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

Barbara Mulloy,† Nian Wu,† Frederick Gyapon-Quast,‡ Lei Lin,§ Fuming Zhang,§ Matthew C. Pickering,‡ Robert J. Linhardt,§ Ten Feizi,† and Wengang Chai†‡§

†Glycosciences Laboratory and ‡Centre for Complement and Inflammation Research, Department of Medicine, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0NN, U.K.
§Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States

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 tetrascarhide and hexascarhide fractions, respectively. ¹H 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, fibroblast growth factors, and the coagulation regulator antithrombin, in addition to pathogenic agents involved in inflammation and amyloid diseases. HS polysaccharides are complex carbohydrate chains. Alternating (1→4)-linked α-N-acetylgalactosamine (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 and has attracted various interest. 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. GlcN amounts to 0.7 to 4% of total glucosamine in HS or 1 to 2 residues in each HS chain depending on the source. The GlcN residue occurs largely near the polysaccharide-protein linkage region of HS chains, with less frequent peripheral location. The GlcN unit has been shown to be part of HS antigens recognized by monoclonal antibodies 10E411, and JM403. The antigen recognized by...
10E4 antibody is closely associated with prion lesions in the brain of mice infected with scrapie.

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.

In the present study, during the preparation of GAG microarrays to include probes from oligosaccharide fractions of HS, we found an unusually high content of free GlcN residue and its recognition by monoclonal antibody 10E4 and lyase III activity toward GlcN residues.

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 was not produced during our enzymatic depolymerization and fractionation procedure, we carried out 1H NMR analysis of the intact HO-01214 polysaccharide in comparison with other HS preparations obtained from bovine kidney and porcine intestine.

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. 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-acetylgalactosamine 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-GlcNS-UA-GlcNS-UA-GlcNS).

This is in complete contrast to the data we obtained in our early study 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 1H NMR analysis of the intact HO-01214 polysaccharide in comparison with other HS preparations obtained from bovine kidney and porcine intestine.

1H 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 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, 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 1H spectrum; a prominent peak at 5.59 ppm in the spectrum of HO-01214 is

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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 \( \text{N} \)-acetylation on the amino sugar is much lower for HO-01214 than is the case for the other two porcine HS samples.1H 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 samples, HS-1B and HO-10095 (0.6 and 0.9, respectively).

Disaccharide-composition analysis28 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 \( \text{N} \)-desulfated heparin (CNDS) as

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. \( \times \), Signal from an unidentified impurity in this sample.
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 HS prepared by Celsus and HS-1A, 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, HS-10095, and HS-10697, (1.6–5.8%) while the content of ΔUA-GlcN 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.

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, 1H NMR spectra of porcine intestinal HS, and anomic region of 13C-1H HSQC NMR spectra; and table of integrated volumes of anomic cross-peaks in the 13C-1H HSQC NMR spectra (PDF)

### AUTHOR INFORMATION

*Corresponding Author*

**Phone:** +44-20 7594 2596. **E-mail:** w.chai@imperial.ac.uk.

**Notes**

The authors declare no competing financial interest.

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### REFERENCES


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**Table 2. Disaccharide Composition Analysis**

<table>
<thead>
<tr>
<th>Samples</th>
<th>GlcNAc-containing</th>
<th>GlcNS-containing</th>
<th>GlcN-containing</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0S</td>
<td>2S/6S</td>
<td>2S6S</td>
</tr>
<tr>
<td>HO-01214</td>
<td>1.7</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>HS-1A</td>
<td>29</td>
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<td>2.4</td>
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<tr>
<td>HO-10095</td>
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<td>3.1</td>
<td>2.1</td>
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<tr>
<td>HS-10697</td>
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<td>3.0</td>
<td>2.2</td>
</tr>
<tr>
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<tr>
<td>heparin</td>
<td>1.6</td>
<td>1.2</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Note: °— not detected; standard deviations are shown in brackets and are based on triplicated measurements.
(42) Zhang, F.; Moniz, H. A.; Walcott, B.; Moremen, K. W.; Linhardt, R. J.; Wang, L. Biochimie 2013, 95, 2345–2353.