UA-GlcN
	-	584.6 (30)	-	1171	ΔUA1.UA2.GlcN1.GlcNS2	ΔUA-GlcN-UA-GlcNS-UA-GlcNS
F4	1026 (25)	512.6	-	1027	?	?
	-	713.2 (30)	-	1428	ΔUA1.UA3.GlcN3.GlcNS1	ΔUA-GlcN-UA-GlcN-UA-GlcN-UA-GlcNS
	-	753.2 (25)	-	1508	ΔUA1.UA3.GlcN2.GlcNS2	ΔUA-GlcN- ?
F5	-	881.7 (50)	587.5 (80)	1765	ΔUA1.UA4.GlcN4.GlcNS1	ΔUA-GlcN-UA-GlcN-UA-GlcN-UA-GlcN-UA-GlcNS
	-	921.7 (30)	614.1	1845	ΔUA1.UA4.GlcN3.GlcNS2	ΔUA-GlcN- ?
	-	961.7 (20)	640.8 (80)	1925	ΔUA1.UA4.GlcN2.GlcNS3	ΔUA-GlcN- ?
	1363 (10)	681.2 (95)	-	1364	?	?

[a]Relative intensity (% relative to the base peak) is shown in parentheses; when the ion is the base peak, relative intensity (100%) was not shown. [b]Although both ΔUA-GlcNS-UA-GlcN and ΔUA-GlcN-UA-GlcNS are possible, lyase III does not favor GlcN-UA for cleavage,[12] and therefore ΔUA-GlcNS-UA-GlcN is not proposed as the main tetrasaccharide product.

10E4 antibody is closely associated with prion lesions in the brain of mice infected with scrapie.[20]

Commercially available HS polysaccharides have been invaluable in many types of research activities. HS isolated from bovine kidney is available from Sigma but is expensive. Porcine intestinal mucosa is a major source for the manufacture of heparin sodium salt used in medicine, and HS can be extracted from the mixture of glycosaminoglycans (GAGs) remaining after the extraction of heparin.[21−23] HS from this source is available from Celsus. We have in the past used Celsus HS to investigate the presence of free GlcN residue and its recognition by monoclonal antibody 10E4[12] and lyase III activity toward GlcN residues.[12]

In the present study, during the preparation of GAG microarrays[24] to include probes from oligosaccharide fractions of HS, we found an unusually high content of free GlcN residue in the batch HO-01214, recently ordered from Celsus. Although we have in the past identified GlcN in HS from Celsus, its incidence in batch HO-01214 was exceptionally high.

For preparation of oligosaccharides, HO-01214 of HS was partially depolymerized by heparin lyase III. The digestion product was fractionated by gel filtration chromatography on Bio-Gel P6 (Supplemental Figure S1) and the oligosaccharide fragments were analyzed by electrospray mass spectrometry (ESI-MS).[25]

The MS results together with the derived composition are listed in Table 1 and representative spectra in Figure 1a−d. Oligosaccharide fraction F1 contains exclusively the disaccharide ΔUA-GlcNS, but to our surprise, a very high content of free GlcN-containing components were found in the tetra- (F2) and larger oligosaccharide fractions, e.g., hexa-, octa-, and decasaccharides (F3, F4, and F5, respectively). In fraction F2a, the main component at m/z 673 (Figure 1b) is a tetrasaccharide which contains two GlcN residues. We could also readily identify up to three GlcN in a hexasaccharide and four GlcN in an octasaccharide sequence (Table 1).

Collision-induced dissociation and tandem MS (ESI-CID-MS/MS) for sequence analysis was carried out to verify the presence of the unusual consecutive GlcN residues in the linear chain.[12] As shown in the spectra, indeed tetrasaccharide (Figure 1e) and hexasaccharide (Supplemental Figure S2a) with exclusive GlcN can be confirmed.

Among the identified main ions in the di- to deca-saccharide fractions, only one component, m/z 715, present in fraction F3, contains a single *N*-acetylglucosamine residue with the sequence of ΔUA-GlcN-UA-GlcNAc (Table 1 and Supplemental Figure S2b). All the other ion species identified contain GlcN and/or GlcNS without GlcNAc. Although sequences with glucosamine fully *N*-sulfated (GlcNS) can be found in the di- and tetrasaccharide fractions (e.g., ΔUA-GlcNS-UA-GlcNS), only partially *N*-sulfated glucosamine were present in the higher oligomeric fractions (e.g., ΔUA-GlcN-UA-GlcN-UA-GlcNS).

