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Characterization of a Heparan Sulfate Octasaccharide That Binds to Herpes Simplex Virus Type 1 Glycoprotein D*

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Herpes simplex virus type 1 utilizes cell surface heparan sulfate as receptors to infect target cells. The unique heparan sulfate saccharide sequence offers the binding site for viral envelope proteins and plays critical roles in assisting viral infections. A specific 3-*O*-sulfated heparan sulfate is known to facilitate the entry of herpes simplex virus 1 into cells. The 3-*O*-sulfated heparan sulfate is generated by the heparan sulfate D-glucosaminyl-3-*O*-sulfotransferase isoform 3 (3-OST-3), and it provides binding sites for viral glycoprotein D (gD). Here, we report the purification and structural characterization of an oligosaccharide that binds to gD. The isolated gD-binding site is an octasaccharide, and has a binding affinity to gD around 18 μM, as determined by affinity coelectrophoresis. The octasaccharide was prepared and purified from a heparan sulfate oligosaccharide library that was modified by purified 3-OST-3 enzyme. The molecular mass of the isolated octasaccharide was determined using both nanoelectrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization mass spectrometry. The results from the sequence analysis suggest that the structure of the octasaccharide is a heptasulfated octasaccharide. The proposed structure of the octasaccharide is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S. Given that the binding of 3-*O*-sulfated heparan sulfate to gD can mediate viral entry, our results provide structural information about heparan sulfate-assisted viral entry.

Heparan sulfates (HS),[1] highly sulfated polysaccharides, are present on the surface of mammalian cells and in the extracellular matrix in large quantities. HS play critical roles in a variety of biological interactions, including assisting viral infection, regulating blood coagulation and embryonic development, suppressing tumor growth, and controlling the eating behavior of mice by interacting with specific regulatory proteins (1–5). HS is initially synthesized as a copolymer of glucuronic acid and *N*-acetylated glucosamine by D-glucuronyl and *N*-acetyl-D-glucosaminyl transferase, followed by various modifications (6). These modifications include C_5-epimerization of glucuronic acid to form iduronic acid residues, 2-*O*-sulfation of iduronic and glucuronic acid, *N*-deacetylation and *N*-sulfation of glucosamine, as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine. Numerous HS biosynthetic enzymes have been cloned and characterized (for review, see Esko and Lindahl (7)).

The specific sulfated saccharide sequences play critical roles in determining the functions of HS. A recent report suggests that the expression levels of various isoforms of each class of HS biosynthetic enzyme contribute to the synthesis of specific saccharide sequences in specific tissues (8). HS *N*-deacetylase/*N*-sulfotransferase, 3-*O*-sulfotransferase, and 6-*O*-sulfotransferase are present in multiple isoforms, and each isoform is believed to recognize the saccharide sequence around the modification site to generate a specific sulfated saccharide sequence (8–10). For instance, HS D-glucosaminyl-3-*O*-sulfotransferase (3-OST) isoforms generate 3-*O*-sulfated glucosamine that is linked to different sulfated uronic acid residues. 3-OST-1 transfers sulfate to the 3-OH position of the *N*-sulfated glucosamine residue that is linked to a glucuronic acid residue at the nonreducing end (GlcUA-GlcNS ± 6S), whereas, 3-OST-3 transfers sulfate to the 3-OH position of the *N*-unsubstituted glucosamine residue that is linked to a 2-*O*-sulfated iduronic acid at the nonreducing end (IdoUA2S-GlcNH$_2$ ± 6S) (11). The differ-

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[1] The abbreviations used are: HS, heparan sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; nESI-MS, nanoelectrospray ionization mass spectrometry; HSV-1, herpes simplex virus type 1; gB, gC, and gD, herpes envelope glycoprotein B, glycoprotein C, and glycoprotein D, respectively; 3-OST; heparan sulfate D-glucosaminyl-3-*O*-sulfotransferase; ΔUA, $\Delta^{4,5}$-unsaturated uronic acid; GlcUA, D-glucuronic acid; IdoUA, α-iduronic acid; GlcNH$_2$, *N*-unsubstituted glucosamine; MWCO, molecular weight cut-off; AnMan, 2,5-anhydromannitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography.

ence in substrate specificity of 3-OSTs results in distinct biological functions of the HS modified by 3-OSTs. For example, HS modified by 3-OST-1 binds to antithrombin and has anticoagulant activity (12), whereas the HS modified by 3-OST-3 binds to herpes simplex 1 envelope glycoprotein D (gD) and assists in viral entry (13).

Herpes simplex virus type 1 (HSV-1) is a member of the herpesvirus family, and infection in humans is prevalent. HSV-1 infection requires a two-step process that can be separated experimentally: attachment to cells and entry into cells (14). It is now known that HS is involved in assisting viral binding as well as viral entry (15). HSV-1 binds to host cells through an interaction of virion envelope glycoprotein C (gC), or in some cases of glycoprotein B (gB), with HS (16–18). Structural analysis of gC-binding HS revealed that a minimum of 10–12 sugar residues containing IdoUA2S and GlcNS(or Ac)6S are necessary (19), and this conclusion was confirmed by another study (20).

A recent report suggests that a specific 3-*O*-sulfated HS is involved in assisting HSV-1 entry (13). The 3-*O*-sulfated HS is generated by 3-OST-3, but not by 3-OST-1. It should be noted that 3-OST-3-modified HS is rarely found in HS from natural sources, suggesting that HSV-1 recognizes a unique saccharide structure (11). In addition, a biochemical study revealed that 3-*O*-sulfated HS provides binding sites for HSV-1 envelope glycoprotein gD, which is a key viral protein involved in entry of HSV-1. It is believed that the interaction between gD and the 3-*O*-sulfated HS triggers the fusion between the virus and the cell in the presence of other viral envelope proteins, including gB, gH, and gL, via an uncharacterized mechanism. The study of the crystal structure of gD and herpes entry receptor HveA suggest that the binding of HveA to gD induces conformational changes in gD (21). This study also predicts a 3-*O*-sulfated HS-binding pocket on gD near the HveA-binding site (21). The exact carbohydrate sequence of the gD-binding site in 3-*O*-sulfated HS remains to be investigated.

In this article, we report the characterization of the structure of a gD-binding octasaccharide. The results from extensive sequencing analysis suggest that the structure of the gD-binding octasaccharide is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S (residue 1 is GlcNH$_2$3S6S and residue 8 is ΔUA). The octasaccharide apparently has two motifs: a relatively low sulfation domain (residues 5–8) that contains two sulfate groups and a high sulfation domain (residue 1 to residue 4) that contains five sulfate groups. Although we still do not know the contribution of each sulfate group to the binding affinity of the octasaccharide and gD, the results from this study will provide the structural information to understand HS-assisted viral infection mechanisms.

EXPERIMENTAL PROCEDURES

Materials

Recombinant 3-OST-3A and 3-OST-1 enzymes were expressed in Sf9 cells using baculovirus expression system. The enzymes were purified by using heparin-Toyopearl and 3′,5′-ADP-agarose chromatographies as described previously (11, 22). [^{35}S]PAPS was prepared by incubating 0.4 mCi/ml [^{35}S]Na_2SO_4 (carrier-free, ICN) and 16 mM ATP with 5 mg/ml dialyzed yeast extract (Sigma) (12). Iduronate-2-sulfatase, α-iduronidase, α-*N*-acetylglucosaminidase, glucosamine-6-sulfatase, and sulfamidase were obtained from Glyko. Recombinant heparin lyase I (EC 4.2.2.7), II (no EC number), and III (E.C. 4.2.2.8) were prepared as described previously (23). $\Delta^{4,5}$-Glycuronidase was isolated from *Flavobacterium heparinum* (24). HS from bovine kidney was obtained from ICN. A truncated form of herpes simplex virus 1 glycoprotein D, gD-1(306t), and monoclonal anti-gD-(DL6) were prepared as previously described (25).

Preparation of gD-binding Octasaccharide

Preparation of the HS Oligosaccharide Library—The library was prepared by incubating HS with limited amounts of heparin lyase III followed by size fractionation on a Bio-Gel P-6 (Bio-Rad) column as described by Pye *et al.* (26). In a typical preparation, HS from bovine kidney (1 mg) was incubated with 2 milliunits of heparin lyase III in 1 ml of buffer containing 50 mM sodium phosphate and 100 μg/ml bovine serum albumin, pH 7.0, at 37 °C overnight. The digestion was terminated by heating at 100 °C for 15 min. The sample was then loaded on a Bio-Gel P-6 (0.75 × 200 cm) equilibrated with 0.5 M ammonium bicarbonate at a flow rate of 5 ml/h and 0.5-ml fractions were collected. The absorbance at 232 nm was measured for each fraction. Peaks corresponding to tetra- to greater than dodecasaccharides were pooled individually, and dialyzed against 50 mM ammonium bicarbonate using MWCO 3,500 membrane. Each pool was dried on a Speed-Vac concentrator (Labconco) and reconstituted in 50 μl of water. The optical density (232 nm) of the resultant solution was about 3. We processed a total of 40 mg of HS to obtain a sufficient amount of gD-binding octasaccharide for the structural analysis.

