CHARACTERIZING THE NON-REDUCING END STRUCTURE OF HEPARAN SULFATE

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The reducing end of heparan sulfate has been known for a long time, but information on the non-reducing end has been lacking. Recent studies indicate that the non-reducing end of heparan sulfate might be the place where FGF signaling complex forms. The non-reducing end also changes with heparanase digestion and thus might serve as a marker for tumor pathology. Using HPLC coupled mass spectrometry, we have identified and characterized the non-reducing end of bovine kidney heparan sulfate. We find that the non-reducing end region is highly sulfated and starts with a GlcA residue. The likely sequences of the non-reducing end hexasaccharides are GlcA-GlcNS-6S-UA±2S-GlcNS±6S-Ido2S-GlcNS±6S. Our data suggests that the non-reducing end of bovine kidney heparan sulfate is not trimmed by heparanase and is capable of supporting FGF signaling complex formation.

Heparan sulfate (HS)\(^1\) is a long linear polysaccharide attached to core proteins in extracellular matrix and the surface of nearly all mammalian cells. The nascent HS chain consists of disaccharide repeats of glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) (1-3). During the process of maturation, several modifications can occur on the HS chain, which include N-deacetylation and N-sulfation of the GlcNAc, epimerization of the GlcA to L-iduronic acid (IdoA), 2-O sulfation of IdoA, and 6-O and 3-O sulfation of the glucosamine (GlcN). These modifications usually focus on separate regions and result in domain structures (4-6). It is the modified domains that various extracellular proteins bind to (2,3). The region at the reducing end of HS is usually un-modified (5,7-9). One major function of HS is to interact with both fibroblast growth factor (FGF) and its cognate receptor and promote the FGF signaling complex formation (10-13). Defects in HS can cause complete losses of FGF as well as Hedgehog, Wingless signaling pathways and lead to severe abnormality in embryonic development (14,15). HS also plays important roles in cell migration and cancer cell metastasis (16).

Despite these known structural information and biological functions of HS, the non-reducing end (NRE) of HS has not been studied. Assuming that each HS chain has similar content of sulfation, the fact that the reducing end of HS is devoid of sulfation (7-9,17) suggests that the NRE of HS might be sulfated and assume biological functions. Indeed, there are indications that HS uses its NRE to direct the FGF signaling complex formation (11,13,18). Consequently, an FGF signaling complex model where two molecules each of FGF and FGFR bind to the NREs of two approaching HS chains has been proposed (13). Whether the NRE of HS has the ability to support the complex formation relies on its structure.

It is known that heparanase activity is closely related to the metastatic potential of tumor-derived cells (19). Because heparanase can trim off the original NRE of cell surface HS, this correlation might be a result of changing the NRE of cell surface HS. Heparanase is an endo-glucuronidase and its action on HS will release oligosaccharides from the NRE and expose a glucosamine residue at the newly formed NRE (20). This change on the NRE of HS may possibly be detected by mass spectrometry, since different end structures could have different molecular masses.
The identification of the NRE of HS may also allow us to establish a new strategy to sequence HS. The positions of internal residues on a HS chain can be assigned based on their distances to the NRE. Previously, HS sequencing strategies were established on purified heparin/HS oligosaccharides (21-24). One shortcoming of these strategies is that the localization of these oligosaccharides on the whole chain of HS could not be pinpointed.

Here, we described the identification and characterization of the NRE of bovine kidney HS.

MATERIALS AND METHODS

Materials - Bovine kidney heparan sulfate, heparinase, heparitinase I and II were obtained from Seikagaku America (Falmouth, MA). These enzymes were reconstituted at 0.3 mU/µl according to the manufacturer’s direction. β-D-glucuronidase was obtained from ProZyme (San Leandro, CA). HS sulfotransferases 6-OST-1 were cloned, expressed and purified with Baculovirus expression system as previously described (25). Stable isotope 34S was from Isonics Corporation (Columbia, MD). PAP34S was prepared as described previously (26).