This is in complete contrast to the data we obtained in our early study[11] using a previous batch of HS from the same source (see below), in which only a single GlcN was identified that was located within an unusual nonsulfated tetrasaccharide sequence. To ensure that in the present study the large amount of GlcN was not produced during our enzymatic depolymerization and fractionation procedure, we carried out ^{1}H NMR analysis of the intact HO-01214 polysaccharide in comparison with other HS preparations obtained from bovine kidney and porcine intestine.

^{1}H NMR spectra of HS from bovine kidney (Sigma), HS batch HO-01214 from Celsus, and samples of porcine mucosal HS 1 from our own laboratory, HS-1B and HS-1A[21] are shown in parts a, b, c, and d of Figure 2, respectively. Resonances at 4.37 ppm, consistent with H1 of either GlcNAc or GlcNS,[26] are seen in the four HS spectra (Figure 2), but signals from IdoA2S are weak in HO-01214. Signals at 3.38 ppm, attributable to H2 of GlcA, are strong in the HS samples, though less marked in HO-01214 (Figure 2b). It is not easy to distinguish between *N*-sulfo and *N*-unsubstituted glucosamine in the ^{1}H spectrum;[27] a prominent peak at 5.59 ppm in the spectrum of HO-01214 is

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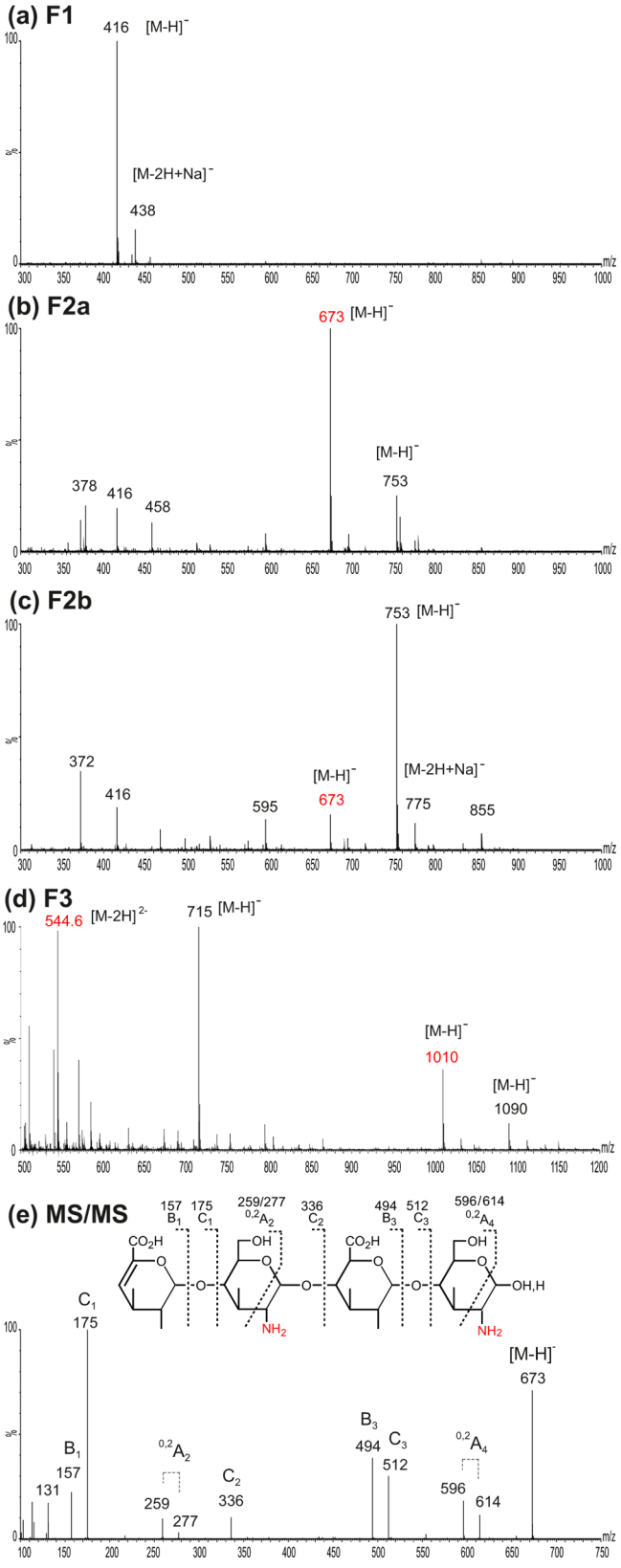

Figure 1. Negative-ion ESI-MS and CID-MS/MS spectra of selected HS-01214 oligosaccharide fractions. Mass spectra of the first four fractions (see Supplemental Figure S1) are shown: (a) Fraction 1; (b) Fraction 2a; (c) Fraction 2b; and (d) Fraction 3. Product-ion spectrum of Fraction F2a using $[M - H]^-$ at m/z 673 as the precursor is shown in part e; the observed fragmentation was consistent to the proposed structure.