Preparation of 3-O-Sulfated Oligosaccharides—To prepare 3-OST-3A-modified HS oligosaccharide, 20 μl of the oligosaccharide library or intact HS (1 μg) was mixed with 240 ng of purified 3-OST-3A enzyme and 10 μM [^{35}S]PAPS (14,000 dpm/pmol) in a buffer containing 50 mM MES, 1% Triton X-100, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 150 mM NaCl, and 168 μg/ml bovine serum albumin, pH 7, in a final volume of 50 μl. The reaction was incubated at 37 °C for 2 h and was then heated at 100 °C for 2 min. The resultant solution was centrifuged at 14,000 rpm for 1 min to remove insoluble materials. The supernatant was dialyzed against 50 mM ammonium bicarbonate using MWCO 3,500 membrane and dried. To prepare 3-OST-1-modified oligosaccharides, we followed nearly identical procedures except for omitting the 150 mM NaCl and using 70 ng of 3-OST-1 enzyme during the enzymatic modification reaction.

Purification of the 3-O-Sulfated Octasaccharides by HPLCs—The 3-OST-3-modified oligosaccharides were applied to a silica-based polyamine (PAMN) HPLC column (0.46 × 25 cm, Waters). The column was eluted with a linear gradient of KH_2PO_4 from 350 mM to 1 M for 60 min followed by an additional wash with 1 M KH_2PO_4 for 20 min at a flow rate of 1 ml/min (11). The fractions containing ^{35}S-radioactivity were pooled separately and resolved on Bio-Gel P-6. The fractions were dialyzed against 25 mM ammonium acetate using MWCO 3,500 membrane and dried. They were further purified by DEAE-NPR HPLC chromatography (0.46 × 7.5 cm, Tosohaas). The DEAE-NPR column was eluted with a linear gradient of NaCl in 50 mM Tris-HCl, pH 7, from 100 to 500 mM in 60 min followed by an additional wash for 20 min with 1 M NaCl in 50 mM Tris-HCl, pH 7, at a flow rate of 0.5 ml/min. The eluted oligosaccharides were monitored by ^{35}S-radioactivity and the absorbance at 232 nm. We obtained about 200 to 300 pmol of purified ^{35}S-labeled oligosaccharides from 20 mg of HS that was partially digested with heparin lyase III. We did observe a UV peak that overlapped with the ^{35}S-radioactive peak when a large amount of 3-*O*-^{35}S-sulfated oligosaccharides (>200 pmol) were injected on DEAE-NPR-HPLC.

Determination of the Binding of 3-O-Sulfated HS Oligosaccharides to gD—The assay for determining the binding of 3-*O*-sulfated HS oligosaccharides to gD was carried out by an immunoprecipitation procedure using gD and anti-gD monoclonal antibody as described previously but at a lower pH (13). Briefly, 3-*O*-sulfated HS (1–10 pmol) was incubated in 50 μl of buffer containing 50 mM MES and 0.01% Triton, pH 6 (binding buffer), and 2 mg/ml gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5 μl) was added and incubated at 4 °C for 1 h followed by addition of the protein A-agarose gel (80 μl of 1:1 slurry) and agitated at 4 °C for an additional hour. The protein A-agarose gel (Pierce) was then washed with 0, 50, 150, and 500 mM NaCl in the above binding buffer.

The binding affinity between 3-*O*-sulfated oligosaccharides and gD was determined using affinity co-electrophoresis, as previously described (13). The gel was dried and analyzed on a PhosphorImager (Amersham Biosciences, Storm 860) to determine the migration of [^{35}S]oligosaccharides. The ^{35}S-intensity was plotted against the migration distance through the separation zone to define the distance migrated in the presence or absence of gD.

Determination of the Structure of a gD-binding Octasaccharide

Enzymatic and Nitrous Acid Degradation of Octasaccharides—The conditions for digestion with $\Delta^{4,5}$-glycuronidase and HS glycuronate-2-sulfatase were described elsewhere (27). The conditions for the nitrous

acid degradations under pH 1.5 and 4.5 were described in a prior publication (8). The degraded octasaccharide was analyzed by DEAE-NPR-HPLC.

N-Acetylation of Oligosaccharides—The oligosaccharide (5×10^5 to 1×10^6 cpm, 36–72 pmol) was dissolved in 20 μl of a solvent containing *N′,N′*-dimethylformamide and triethylamine (1:1, v/v) and 5 μl of acetic anhydride, and incubated on ice for 1 h. Tris (20 μl of 50 mM) was then added and the reaction mixture was incubated on ice for an additional hour. The sample was then diluted with 10 volumes of water and dialyzed against 50 mM ammonium bicarbonate using a MWCO 3,500 membrane.

Derivatizations—Derivatizations were carried out by reacting 5 μl of oligosaccharide solution with 5 μl of 50 mM semicarbazide and 60 mM Tris acetic acid (pH 7.0, prepared fresh daily) for 16 h at 30 °C.

Analysis of HS Oligosaccharides Using Mass Spectrometry—Two mass spectrometry techniques, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nanoelectrospray ionization mass spectrometry (nESI-MS), were employed. The MALDI-MS spectra were acquired in the linear mode using a Perseptive Biosystems Voyager Elite reflectron time-of-flight instrument fitted with a 337-nm laser as described elsewhere (28). The nESI-MS analysis was carried out using a Micromass Quattro II with QhQ geometry, a Z-spray source, and pulled borosilicate glass nanovials (29). In the neutral loss scan, MS/MS spectra were obtained by scanning Q1 and Q3 with an offset of 26.7 or 20 m/z in their scan cycles, corresponding to the loss of sulfate from the triple or quadruple charged octasaccharide, respectively (29). To obtain a high quality nESI-MS spectrum, the purified octasaccharide was further dialyzed against 25 mM ammonium acetate (purity of ammonium acetate is 99.9999%, Aldrich) using MWCO 13,000 hollow fiber dialysis tubing (Spectrum). Control studies showed that 80–95% of 3-*O*-[^{35}S]pentasaccharide (M_r = 1507) could be recovered using this dialysis tubing.

Analysis of Oligosaccharides by Capillary Electrophoresis—The approach for the analysis of oligosaccharides followed a previously described method with modifications (30). Briefly, the analysis was carried out on a Beckman P/ACE MDQ unit using an uncoated fused silica capillary (inner diameter = 75 μm; L_{tot} = 106 cm). Hydrodynamic injection was employed under 9.5 p.s.i. for 5 s. About 274 nl of the sample was calculated to be injected by *CE Expert* software. The electrolyte was a solution of 10 μM dextran sulfate and 50 mM Tris-phosphoric acid, pH 2.5. Separation was carried out at 25 kV.

TABLE I
The binding of 3-O-sulfated oligosaccharides to gD

The binding of HS and oligosaccharides to gD was carried out at pH 6 by using an immunoprecipitation approach as described under "Experimental Procedures."

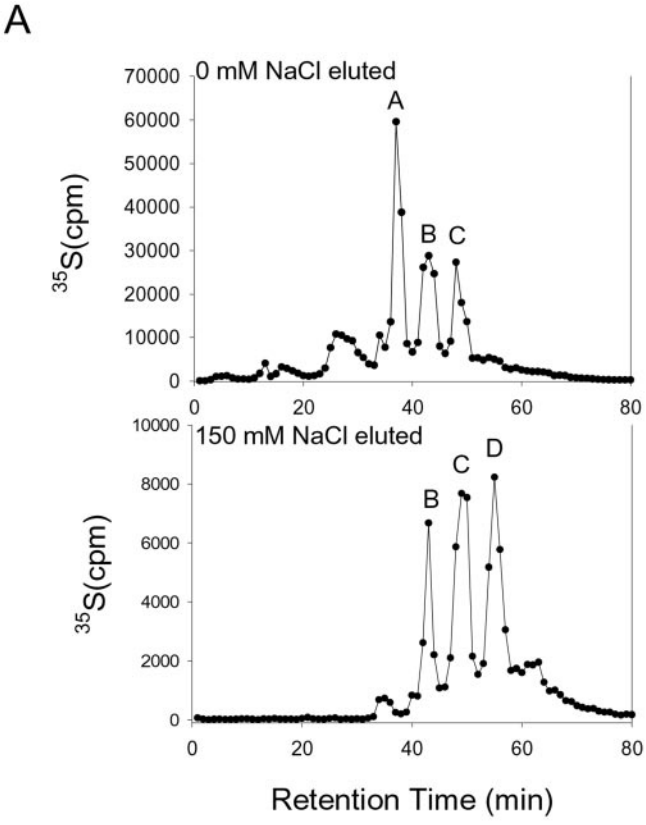
Size of the oligosaccharides	gD-binding	
	3-OST-1-modified	3-OST-3A-modified
	%	
Intact HS	8.8	22.9
>Dodecasaccharides	2.0	7.2
Dodecasaccharides	2.4	7.6
Decasaccharides	5.3	7.0
Octasaccharides	1.3	5.4
Hexasaccharides[a]	Not determined	3.4

[a] Because 3-OST-1 sulfated hexasaccharides very poorly, we could not obtain sufficient amount of 3-OST-1-modified hexasaccharides for the binding experiment. Therefore, we were unable to compare the binding of 3-OST-1-modified hexasaccharides and 3-OST-3A-modified hexasaccharides. Nevertheless, a gD-binding octasaccharide was isolated from the 3-OST-3 modified-hexasaccharide library as described in the text.