Stable isotope labeling of heparan sulfate 34S incorporation by 6-OST-1 was done as previously described (26). The labeling buffer (2X) contained 50 mM MES (pH 7.0), 1% (W/V) triton X-100, 5 mM MgCl2, 5 mM MnCl2, 2.5 mM CaCl2, 0.075 mg/ml protamine chloride and 1.5 mg/ml BSA. To label HS with 34S, 10 µg of HS, 10 µl of labeling buffer, 1µl of 6-OST-1, 2 µl of PAP34S (3 mM) and appropriate amount of water were mixed to the total volume of 20 µl. The reaction was incubated for two hours at 37°C. The modified HS was purified on DEAE column.

Heparan sulfate lyase digestion - For analyzing HS end structures, HS samples with or without 34S labeling were digested thoroughly with heparinase, heparitinase I and II individually or in combination at 37 °C for 2 hours. For each case, 10 µg of HS sample was digested in 20 µl of a buffer containing 40 mM NH4Ac (pH 7.0), 1 mM CaCl2 and 1 mM each of the enzymes. One µg of the digestion product was then used for LC/MS analysis.

Heparan sulfate oligosaccharide β-glucuronidase digestion - Briefly, 10 µg of HS lyase digest was treated with 1 µl of β-glucuronidase (50 units) in 15 µl of digestion buffer (0.05 mM NaAc pH 5.0, 0.1 mg/ml bovine serum albumin). The reaction was incubated for overnight at 37°C.

Capillary liquid chromatography and mass spectrometry - HPLC and mass spectrometry analysis was described previously (26,27). Separations were performed on an Ultimate capillary HPLC workstation (Dionex, Sunnyvale, USA), employing dibutylamine (DBA) as an ion-pairing agent. A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% aqueous methanol (eluent B), both containing 8 mM acetic acid and 5 mM ion-pairing agent. HPLC separations were performed on a 0.3 mm × 250 mm C18 column (MS 5 µm) at rate of 5 µl per minute and a sample volume of 6.3 µl. The elution profile was 0% B for 5 minutes, 6% B for 19 minutes, 18% B for 17 minutes, 34% B for 13 minutes and 55% B for 16 minutes. After each run, the column was washed with 90% B for 15 minutes and equilibrated with 100% A for 28 minutes.

Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). Nitrogen was used as a desolvation gas as well as a nebulizer. Conditions for ESI-MS were as follows: nebulizer gas (N2) flow rate 1 l/min, nozzle temperature 140 °C, drying gas (N2) flow rate 0.6 l/min, spray tip potential 2.8 kV and nozzle potential 70 V. Negative ion spectra were acquired every 4 seconds by scanning m/z from 40 to 4000. Total ion chromatograms and mass spectra were processed with the Data Explorer software version 3.0.
RESULTS

Theoretical calculation of the monoisotopic m/z value of a terminal oligosaccharide released from the non-reducing end of an HS chain by bacterial lyases

An oligosaccharide released from internal sections of an HS chain with bacterial lyases begins with a ∆₄,₅-uronic acid (ΔUA); however, the terminal oligosaccharide released from the NRE of an HS chain should begin with a saturated residue (Fig. 1). If the NRE of an HS chain is a uronic acid (UA), the molecular mass of the terminal oligosaccharide will be 18.01 Da (addition of H₂O₁) more than its ΔUA-containing internal counterpart; if the NRE of an HS chain is a GlcN, the molecular mass of the terminal oligosaccharide will be at least 181.09 Da (addition of C₆H₁₅O₅N₁) more than the ΔUA-containing internal counterpart (Fig. 1). In negative mode mass spectrometry, the molecular formula of a ΔUA-containing oligosaccharide can be identified with the equation (26),

\[ m/z = \frac{(337.09n + 42.01p + 79.96q - z)}{z} \]  
(Eq. 1)

where:
- \( n \) is the number of disaccharides,
- \( p \) is the number of acetyl groups,
- \( q \) is the number of sulfates.