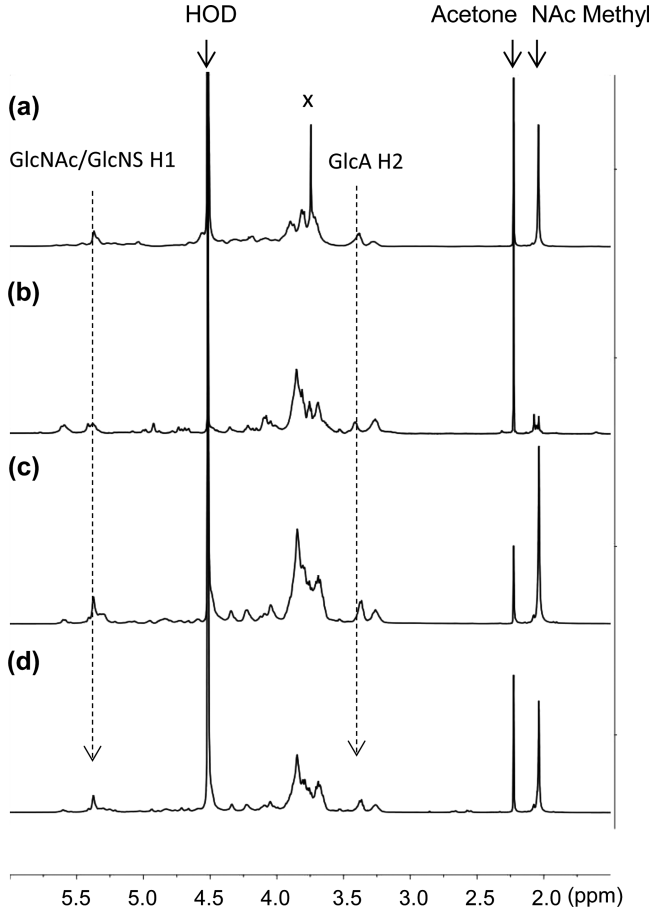

Figure 2. ^{1}H NMR spectra of different HS preparations: (a) bovine kidney HS obtained from Sigma; (b) porcine intestinal HS, HO-01214, obtained from Celsus Laboratories; (c) porcine intestinal HS-1B; and (d) porcine intestinal HS-1A. ×, Signal from an unidentified impurity in this sample.

consistent with nonacetylated GlcN or GlcNS linked to GlcA.[27] The most obvious difference between HO-01214 and the other two HS spectra lies in the methyl signal of the acetyl group at about 2.04 ppm. The intensity of this peak, compared with that of the anomeric and ring proton signals, is high for the HS spectra in Figure 2a,c,d but weak in the HO-01214 spectrum (Figure 2b). This indicates that the degree of *N*-acetylation on the amino sugar is much lower for HO-01214 than is the case for the other two porcine HS samples. ^{1}H NMR spectra for three preparations of porcine mucosal HS-1C, prepared as described by Casu et al.,[21] and HO-10697 and HO-10095, very old batches from Celsus, are also shown in Supplemental Figure S3; all of them contain the same prominent acetyl methyl signal at 2.04 ppm.

The ratios of IdoA/GlcA for the abnormal HO-01214 and two other porcine HS preparations (HS-1B and HO-10095) were calculated based on the integrated anomeric cross-peaks in their ^{13}C−^{1}H HSQC NMR spectra (Supplemental Figure S4). The ratio of IdoA/GlcA for HO-01214 at 0.7 (Supplemental Table S1) is similar to that of the two normal porcine HS preparations, HS-1B and HO-10095 (0.6 and 0.9, respectively).