RESULTS

Isolation of the gD-binding Octasaccharide

A gD-binding octasaccharide was purified from a 3-OST-3A-modified HS oligosaccharide library. The HS oligosaccharide library was prepared by incubating HS with a limited amount of heparin lyase III. The resultant material was fractionated by a Bio-Gel P-6 column based upon the size of the oligosaccharides, obtaining di-, tetra,-, . . . , dodeca-, and >dodecasaccha-



B

The binding of the purified 3-*O*-[^{35}S] sulfated oligosaccharides and gD

Samples	gD-binding (%)
Fraction A	9.1± 3% (n=2)
Fraction B	10.8%
Fraction C	11.4%
Fraction D	32.4 ± 6% (n=6)

FIG. 1. **Purification of a gD-binding oligosaccharide by PAMN-HPLC.** *Panel A* shows the profiles of the 3-*O*-^{35}S-sulfated oligosaccharides eluted from a protein A-agarose column with different concentrations of NaCl. The *top chromatogram* is the profile of the fractions that were eluted without sodium chloride; the *bottom chromatogram* is the profile of the fractions that were eluted with 150 mM NaCl. *Panel B* shows the binding of purified oligosaccharides and gD using an immunoprecipitation approach.

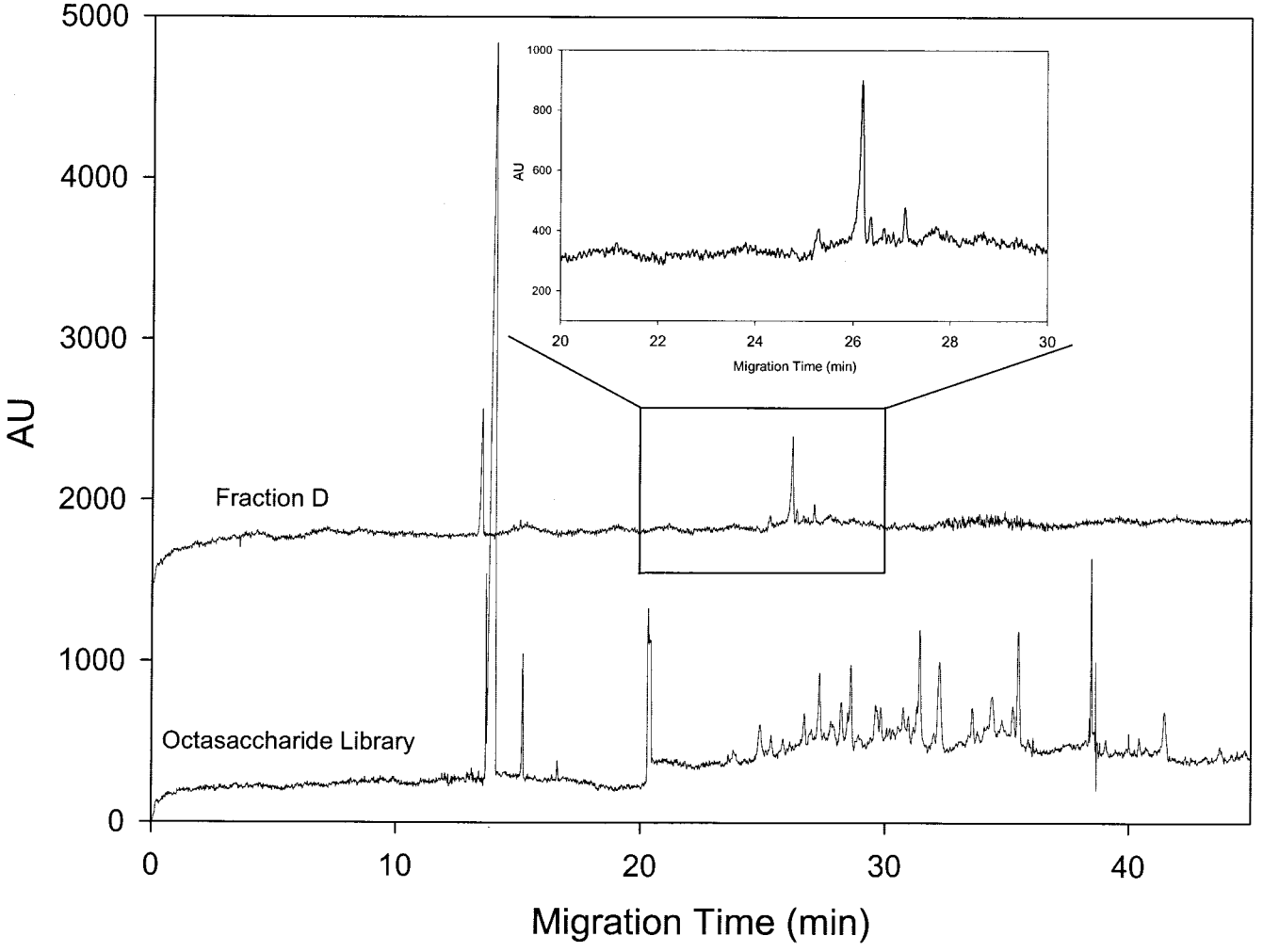


FIG. 2. **The electrophoretogram of fraction D analysis with capillary electrophoresis.** Purified fraction D was analyzed on capillary electrophoresis with an on-line UV detector at 230 nm under reverse polarity conditions. The *bottom electrophoretogram* shows the separation of HS octasaccharide library. The *top electrophoretogram* shows the separation of fraction D. The *inset* shows the enlarged region where fraction D migrated.

rides (data not shown), and a similar approach to prepare a HS oligosaccharide library was reported by Pye and colleagues (26). Because of the limited resolution capability of Bio-Gel P-6, each oligosaccharide library undoubtedly contained the oligosaccharides with different sizes. These fractions were then subjected to 3-OST-3A modification and assayed for gD binding (Table I). Because 3-OST-1-modified HS does not bind to gD, we utilized the 3-OST-1-modified oligiosaccharides as a negative control (13). As shown in Table I, the gD-binding percentage of 3-OST-3A-modified oligosaccharides was about 3-fold higher than that of the 3-OST-1-modified counterparts. We chose to purify a gD-binding oligosaccharide from the 3-OST-3A-modified hexasaccharide pool based upon the following two reasons: 1) the purification of hexasaccharides or octasaccharides can be achieved by anion exchange HPLC; 2) sequencing analysis for hexa- or octasaccharide is significantly less complex than larger oligosaccharides.

We purified a gD-binding oligosaccharide by successive anion-exchange HPLC, PAMN-, and DEAE-NPR-HPLC. Five major 3-*O*-^{35}S-sulfated oligosaccharides were resolved by PAMN-HPLC (data not shown). To identify which [^{35}S]oligosaccharide has the highest binding affinity for gD, the 3-OST-3A-modified oligosaccharides were fractionated by an immunoprecipitation approach as described under "Experimental Procedures." The eluents were analyzed by PAMN-HPLC (Fig. 1*A*). Comparing the chromatograms of the 3-*O*-^{35}S-sulfated oligosaccharides eluted from protein A-agarose under different concentrations of sodium chloride, we found that fraction D was present when the protein A-agarose was eluted with 150 mM NaCl (Fig. 1*A, bottom chromatogram*). In contrast, fraction A was present when the protein A-agarose was eluted with the buffer without NaCl (Fig. 1*A, top chromatogram*). This result suggests that fraction D has higher affinity for gD than fraction A. As indicated, it was observed that 32% of fraction D binds gD, whereas only 9% of fraction A binds to gD (Fig. 1*B*). Thus, we designated fraction D as a gD-binding oligosaccharide and fraction A as a gD-nonbinding oligosaccharide. Fraction B and fraction C were considered gD-nonbinding oligosaccharides, and were not subject to further structural study as their binding percentages to gD are similar to that of fraction A (Fig. 1*B*). Additional ^{35}S-labeled molecules were eluted from the protein A-agarose column with 500 mM sodium chloride. However, those molecules did not give sharp peaks on PAMN-HPLC. In addition, those molecules migrated as the oligosaccharides that were much larger than octasaccharides on Bio-Gel P-6. It is possible that these molecules represented the ^{35}S-labeled oligosaccharide contaminants that were larger than octasaccharides in the oligosaccharide library. Fraction D was further purified on DEAE-NPR-HPLC.