The molecular formula of a terminal oligosaccharide with a UA at its NRE can be identified with,

\[ m/z = \frac{(337.09n + 42.01p + 79.96q - z + 18.01)}{z} \]  
(Eq. 2)

The molecular formula of a terminal oligosaccharide with a GlcN at its NRE can be identified with,

\[ m/z = \frac{(337.09n + 42.01p + 79.96q - z + 181.09)}{z} \]  
(Eq. 3)

Identification of the non-reducing end of bovine kidney HS

A bovine kidney HS sample was first subject to complete digestion with HS lyase mixture and then analyzed with LC/MS (Fig. 2). Most of the m/z values in the integrated mass spectrum had solutions with Eq. 1 (Table 1) and therefore should belong to internal oligosaccharides. Only m/z 514.05 had solution with Eq. 2, suggesting that it belongs to a terminal oligosaccharide with a UA at the NRE. This terminal oligosaccharide was then designated as a dp2-2S* to distinguish it from internal disaccharides dp2-2S at m/z 496.04 (Fig. 2B). Because the NRE UA had no preceding glucosamine residue that was required for its modification (1), this UA was likely a GlcA. The following GlcN residue could not be labeled with 6-OSTs on the whole chain of HS (supplemental 1), suggesting that it might have been sulfated at its 6-O position. Also considering the rareness and positioning of 3-O sulfate and free amino group (28,29), this dp2-2S* was likely to be GlcA-GlcNS6S. No m/z value was found to have solution with Eq. 3, suggesting that no HS chain terminated on GlcN. Because the dp2-2S and dp2-2S* had similar chemical structures, the ratio of their m/z signals would quantitatively reflect their molar ratio. This ratio was found to be about 15 (Fig. 2B), suggesting each bovine kidney HS chain contains about 15 dp2-2S.

Confirmation of the non-reducing end by exoglycosidase treatment

To confirm that the dp2-2S* did begin with a GlcA residue, the above HS lysate was further digested with β-D-glucuronidase and analyzed with LC/MS. Extracted Ion current chromatogram (XIC) was obtained at m/z 514.05 and 576.00 from the total ion chromatogram (TIC) (Fig. 3). M/z 576.00 belonged to internal tri-sulfated disaccharides dp2-3S and caused two peaks in the XIC. M/z 514.05 caused a single peak at elution time of 37.6 minute. Because the dp2-3S began with ΔUA and were resistant to β-D-glucuronidase digestion, they could serve as controls. The β-D-glucuronidase treatment eliminated the peak of dp2-2S* but not the peaks of the dp2-3S (Fig. 3B), indicating that the dp2-2S* did start with a GlcA residue.

Determination of the non-reducing end structure of HS at tetrasaccharide level

Bovine kidney HS samples were first labeled with ⁷⁵S by 6-OST-1 and then digested with various lyases individually or in combination. It was found that heparinase was able to release tetrasaccharides
from the NRE. When the heparinase digest was analyzed with LC/MS, peaks at m/z 465.07, 486.08, 505.05 and 545.03 were found in the integrated mass spectrum (Fig. 4). These values had solutions with Eq.2 and corresponded to NRE terminal tetrasaccharides dp4-3S*, dp4-1Ac-3S*, dp4-4S* and dp4-5S* respectively (Table 2). Corresponding internal tetrasaccharides dp4-3S, dp4-1Ac-3S, dp4-4S and dp4-5S (m/z 456.07, 477.07, 496.05 and 536.03 respectively) were also detected.

Among these terminal tetrasaccharides, dp4-4S* accounted the majority and had 34S incorporation (Fig. 4B). To see if dp4-4S* contained a single compound, XIC at m/z 505.05 ± 0.10 was obtained. Two peaks at 63.9 and 64.3 minute were found, indicating that dp4-4S* had two structural isomers (Fig. 5A). Only the isomer of the second peak had 34S incorporation. Because the first disaccharide from the NRE of bovine kidney HS could not be labeled by 6-OST-1 (supplemental 1), the 34S labeling should occur at the 6-O position of the second disaccharide. Considering the rareness and unique positioning of free amino group and 3-O sulfate on HS (28,29), the 34S-labeled dp4-4S* isomer was likely to be GlcA-GlcNS6S-UA-GlcNS. The dp4-3S* had a single peak in its extracted ion chromatogram (data not shown) and was likely to have a single structure of GlcA-GlcNS6S-UA-GlcNS. Only trace amounts of dp4-1Ac-3S* and dp4-5S* were detected.