Disaccharide-composition analysis[28] was next carried out to quantitatively assess the content of the free GlcN in HO-01214 in comparison with three porcine intestinal HS samples, HS-1A, HO-10095, and HO-10697, together with US Pharmacopeia heparin and chemically *N*-desulfated heparin (CNDS) as

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Table 2. Disaccharide Composition Analysis

Samples	GlcNAc[a]-containing			GlcNS[b]-containing			GlcN[c]-containing			Total free GlcN (%)
	0S	2S/6S	2S6S	NS	NS2S/NS6S	TriS	0S-(GlcN)	2S-(GlcN)/6S-(GlcN)	2S6S-(GlcN)	
HO-01214	1.7	0.2	2.3	50	16	0.4	26	3.8	0.02	30
HS-1A	29	3.6	2.4	35	19	9.0	1.0	0.2	0.4	1.6
HO-10095	19	3.1	2.1	41	18	12	3.5	0.7	0.3	4.5
HO-10697	18	3.0	2.2	42	18	10	4.6	1.0	0.2	5.8
CNDS	1.1	1.5	3.5	0.1	0.4	0.7	1.4	4.9	86	93
heparin	1.6 (±0.0)	1.2 (±0.0)	2.9 (±0.02)	1.3 (±0.0)	18 (±0.06)	74 (±0.06)	0.6 (±0.02)	-[d]	0.03 (±0.0)	0.7 (±0.02)

[a]GlcNAc-containing: 0S, ΔUA-GlcNAc; 2S, ΔUA(2S)-GlcNAc; 6S, ΔUA-GlcNAc(6S); 2S6S, ΔUA(2S)-GlcNAc(6S). [b]GlcNS-containing: NS, ΔUA-GlcNS; NS2S, ΔUA(2S)-GlcNS; NS6S, ΔUA-GlcNS(6S); TriS, ΔUA(2S)-GlcNS(6S). [c]GlcN-containing: 0S-(GlcN), ΔUA-GlcN; 2S-(GlcN), ΔUA(2S)-GlcN; 6S-(GlcN), ΔUA-GlcN(6S); 2S6S-(GlcN), ΔUA(2S)-GlcN(6S). [d]"–": not detected; standard deviations are shown in brackets and are based on triplicated measurements.

controls. The percentages of the 12 potential disaccharide constituents were obtained (Table 2) and these include four GlcNAc-containing, 4 GlcNS-containing, and 4 possible GlcN-containing disaccharides. A total of 30% of GlcN was found in HO-01214, mainly in the form of ΔUA-GlcN (26%) together with some minor (3.8%) ΔUA(2S)-GlcN(6S). This is very high compared with the other three porcine intestinal HS, HS-1A, HO-10095, and HO-10697, (1.6–5.8%) while the content of ΔUA-GlcNAc is extremely low (1.7%, Table 2) that is identical to the CNDS.

It became clear that the HO-01214 of HS received from Celsus is very different from all other HS preparations analyzed. The content of GlcNAc is much below 75% as defined by Celsus in the product information. The very high content of GlcN and low content of GlcNAc may indicate a *N*-deacetylation and/or *N*-desulfation procedure has taken place.

The unusual structure feature of HS HO-01214 identified here could not simply be a batch-to-batch variation issue. According to the manufacturer's product information, HS is a "fraction of crude heparin of porcine mucosal tissue" and "comprising primarily ΔUA-GlcNAc (~75%)". On the basis of the data obtained from MS sequence analysis of the oligosaccharide fragments, NMR of the polysaccharide, and disaccharide composition analysis described above, HO-01214 does not meet this criterion. NMR spectroscopy also shows that it is considerably different from other sources of porcine intestinal HS.[21,22,27,29]

HS prepared by Celsus has been valuable to the scientific community in analytical applications to develop methods for chromatography[30,31] and sequencing of the complex molecule,[32−35] as a standard for quantitation[36] and stability studies,[37] in biomedical application to investigate its activity to interact with oligopeptides[38,39] and a number of proteins[40−42] including growth factors,[43,44] endostatin,[45,46] and HIV gp120,[47] and as controls in the investigation of HS structural transition in embryonic stem cells.[48]

Complex polysaccharides isolated from natural sources offered for use in research laboratories are not regulated as closely as similar preparations used in medicine, such as the HS-related compound heparin.[49] Manufacturers need to implement careful control of this complex natural product and care has to be taken by users in drawing conclusions particularly from investigations of structural features of HS and specificities of HS-protein interactions when HS is from a commercial source. Pending the availability of a validated commercial HS reference preparation, it is hoped that our data will be useful for members of the scientific community who may have used the present preparation in biological and biochemical studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01662.

Full experimental procedure; figures of gel filtration chromatography of HS oligosaccharides, negative-ion ESI-CID-MS/MS spectra of hexa- and tetrasaccharide components in F3, ^{1}H NMR spectra of porcine intestinal HS, and anomeric region of ^{13}C-^{1}H HSQC NMR spectra; and table of integrated volumes of anomeric cross-peaks in the ^{13}C-^{1}H HSQC NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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