To confirm the purity, fraction D was analyzed by capillary electrophoresis using a UV 230 nm on-line detector. As shown in Fig. 2 (*bottom electrophoretogram*), the octasaccharide li-

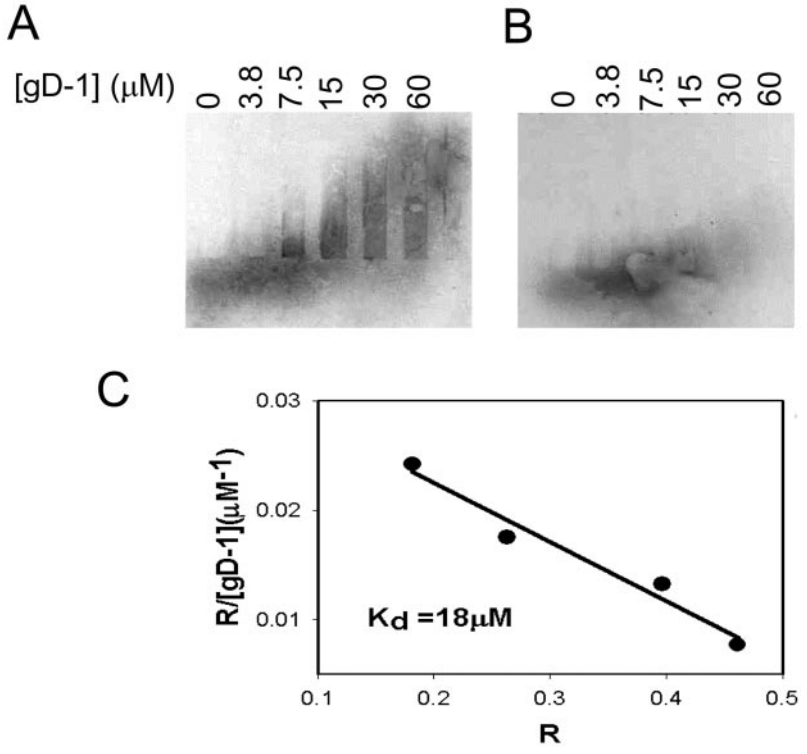


FIG. 3. **Binding constant (K_d) for the interaction between fraction D and gD-1.** *Panel A* presents the autoradiograph of the agarose gel in which purified fraction D was subjected to electrophoresis through zones containing gD-1 at the concentrations indicated. Approximately 30,000 cpm (3×10^{-12} mol)/lane of [^{35}S]fraction D was loaded in each separation zone. *Panel B* presents the autoradiograph of the agarose gel in which purified [^{35}S]fraction A (15,000 cpm, 1.5×10^{-12} mol/lane) was subjected to electrophoresis through zones containing gD at the concentrations indicated. *Panel C* represents the plot of $R/[gD]_{total}$ *versus* R, where the retardation coefficient, $R = (M_0\text{-}M)/M_0$. M_0 is the migration of free [^{35}S]fraction D, and M is the observed migration of [^{35}S]fraction D in the presence of gD-1. Assuming that [^{35}S]fraction D and gD-1 form a 1:1 complex and gD-1 is in great excess, this plot should yield a straight line with a slope of $-1/K_d$ according to the Scatchard equation. The linear coefficient value of the plot is 0.98, and the calculated K_d is 18 μM. Because there was no obvious retardation for fraction A under the assay conditions, we were unable to perform a graphical analysis to determine the binding constant (K_d) between gD and fraction A.

brary was well resolved by capillary electrophoresis, suggesting that the resolution of the oligosaccharides on capillary electrophoresis is high. Fraction D migrated predominantly as a single peak under such conditions (Fig. 2, *top electrophoretogram*). In addition, the area of the major UV peak is consistent with the estimated concentration of the octasaccharide based upon the specific ^{35}S-radioactivity. Having considered the minor UV peaks resulting from contaminants, we calculated the purity of fraction D to be greater than 80%.

We also determined the binding affinity (K_d) between fraction D and gD using affinity coelectrophoresis as described by Lee and Lander (31). Fraction D was separated under electrophoresis in an agarose gel through zones containing gD at various concentrations (Fig. 3*A*). From these data, the K_d for fraction D and gD was determined to be 18 μM (Fig. 3*C*), which is somewhat higher than the K_d of intact 3-*O*-sulfated HS and gD (2 μM) (13). We also attempted to determine the K_d between fraction A (gD-nonbinding oligosaccharide) and gD using this method. We failed to observe any obvious retarded migration of fraction A, suggesting that the binding affinity between fraction A and gD is low (Fig. 3*B*). We estimated that the K_d for fraction A and gD is greater than 200 μM.

Structural Characterization of Fraction D

Analysis of Fraction D by nESI-MS and MALDI-MS—The molecular mass of fraction D was determined by both nESI-MS and MALDI-MS. The nESI-MS spectrum of fraction D is shown in Fig. 4. The sample shows a triple charged ion, $[M\text{-}3H]^{3-}$, at *m/z* 648.8 and a strong quadruple charged ion, $[M\text{-}4H]^{4-}$, at *m/z* 486.4 (Fig. 4*A*). We confirmed that the signals at *m/z* 648.8 and 486.4 contain sulfate groups by using neutral loss experiments as described in a prior publication (29). Briefly, the isolated ions were sequentially admitted to a collision cell filled with argon under controlled energy conditions, resulting in limited dissociation. The linkage between the sulfate and the hydroxyl group is labile, and the products from a series of sulfate losses within the collision cells are common. The experiments were designed to detect molecular ions that lose 20 *m/z* (corresponding to the loss of the sulfate from the quadruple charged oligosaccharide), and signals were detected 486.4, 491.9, and 495.9 *m/z* (Fig. 4*B*). Similarly, neutral loss scans looked for oligosaccharides that lose 26.7 *m/z* (corresponding to the loss of the sulfate from the triple charged oligosaccharide), and signals were detected at 648.9, 656.0, and 661.4 *m/z* (Fig. 4*C*). Taken together, these data suggest that the signals at 648.8 and 486.4 *m/z* were likely derived from triple and quadruple charged oligosaccharides, respectively. From these data, the molecular mass of fraction D was calculated to be 1949.5 Da (from the triple charged ion, the molecular mass of fraction D is $648.8 \times 3 + 3 = 1949.4$; from the quadruple charged ion, the molecular mass of fraction D is $486.4 \times 4 + 4 = 1949.6$). The determined molecular mass for fraction D was very close to the theoretical value (1950.1 Da) for a heptasulfated octasaccharide with one *N*-acetylated glucosamine residue ($\Delta UA(UA)_3(GlcN)_3\ GlcNAc(SO_3H)_7$, $C_{50}H_{78}O_{62}N_4S_7$).[2]

We also determined the molecular mass of fraction D by using MALDI-MS. MALDI-MS requires that a complex between fraction D and a synthetic peptide $(Arg\text{-}Gly)_{19}\text{-}Arg$ be formed (32). After subtracting the contribution of the protonated peptide, the molecular mass of fraction D was calculated to be 1951.2 Da (spectrum not shown).[3] Thus, the result of MALDI-MS is consistent with the result of nESI-MS.

Sequencing Analysis of Fraction D—Because we had demonstrated that fraction D is a heptasulfated octasaccharide with an *N*-acetylated glucosamine residue, we conducted sequencing analysis to identify the position of the sulfate groups in each residue and to determine the identity of uronic acid residues (*i.e.* glucuronic acid or iduronic acid).

[^{35}S]Disaccharide Analysis of Fraction D—A disaccharide analysis was performed to determine the identity of the disaccharide with a 3-*O*-[^{35}S]sulfate group. In this experiment, frac-

[2] We calculated the molecular weight of fraction D based on ^{32}S, because [^{35}S]sulfate represents less than 0.4% of total 3-*O*-sulfation.

[3] The molecular mass of fraction A was determined to be 1834 Da by MALDI-MS. The molecular mass is consistent with an octasaccharide with five sulfate groups and two *N*-acetylated glucosamine residues.