Because heparitinase II was known to have overlapping substrate specificity with heparinase, heparitinase II digested HS sample was also examined in parallel. Both enzymes were able to release dp2-3S, but otherwise they were different. The major NRE oligosaccharides released by heparitinase II and heparinase were dp2-2S* and dp4-4S* respectively (Fig. 6). These data suggest that the two enzymes have overlapping but distinct substrate specificities, thus are able to release different NRE oligosaccharides.

**DISCUSSION**

We have identified and characterized the NRE structures of bovine kidney HS. The first NRE disaccharide of bovine kidney HS was likely to be GlcA-GlcNS6S. The second disaccharide was variable and could be UA-GlcNS6S, IdoA2S-GlcNS or UA-GlcNS. Because heparinase that released tetrasaccharides from the NRE has a preference to cleave the glycosidic bond between GlcNS and IdoA2S-GlcNS±6S (30), the third disaccharide was likely to be IdoA2S-GlcNS±6S (Fig. 7). Chinese hamster ovary cell HS and porcine intestine HS were also examined, same end structures were found at disaccharide level (supplemental 2). Overall, limited sequence variation was observed at the NRE of bovine kidney HS, which suggests that the sequences at the NRE of bovine kidney HS are probably strictly controlled.

It would be interesting to know if this restricted sequence variation is an overall theme of the whole chain of bovine kidney HS. The sequences of sulfated domains of fibroblast HS were shown to be strictly limited (31). The reducing end regions of HS samples from various sources were shown to be unmodified (5,7-9). These together suggest that HS could have limited sequences.

Great technical progresses have been made for HS sequencing and structural determination in recent years (21,24,26,31-33), but HS whole chain sequencing has not been attempted yet. The identification of the NRE of HS may open a door to this avenue. The NRE of an HS is a logical starting point to read sequences on HS chain. The NRE oligosaccharides can be easily distinguished from various internal oligosaccharides due to their unique molecular masses. LC/MS also allows us to monitor HS oligosaccharides without radioisotope or chemical-labeling. With these advantages, it might be possible for us to sequence HS whole chain in the future. Current study represents an initial effort on sequencing HS whole chain from its NRE.

Interestingly, only GlcA residue was observed at the NRE of bovine kidney HS. A GlcN residue was expected at the NRE of an HS chain if the chain had gone through heparanase
digestion in vivo (Fig. 1C), because heparanase is an endo-glucuronidase that cleaves glucuronidic linkages (20,34). The fact that no GlcN residue was observed at the NRE of bovine kidney HS suggests that heparanase activity in bovine kidney was negligible or had different subcellular localizations. Consistently, heparanase was shown to be localized mainly within lysosome and responsible for HS turnover under normal condition (35,36). Recently, secreted heparanase protein was detected in tumor tissues and was related to metastatic potential of tumor-derived cells (19). It will be interesting to see if HS from tumor tissues or tumor-derived cells has GlcN at its NRE. In this sense, the NRE of HS could serve as a convenient marker for secreted heparanase activity and thus tumor pathology.

Based on crystal structure (11) and biochemical data (13,18), an end model for FGF signaling complex formation at the NREs of two approaching HS chains had been proposed previously (13). Our current data prove that a hexasaccharide at the NRE of bovine kidney HS contains five to seven sulfates. Among them, three are N-sulfates, one or two are 2-O sulfates, and at least one is 6-O sulfate. These sulfates are pivotal and maybe sufficient (11,13,37) for the FGF signaling complex formation; therefore, our structural information on the NRE of HS provides strong evidence to support the end model of FGF signaling complex (13). The fact that bovine kidney HS contains a highly sulfated NRE and a non-sulfated reducing end (9) also suggests a sulfation gradient on HS chain from the reducing end to the NRE. This gradient may facilitate the movement of binding protein towards the NRE on HS and explains the mechanism of FGF signaling complex formation (13). In this way, HS can be considered as a regulator of molecular encounter for FGF and its receptor (38).