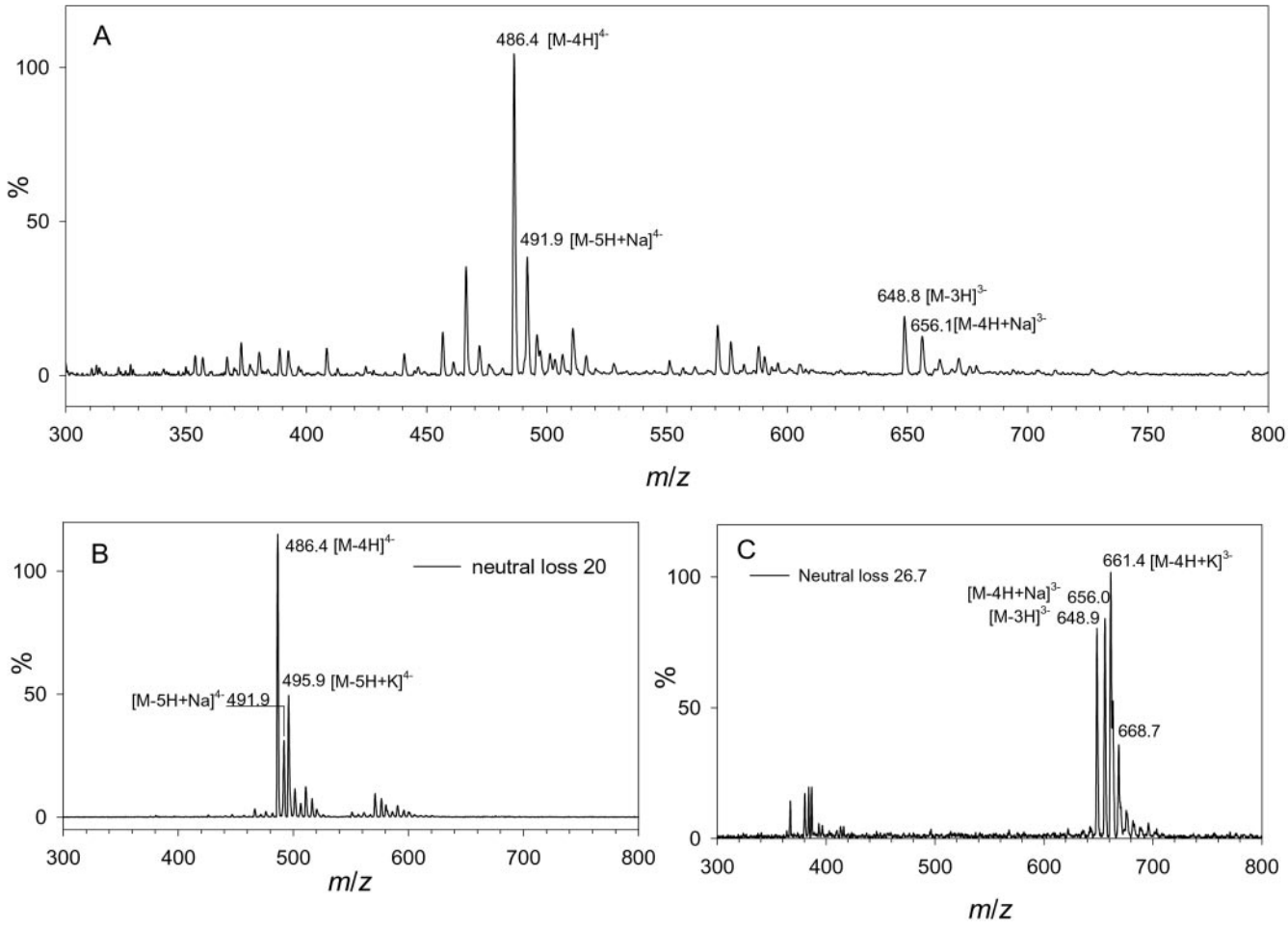


FIG. 4. **nESI-MS spectra of fraction D.** The mass spectrum of fraction D is shown in *panel A*. A neutral loss scan for the loss of 20 *m*/*z* is shown in *panel B*. A neutral loss scan for the loss of 26.7 *m*/*z* is shown in *panel C*.

tion D was degraded with nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant disaccharides were analyzed by reverse-phase ion pairing HPLC (11). We found that nearly 90% of the [^{35}S]disaccharide was IdoUA2S-[3-^{35}S]AnMan3S6S (Fig. 5).[4] The result suggests that the 3-*O*-^{35}S-sulfation site is present in a disaccharide with a structure of -IdoUA2S-GlcNH$_2$3S6S-, provided that a previous report (11) demonstrated that 3-OST-3A sulfates an *N*-unsubstituted glucosamine residue.

Sequencing Analysis from the Reducing End—Fraction D was reacted with semicarbazide to form a semicarbazone at the reducing end, as illustrated in Fig. 6*C* (30). This reaction increases the molecular mass of the oligosaccharide by 56.1 Da. The semicarbazone moiety serves as a mass tag during sequence analysis to differentiate oligosaccharides derived from the reducing end of the parent compound. By capillary electrophoresis, we confirmed that greater than 95% of Fraction D was labeled under the standard derivatization conditions (data not shown). The molecular mass of the derivatized fraction D was determined to be 2008 Da using MALDI-MS (Fig. 6*A*), confirming that the tag was present. Upon treatment of the derivatized oligosaccharide with heparin lyase II, a tetrasaccharide of mass 1131.1 was observed (Fig. 6*B*). This mass corresponds to a pentasulfated tetrasaccharide labeled with the mass tag (1075.0 + 56.1 Da). By capillary electrophoresis, this tetrasaccharide had an identical migration time to the standard ΔUA2S-GlcNS-IdoA2S-GlcNH$_2$3S6S-semicarbazide (11) (data not shown). We further strengthened the conclusion by demonstrating that residue 4 (ΔUA2S) carries the 2-*O*-sulfate group as described below.

We examined the susceptibility of the reducing end tetrasaccharide (residues 1–4) to HS glycuronate-2-sulfatase (Fig. 7). The reducing end tetrasaccharide was prepared by subjecting the octasaccharide to digestion by heparin lyase II, because it was reported that the tetrasaccharides containing a 3-*O*-sulfated glucosamine residue is resistant to digestion (11, 33). Comparing the elution times of the undigested and digested reducing end tetrasaccharide, we found that the tetrasaccharide was susceptible to HS glycuronate-2-sulfatase digestion. Thus, the reducing end residue (residue 4) of the tetrasaccharide is a 2-*O*-sulfated $\Delta^{4,5}$-uronic acid, provided that HS glycuronate-2-sulfatase specifically reacts with this residue (24).[5] Taken together, these results demonstrate that fraction D contains a tetrasaccharide with a structure of ΔUA2S-GlcNS-

[4] Two minor [^{35}S]peaks were also observed, representing [^{35}S]sulfate and IdoUA2S-AnMan3S, respectively. The IdoUA2S-AnMan3S is probably the partial desulfated IdoUA2S-AnMan3S6S because of nitrous acid degradation or the product of the minor contaminants in the fraction D.

[5] It should be noted that we identified the positions of five sulfate groups in the reducing end tetrasaccharide to this end. Because low-pH nitrous, pH 1.5, degradation of fraction D resulted in a trisulfated disaccharide (IdoUA2S-AnMan3S6S) (Fig. 5), the octasaccharide must contain a trisaccharide carrying four sulfate groups with a structure of –GlcNS-IdoUA2S-GlcNH$_2$3S6S–. Another sulfate group is located at the 2-OH position of residue 4.

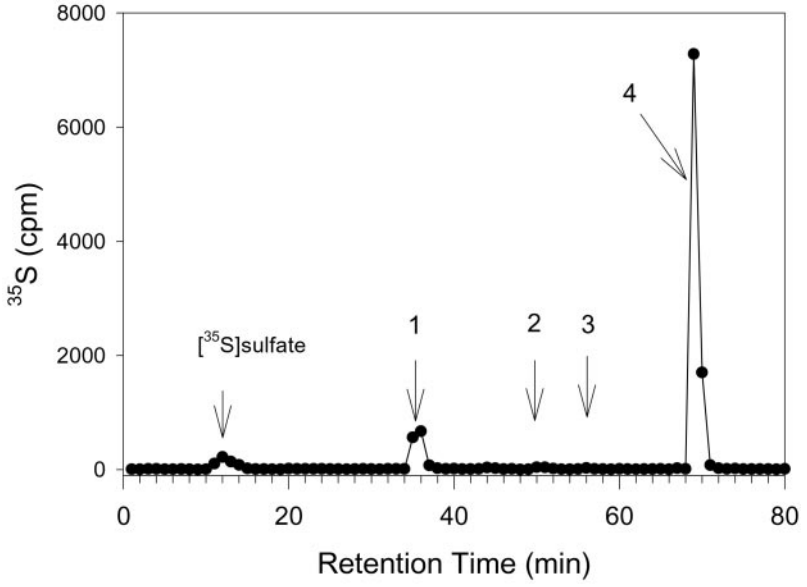


FIG. 5. **RPIP-HPLC chromatogram of low-pH nitrous acid degraded fraction D.** Fraction D was treated with nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant disaccharides were resolved on RPIP-HPLC. *Arrows* indicate the elution positions of the disaccharide standards. *1* represents IdoUA2S-AnMan3S; *2* represents GlcUA-AnMan3S6S; *3* represents IdoUA2S-AnMan6S; and *4* represents IdoUA2S-AnMan3S6S.