One concern about the current study is that if we have covered all possible NRE structures. If some NRE structures were resistant to lyase digestion and could not be released as short oligosaccharides, they could have been missed in our detection. Our current mass spectrometry coupled HPLC can not separate large and highly sulfated oligosaccharides, such as, dp6-8S to dp6-12S, dp8-8S to dp8-16S, dp10-7S to dp10-20S, et al. Since these large and highly sulfated oligosaccharides are either unlikely to occur on HS or unlikely to be resistant to lyase digestion, we think that we have covered most if not all possible NRE structures.

REFERENCES
1. Esko, J. D., and Selleck, S. B. (2002) Annu Rev Biochem 71, 435-471
2. Gallagher, J. T. (2001) J Clin Invest 108, 357-361
3. Lindahl, U., Kutsche-Gullberg, M., and Kjellen, L. (1998) J Biol Chem 273, 24979-24982
4. Turnbull, J. E., and Gallagher, J. T. (1991) Biochem J 273 (Pt 3), 553-559
5. Lyon, M., Deakin, J. A., and Gallagher, J. T. (1994) J Biol Chem 269, 11208-11215
6. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) J Biol Chem 271, 17804-17810
7. Turnbull, J. E., and Gallagher, J. T. (1991) Biochem J 277 (Pt 2), 297-303
8. Knecht, J., Cifonelli, J. A., and Dorfman, A. (1967) J Biol Chem 242, 4652-4661
9. Parthasarathy, N., and Spiro, R. G. (1984) J Biol Chem 259, 12749-12755
10. Rapraeger, A. C., Krufta, A., and Olwin, B. B. (1991) Science 252, 1705-1708
11. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkovka, A. V., Yeh, B. K., Yayan, A., Linhardt, R. J., and Mohammadi, M. (2000) Mol Cell 6, 743-750.
12. Yayan, A., Klagsbrun, M, Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841-848
13. Wu, Z. L., Zhang, L., Yabe, T., Kuberan, B., Beeler, D. L., Love, A., and Rosenberg, R. D. (2003) J Biol Chem 278, 17121-17129
14. Perrimon, N., and Hacker, U. (2004) Development 131, 2509-2511; author reply 2511-2503
15. Lin, X. (2004) Development 131, 6009-6021
16. Sanderson, R. D., Yang, Y., Suva, L. J., and Kelly, T. (2004) Matrix Biol 23, 341-352
17. Lyon, M., Steward, W. P., Hampson, I. N., and Gallagher, J. T. (1987) Biochem J 242, 493-498
18. Zhang, Z., Coomans, C., and David, G. (2001) J Biol Chem 276, 41921-41929
19. Vlodavsky, I., and Friedmann, Y. (2001) *J Clin Invest* **108**, 341-347
20. Pikas, D. S., Li, J. P., Vlodavsky, I., and Lindahl, U. (1998) *J Biol Chem* **273**, 18770-18777
21. Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R. (1999) *Science* **286**, 537-542
22. Turnbull, J. E. (2001) *Methods Mol Biol* **171**, 129-139
23. Stringer, S. E., Kandola, B. S., Pye, D. A., and Gallagher, J. T. (2003) *Glycobiology* **13**, 97-107
24. Liu, J., Shriver, Z., Pope, R. M., Thorp, S. C., Duncan, M. B., Copeland, R. J., Raska, C. S., Yoshida, K., Eisenberg, R. J., Cohen, G., Linhardt, R. J., and Sasisekharan, R. (2002) *J Biol Chem* **277**, 33456-33467
25. Zhang, L., Beeler, D. L., Lawrence, R., Lech, M., Liu, J., Davis, J. C., Shriver, Z., Sasisekharan, R., and Rosenberg, R. D. (2001) *Arch Biochem Biol* **385**, 451-459
26. Westling, C., and Lindahl, U. (2002) *J Biol Chem* **277**, 49247-49255
27. Linker, A., and Hovingh, P. (1984) *Carbohydr Res* **127**, 75-94
28. Merry, C. L., Lyon, M., Deakin, G. A., Hopwood, J. J., and Gallagher, J. T. (1999) *J Biol Chem* **274**, 18455-18462
29. Bame, K. J. (2001) *Glycobiology* **11**, 91R-98R
30. Zetser, A., Levy-Adam, F., Kaplan, V., Gingis-Velitski, S., Bashenko, Y., Schubert, S., Flugelman, M. Y., Vlodavsky, I., and Ilan, N. (2004) *J Cell Sci* **117**, 2249-2258
31. Goldshmidth, O., Nadav, L., Aingorn, H., Irit, C., Feinstein, N., Ilan, N., Zamir, E., Geiger, B., Vlodavsky, I., and Katz, B. Z. (2002) *Exp Cell Res* **281**, 50-62
32. Kreuger, J., Jemth, P., Sanders-Lindberg, E., Eliahu, L., Ron, D., Basilico, C., Salviorla, M., and Lindahl, U. (2005) *Biochem J* **278**, 465-472.

**FOOTNOTES**

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\* The abbreviations used are: dp\(X\), oligosaccharides containing \(X\) number of sugar residues, e.g. dp2 = disaccharides; dp\(X\)-pAc-\(q\)S, dp\(X\) contained \(p\) number of acetyl groups and \(q\) number of sulfates; FGF, fibroblast growth factor; GlcA, glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; GlcNS, N-sulfate-D-glucosamine; GlcN, glucosamine with unspecified N-substituent; HS, heparan sulfate; IdoA, iduronic acid; LC/MS, liquid chromatography coupled mass spectrometry; NRE, non-reducing end, 6-OST-1, heparan sulfate 6-O sulfotransferase-1; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; UA, unspecified saturated uronic acid; ∆UA, ∆\(^4\)-uronic acid.
Table 1. The oligosaccharides in Figure 2
The monoisotopic m/z values were related to the numbers of charges (z), disaccharides (n), acetyl groups (p) and sulfates (q) with either Eq.1 or Eq.2 or Eq.3. The identified NRE terminal oligosaccharide was in bold and its formula was distinguished with an asterisk (*).

| m/z   | Elution time (minutes) | z  | n  | p  | q  | Formula   |
|-------|------------------------|----|----|----|----|-----------|
| 378.13 | 3.0                    | 1  | 1  | 1  | 0  | dp2-1Ac   |
| 458.10 | 16.6                   | 1  | 1  | 1  | 1  | dp2-1Ac-1S|
| 416.08 | 20.8, 25.6             | 1  | 1  | 0  | 1  | dp2-1S    |
| 496.04 | 38.3, 39.7, 41.5        | 1  | 1  | 0  | 2  | dp2-2S    |
| 514.05 | 37.6                   | 1  | 1  | 0  | 2  | dp2-2S*   |
| 576.00 | 52.3, 53.3             | 1  | 1  | 0  | 3  | dp2-3S    |
| 705.16a| 53.3                   | 1  | 1  | 0  | 3  | dp2-3S:1DBA|
| 416.08 | 37.2                   | 2  | 2  | 0  | 2  | dp4-2S    |
| 456.06 | 56.6                   | 2  | 2  | 0  | 3  | dp4-3S    |
| 496.04 | 64.2                   | 2  | 2  | 0  | 4  | dp4-4S    |
| 536.03 | 66.2, 66.8             | 2  | 2  | 0  | 5  | dp4-5S    |
| 437.09 | 52.6                   | 2  | 2  | 1  | 2  | dp4-1Ac-2S|
| 477.07 | 58.9                   | 2  | 2  | 1  | 3  | dp4-1Ac-3S|
| 517.05 | 64.8                   | 2  | 2  | 1  | 4  | dp4-1Ac-4S|
| 645.63 | 61.7                   | 2  | 3  | 1  | 3  | dp6-1Ac-2S|
| 685.61 | 65.4                   | 2  | 3  | 1  | 4  | dp6-1Ac-3S|