IdoUA2S-GlcNH$_2$3S6S; the location of this tetrasaccharide is from residue 1 to 4.[6]

Sequencing Analysis from the Nonreducing End—Sequencing from the nonreducing end was accomplished by using various exoenzymes followed by chromatography on DEAE-NPR-HPLC. The goal of the nonreducing sequencing analysis was to identify the position of the two remaining sulfate groups within residues 5–8, given that five sulfate groups were determined to be present on residues 1–4.

The sequencing strategy from the nonreducing end and the experimental data are shown in Fig. 8. The retention time of fraction D was shifted from 46 to 38 min after digestion with HS $\Delta^{4,5}$-glycuronidase (Fig. 8*B*). This result suggests that a $\Delta^{4,5}$-unsaturated uronic acid residue was at the nonreducing end, based upon the substrate specificity of HS glycuronidase (24, 34). The minor unknown product was removed prior to the sulfamidase digestion. The resultant heptasaccharide was susceptible to digestion by sulfamidase, as observed by a shift in the retention time from 38 to 30 min on DEAE-NPR-HPLC (Fig. 8*C*), suggesting that it contains an *N*-sulfated glucosamine residue. The sample was then subjected to *N*-acetylation by incubating with acetic anhydride to generate an *N*-acetylated glucosamine residue at the nonreducing end, because α-*N*-acetylglucosaminidase does not react with an *N*-unsubstituted glucosamine. A successful *N*-acetylation on the *N*-unsubstituted glucosamine residue of the heptasaccharide after treatment with acetic anhydride was confirmed, as a shift in the retention time on DEAE-NPR-HPLC was observed (Fig. 8*D*). The *N*-acetylated heptasaccharide was susceptible to α-*N*-acetylglucosaminidase digestion as a shift of retention time from 33 to 38 min was observed (Fig. 8*E*).[7] At this point, fraction D was converted to a hexasaccharide carrying six sulfate groups. The undigested product was removed after purification by DEAE-NPR-HPLC. The resultant hexasaccharide was susceptible to iduronate-2-sulfatase digestion as the retention time was shifted from 38 to 27 min after treatment (Fig. 8*F*). Furthermore, the resultant oligosaccharide was susceptible to α-iduronidase digestion (Fig. 8*G*). The results from the susceptibilities to digestions by iduronate-2-sulfatase and α-iduronidase suggest that residue 6 is a 2-*O*-sulfated iduronic acid residue. The positions of all sulfate groups were therefore determined. The proposed structure is: ΔUA-GlcNS-IdoUA2S-GlcNAc-GlcUA2S (or IdoUA2S)-GlcNS-IdoUA2S-GlcNH$_2$3S6S.

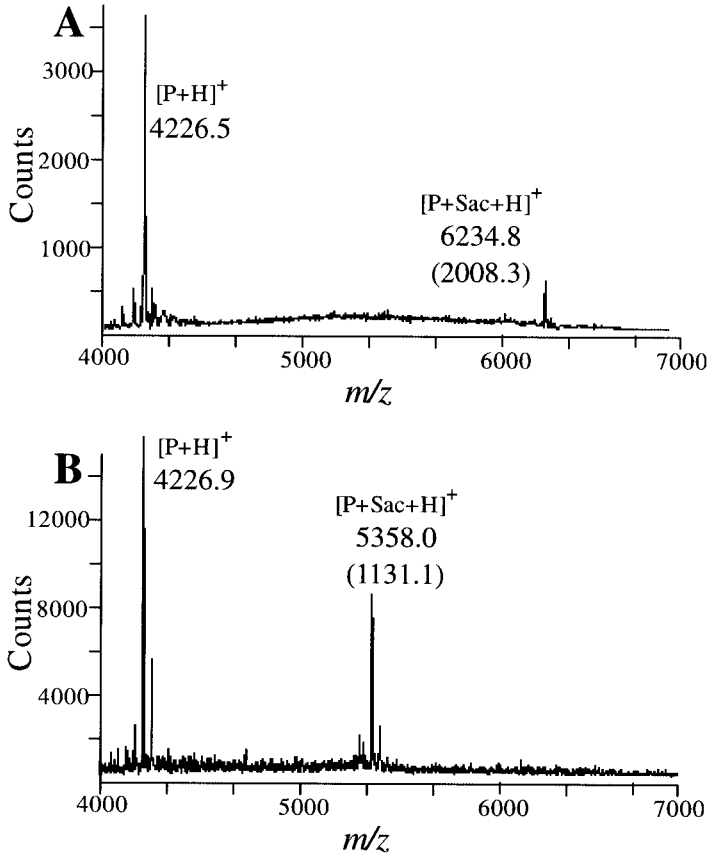
To further confirm the results from nonreducing sequencing analysis, fraction D was treated with heparin lyase II and analyzed by capillary electrophoresis. A tetrasaccharide (residues 1–4) and two disaccharides were generated after digestion as the tetrasaccharide carrying the 3,6-disulfated glucosamine was not susceptible to digestion by heparin lyase II as described above. Enzyme-produced tetrasaccharide coeluted with a tetrasaccharide standard with a structure of ΔUA-GlcNS-IdoUA2S-GlcNH$_2$6S3S (corresponding residues 1–4, data not shown). The preparation of this tetrasaccharide standard was published previously (11). In addition, two resultant disaccharides were identified to be ΔUA-GlcNS and ΔUA2S-GlcNAc (data not shown). Again, the results from heparin lyase II digestion are consistent with the proposed structure for fraction D.

We attempted to determine the identity of residue 4. As described above, we concluded that residue 4 carries a 2-*O*-sulfate group. To address the identity of residue 4, a tetrasaccharide, representing residues 1–4, was prepared by deacetylation followed by nitrous acid degradation at pH 4.5.[8] The resultant tetrasaccharide, which carried a 2-*O*-sulfated uronic

[6] We also attempted to prove the presence of an *N*-unsubstituted glucosamine residue at the reducing end by treating the octasaccharide with nitrous acid at pH 4.5 followed by sodium borohydride reduction. The molecular mass of the resultant octasaccharide was determined using nESI. We found that the molecular mass of the high pH nitrous-treated octasaccharide is 1855 Da, a loss of 95 Da. A reduction of 95 Da is consistent with the deamination (−15 Da) and a loss of sulfate (−80 Da). Our result suggested that desulfation also occurred during nitrous acid, pH 4.5, treatment or during analysis of nESI-MS. Nevertheless, the result is consistent with the fact that the octasaccharide contains an *N*-unsubstituted glucosamine residue.

[7] We observed that only 50% of the heptasaccharide was digested by α-*N*-acetylglucosaminidase. A similar incomplete digestion was observed for a pentasaccharide with a structure of GlcNAc-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe digested by α-*N*-acetylglucosaminidase. The pentasaccharide was prepared by incubating acetic anhydride with a pentasaccharide with a structure of GlcNH$_2$-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe. The latter pentasaccharide was generated from GlcNS6S-GlcUA-[3-^{35}S]GlcNS3S6S-IdoUA2S-GlcNS6SOMe (29) by sequential digestions by glucosamine-6-sulfatase and sulfamidase.

[8] The tetrasaccharide was prepared by incubating purified fraction D with hydrazine for deacetylation. The deacetylated octasaccharide was degraded by nitrous acid at pH 4.5 and sodium borohydride reduction. The resultant oligosaccharide migrated as a tetrasaccharide on Bio-Gel P-6, and was purified by DEAE-NPR HPLC. The proposed structure of this tetrasaccharide is UA2S-GlcNS-IdoUA2S-AnMan3S6S.