Note: a DBA (monoisotopic mass 129.15 Da) adduct of dp2-3S.
Table 2. The oligosaccharides in Figure 4
The monoisotopic $m/z$ values were related to the numbers of charges ($z$), disaccharides ($n$), acetyl groups ($p$) and of sulfates ($q$) with either Eq.1 or Eq.2 or Eq.3. The identified NRE terminal oligosaccharides were in bold and their formula were distinguished with asterisks (*).

| $m/z$ | Elution time (minutes) | $z$ | $n$ | $p$ | $q$ | Formula          |
|-------|------------------------|-----|-----|-----|-----|------------------|
| 416.03| 58.4                   | 2   | 2   | 0   | 2   | dp4-2S           |
| 456.06| 58.0, 64.4             | 2   | 2   | 0   | 3   | dp4-3S           |
| **465.07** | 57.5                  | 2   | 2   | 0   | 3   | **dp4-3S***     |
| 477.07| 60.0                   | 2   | 2   | 1   | 3   | dp4-1Ac-3S       |
| **486.08** | 58.3                  | 2   | 2   | 1   | 3   | **dp4-1Ac-3S*** |
| 496.04| 64.4                   | 2   | 2   | 0   | 4   | dp4-4S           |
| **505.05** | 63.9, 64.3             | 2   | 2   | 0   | 4   | **dp4-4S***     |
| 536.03| 66.2, 66.8             | 2   | 2   | 0   | 5   | dp4-5S           |
| **545.03** | 66.2                  | 2   | 2   | 0   | 5   | **dp4-5S***     |
| 576.00| 53.9                   | 1   | 1   | 0   | 3   | dp2-3S           |
| 685.61| 65.4                   | 2   | 3   | 1   | 4   | dp6-1Ac-3S       |
| 705.16$^a$ | 53.9                  | 1   | 1   | 0   | 3   | dp2-3S:1DBA      |

Note: $^a$ DBA adduct of dp2-3S. It had $^{34}$S incorporation and only the isotopic peak $m/z$ 707.16 was labeled in the figure.
FIGURE LEGENDS

Fig. 1. **Structures of HS oligosaccharides released by HS lyases.** *A,* Internal oligosaccharides. *B,* Possible NRE terminal oligosaccharides starting with a GlcA residue. *C,* Possible NRE terminal oligosaccharides starting with a glucosamine residue. R, sugar residues; R1, -COCH₃, -SO₃H or –H; R2, –OH or -SO₃H. Epimerization of uronic acid is not depicted here for simplicity.

Fig. 2. **Identifying the NRE terminal disaccharide of bovine kidney HS.** *A,* Total ion current chromatogram of a bovine kidney HS digest separated on a reverse phase C-18 column. Bovine kidney HS was first digested with heparinase, heparitinase I and II mixture and then analyzed with LC/MS. The NRE terminal disaccharide of dp2-2S* caused a peak at elution time 37.6 minute. *B,* Integrated mass spectrum of the entire separation. Each m/z peak could be assigned to certain oligosaccharides (Table 1). Monoisotopic peak at m/z 514.05 was assigned to a NRE terminal disaccharide of dp2-2S*. Monoisotopic peak at m/z 496.04 was assigned to internal disaccharides of dp2-2S. The isotopic cluster of dp4-1Ac-4S (at m/z 517.05) happened to be in close proximity with that of dp2-2S*.

Fig. 3. **Confirming the NRE terminal residue with β-D-glucuronidase.** *A,* Extracted ion current chromatogram at m/z 514.05±0.20 and 576.00 ± 0.20 from the total ion current chromatogram of Fig. 2A. Peak at 37.6 minute was caused by the dp2-2S* at m/z 514.05. Peaks at 52.3 and 53.3 minute were caused by two dp2-3S disaccharides at m/z 576.00. *B,* Ion current chromatogram extracted at m/z 514.05 ± 0.20 and 576.00 ± 0.20 after the HS lysate was further treated with β-D-glucuronidase.