C

ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S

(MW = 1950 Da)

Semicarbazide

ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S—Semicarbazide

(MW = 2006 Da, measured MW = 2008 Da)

Heparin lyase II digestion

Disaccharides

ΔUA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S—Semicarbazide

(MW = 1130 Da, measured MW = 1131 Da)

FIG. 6. **MALDI-MS spectra of the reducing end sequencing analysis of fraction D.** *Panel A* shows the mass spectrum of the analysis of the intact octasaccharide after derivatization. The observed mass of 2008.3 is consistent with a single label attached to the reducing end. *Panel B* shows the MALDI-MS spectrum of the end-labeled tetrasaccharide arising from heparin lyase II digestion of the semicarbazide-labeled octasaccharide. *Panel C* shows the chemical reactions of the reducing end sequencing analysis. *P* represents peptide $(Arg\text{-}Gly)_{19}$-Arg; *Sac* + *P* represents the complex of peptide and octasaccharide.

acid residue at the nonreducing end, was subjected to exolytic enzyme digestions. We found that the tetrasaccharide was resistant to the digestions by iduronate-2-sulfatase, α-iduronidase, and β-glucuronidase (data not shown). The result suggests that residue 4 is a 2-*O*-sulfated glucuronic acid as it is known to be unsusceptible to digestions by any of these enzymes. It is known that glucuronate-2-sulfatase specifically removes the sulfate from 2-*O*-sulfated glucuronic acid residue (36). However, because of the unavailability of glucuronate-2-sulfatase, we could not determine whether the resultant tetrasaccharide was susceptible to digestion. We also found that fraction D was resistant to heparin lyase II mutant (C348A) digestion (data not shown). The mutated enzyme cleaves glycosidic linkages containing unsulfated uronic acids but not those containing sulfated iduronic acids (35) and sulfated glucuronic acids.[9] The latter result hinted that residue 4 is a 2-*O*-sulfate glucuronic acid residue. However, we also found that an octasaccharide, containing the linkage of

[9] Z. Shriver and R. Sasisekharan, unpublished observation.

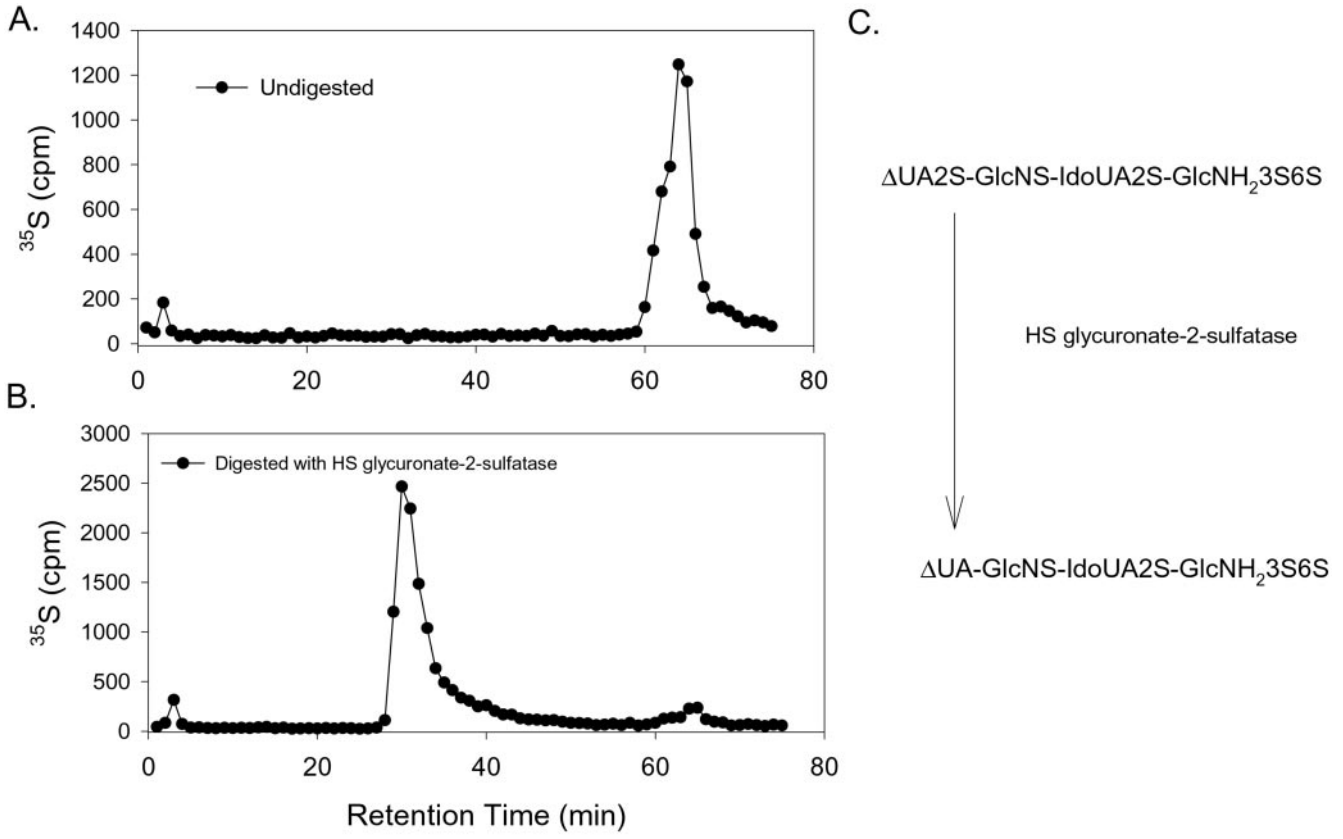


FIG. 7. **DEAE-NPR-HPLC chromatograms of the reducing end tetrasaccharide (residues 1–4) before and after HS glycuronate-2-sulfatase digestion.** The reducing tetrasaccharide was obtained by subjecting fraction D to heparin lyase II digestion. The resultant tetrasaccharide was then digested with HS glycuronate-2-sulfatase. *Panel A* shows the chromatogram of the undigested tetrasaccharide. *Panel B* shows the chromatogram of glycuronate-2-sulfatase-digested tetrasaccharide. *Panel C* depicts action of the HS glycuronate-2-sulfatase digestion.

–GlcNAc-IdoUA2S-, was resistant to digestion by the heparin lyase II mutant (C348A).[10]

DISCUSSION

HS is a common receptor for numerous viruses. It is believed that the defined sulfated sequences determine the specificity for herpes simplex virus (13, 19, 20). Because of the structural complexity of HS, the structural specificity of HS-based receptors is still unknown. It is apparent that understanding the relationship between the saccharide sequences and their activities in promoting viral infection will permit us to delineate HS-assisted viral infections at the molecular level. A previous study has shown that the interaction of 3-*O*-sulfated HS, generated by 3-OST-3 but not by 3-OST-1, interacts with gD to induce the entry of HSV-1 into target cells (13). In the present study, a gD-binding octasaccharide was prepared and purified from a HS oligosaccharide library that was modified by 3-OST-3. The sequence of the isolated gD-binding site is ΔUA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S. To our knowledge, this structure has not been previously reported in HS or heparin. As expected, a 3-*O*-sulfated *N*-unsubstituted glucosamine residue was found (residue 1). The result is consistent with the previously characterized substrate specificity of 3-OST-3A (11).

The binding affinity between gD and the purified octasaccharide was determined to be 18 μM. We noted that the gD-binding affinity of the octasaccharide is about 10-fold lower than that of the intact 3-OST-3-modified HS. Two possibilities may contribute to the lower binding affinity. First, two domains in gD for binding to 3-*O*-sulfated HS were predicted by Carfi and colleagues (21). It is possible that the HS polysaccharide interacts with both sites, whereas the octasaccharide is insufficiently large to bind at both sites. Second, both α- and β-anomeric isomers of octasaccharides are likely generated by heparin lyase III depolymerization. The binding affinities of the α- and β-anomeric isomers of the octasaccharide may be different. At the polysaccharide level, the glucosamine residue is present in only the α-form.

The results from the sequencing analysis of the octasaccharide suggested that residue 4 is a 2-*O*-sulfated uronic acid. Two lines of evidence hinted that residue 4 is a 2-*O*-sulfated glucuronic acid residue. First, our results showed that residue 4 is resistant to digestion by β-glucuronidase, α-iduronidase, or iduronate-2-sulfatase. We noted that 2-*O*-sulfated glucuronic acid is resistant to digestions by these enzymes. Second, the octasaccharide is susceptible to the wild type heparin lyase II digestion, but not susceptible to digestion by a heparin lyase II mutant (C348A). Heparin lyase II cleaves the linkages that contain 2-*O*-sulfated glucuronic acid residue, whereas the mutated enzyme, heparin lyase II (C348A), does not cleave this type of linkage.[11] In addition, a previous report demonstrated

[10] In addition to the results presented in a prior publication (35), we tested the susceptibilities of three oligosaccharides to the digestion by heparin lyase II mutant (C348A). Two of them were tetrasaccharides with structures of ΔUA2S-GlcNS6S-GlcUA2S-GlcNS and ΔUA2S-GlcNS6S-GlcUA2S-GlcNS6S (43). One is an octasaccharide with a structure of ΔUA2S-GlcNAc-IdoUA2S-GlcNAc-IdoUA2S-GlcNAc-IdoUA2S-GlcNAc (38). All three of the oligosaccharides were resistant to the digestion.

[11] We failed to detect any ^{3}H-labeled monosulfated disaccharides from the high pH (4.5) and low pH (1.5) nitrous acid-degraded fraction D (10 pmol) followed by [^{3}H]sodium borohydride (5–15 Ci/mmol) reduction despite the fact that IdoUA2S-[^{3}H]AnMan3S6S was detected. It is mainly because of ^{3}H-labeled contaminants in the sodium [^{3}H]borohydride. Those contaminants were eluted near the region where ^{3}H-labeled monosulfated disaccharides were eluted on RPIP-HPLC.

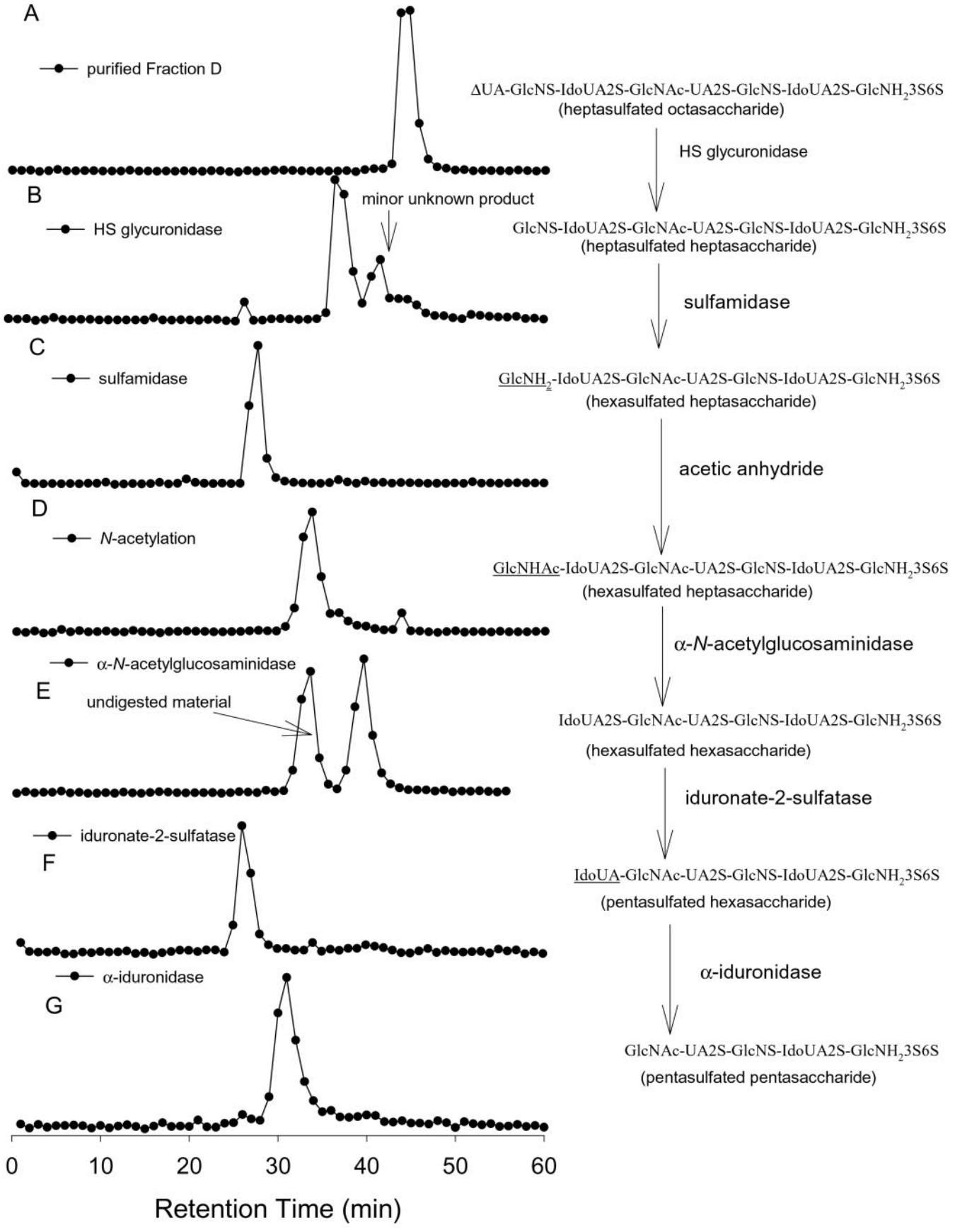


FIG. 8. **Nonreducing end sequencing analysis of fraction D.** *Panels A–G* shows the profiles of enzymatically digested fraction D on DEAE-NPR-HPLC. Between the steps of the sequencing analysis, the designated product was purified by DEAE-NPR-HPLC followed by desalting. The action of the enzymatic digestions at each sequencing step is also indicated. The "minor unknown product" (*panel B*) was removed by HPLC before sulfaminidase digestion. Likewise, the "undigested material" (*panel E*) was removed HPLC before iduronate-2-sulfatase digestion.

that the 2-*O*-sulfated glucuronic acid is present in the HS that is isolated from bovine kidney (37). More convincing evidence is required to conclude with certainty that residue 4 is a 2-*O*-sulfated glucuronic acid. Whereas high resolution NMR spectroscopy could solve this question, it requires significantly larger quantities of octasaccharide than is currently available (38).

It is also very important to note that the disaccharide sequence of –GlcNAc-IdoUA2S– was specifically excluded from the HS or heparin from mammalian cells (39). This conclusion is based on the substrate specificity of HS epimerase that converts glucuronic acid to iduronic acid (40). It is known that an *N*-sulfated glucosamine residue that is linked to a glucuronic acid at the nonreducing end is "absolutely" required for the action of epimerase (39). Thus, the sequences of –GlcNS-IdoUA– and –GlcNS-IdoUA2S– are present in HS, whereas, the sequences of –GlcNAc-IdoUA- and –GlcNAc-IdoUA2S– are not. It should be noted that the sequence of –GlcNAc-IdoUA2S– was isolated from *Achatina fulica*, suggesting that the epimerase may have different substrate specificities from different organisms (38).

We also noted that –GlcNAc-GlcUA2S– has not been discovered in HS that is isolated from natural sources. The 2-*O*-sulfated glucuronic acid residue is a rare constituent of HS, and was found in HS isolated from the adult human cerebral cortex, a nuclear fraction from hepatocytes, HS from bovine kidney, and heparin from porcine intestines (41–43). This residue is synthesized by HS 2-*O*-sulfotransferase, although the enzyme preferably generates 2-*O*-sulfated iduronic acid (IdoUA2S) (44). To this end, it is still not known whether the presence of a 2-*O*-sulfated glucuronic acid, or possibly a 2-*O*-sulfated iduronic acid, residue is essential for gD binding. A comprehensive study of the relationship of the saccharide sequences and gD-binding affinity remains to be investigated when a series of structurally defined HS oligosaccharides are available. The structural information from this study will serve as a lead compound for the chemical synthesis of HS oligosaccharides for further investigation (45).

It is now widely accepted that HS contains both high and low sulfated domains (46). The highly sulfated domains, containing the repeating trisulfated disaccharides of –IdoUA2S-GlcNS6S–, has been the focus of a number of studies investigating HS-related biological functions. For example, the highly sulfated domains bind to fibroblast growth factors and fibroblast growth factor receptors to exhibit various biological functions. Furthermore, previous reports suggest that the antithrombin-binding site is also located within highly sulfated domains (27, 47). It is interesting to note that the gD-binding octasaccharide contains two motifs with distinct sulfation levels. The low sulfated domain, residues 5–8, is composed of one sulfate group per disaccharide, and the highly sulfated domain, residues 1–4, is composed of an average of 2.5 sulfate groups per disaccharide. Thus, these results suggest that the gD-binding site contains both a highly sulfated domain and a low sulfated domain. The crystal structure of gD and HveA, a previously characterized herpes simplex viral entry receptor, predicts two potential 3-*O*-sulfated HS-binding sites (21). One binding site is located in a deep surface pocket with only three basic amino acid residues. This proposed binding site is very close to the binding site of gD and HveA, suggesting that this site might be involved in functional changes in gD. Their observation suggests that the binding of 3-*O*-sulfated HS to this site might involve a small number of positive amino acid residues (from gD) and small number of negatively charged sulfate groups (from HS). A second 3-*O*-sulfated HS-binding site was identified on a relatively flat surface with numerous basic amino acid residues. The second site is away from the HveA-binding site on gD. Carfi and colleagues (21) suggest that the second site in gD might provide the ionic interaction sites to bind to 3-*O*-sulfated HS. It remains to be investigated at which binding sites in gD the isolated octasaccharide interacts.

In summary, an approach to isolate and characterize an octasaccharide that binds to gD was described. It is still not known if this octasaccharide is the minimum necessary sequence for assisting HSV-1 entry into cells. Nevertheless, because the interaction of gD and 3-*O*-sulfated HS is a key step for triggering the fusion of virus and cells, our study provides valuable structural information for determining the specific roles of HS in assisting HSV-1 infections, as well as in the development of therapeutic agents for treating HSV-1 infection.

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