Fig. 4. **Identifying the NRE terminal tetrasaccharides of bovine kidney HS.** *A,* Total ion current chromatogram of a heparinase digested ³⁴S/6-OST-1 labeled bovine kidney HS sample. *B,* The integrated mass spectrum of the entire chromatogram. Monoisotopic m/z 465.07, 486.08, 505.05 and 545.03 corresponded to dp4-3S*, dp4-1Ac-3S*, dp4-4S* and dp4-5S* respectively (Table 2). dp4-4S* accounted more than 80% of the total end structures and showed significant ³⁴S incorporation. The isotopic cluster of dp4-4S* resembled to that of dp4-4S (m/z 496.05) (lower panel), indicating that they have similar molecular composition.

Fig. 5. **The major end structure dp4-4S* contained two isomers.** *A,* Ion current chromatogram extracted at m/z 505.05 ± 0.10 from the total ion current chromatogram of Fig. 4 and the corresponding mass spectra. Two overlapping peaks at 63.9 and 64.3 minute were observed. The second peak exhibited ³⁴S incorporation. *B,* Simulated m/z cluster of dp4-4S* according to natural isotope abundance.

Fig. 6. **Heparitinase II and heparinase released different NRE structures.** These two enzymes both released trisulfated disaccharide but were very different in the composition of the oligosaccharides that they released, reflecting similar but distinct substrate specificities. *A,* Total ion current chromatogram of heparitinase II digested ³⁴S/6-OST-1 labeled bovine kidney HS sample and corresponding integrated mass spectrum. The major NRE oligosaccharide was dp2-2S*. *B,* Total ion current chromatogram of heparinase digested ³⁴S/6-OST-1 labeled bovine kidney HS sample and corresponding integrated mass spectrum. The major NRE oligosaccharides were dp4-4S*.
Fig. 7. **Major possible end structures of bovine kidney HS.** The first disaccharide was conserved. The second disaccharide was variable. The third disaccharide was proposed based on the substrate specificity of heparinase, which cleaved the linkage between the second and third disaccharide. The size of the reducing end NA domain was estimated based on previous study on HS prepared from bovine kidney glomerular basement membrane (9).
Figure 1
Figure 2

(A) TIC

(B) Integrated mass spectrum

Retention Time (Minute)

Mass (m/z)

Relative Intensity

Mass (m/z)
Figure 3
Figure 4
Figure 5

Retention Time (Min)

XIC 505.05±0.10

Mass (m/z)

ISO:C24H40O33N2S4 - (H)2
Figure 6
Figure 7
Supplemental 1. The non-reducing end disaccharide could not be labeled by 6-OSTs. The HS sample was first labeled with 34S by 6-OST-1, then digested with heparin lyase mixture and the lysate was analyzed with LC/MS. Peak b, c, d, e, f and g in the TIC contained stable isotope incorporation, but the non-reducing end disaccharide (peak a) could not be labeled, as shown with the corresponding mass spectra. Experiment with other 6-OSTs showed the same results.
Supplemental 2A. Bovine kidney HS from Sigma-Aldrich had only dp2-2S* at its non-reducing end. The dp2-1S* in the mass spectrum must be caused by a sulfate loss from the dp2-2S*, because they had same elution time. HS sample was digested with heparin lyase mixture.
Supplemental 2B. Porcine intestine HS had only dp2-2S* at its non-reducing end. dp2-2S* with m/z 514.06 was the only found non-reducing end disaccharide. HS sample was digested with heparin lyase mixture.
Supplemental 2C. CHO cell HS had dp2-2S* at its non-reducing end. dp2-2S* with m/z 514.07 was the only found non-reducing end disaccharide. HS sample was digested with heparin lyase mixture.
Supplemental 2D. Heparin had dp2-3S* at its non-reducing end. Because heparin is closely related to heparan sulfate, its NRE disaccharide was analyzed. dp2-3S* with m/z 594.05 was found to be the major non-reducing end disaccharide. The dp2-2S* in the mass spectrum was caused by a sulfate loss from the dp2-3S*, because they had same elution time.
Characterizing the non-reducing end structure of heparan sulfate
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