Relative Susceptibilities of the Glucosamine−Glucuronic Acid and *N*-Acetylglucosamine−Glucuronic Acid Linkages to Heparin Lyase III[†]

Wengang Chai,*[,‡] Christine Leteux,[‡] Camilla Westling,[§] Ulf Lindahl,[§] and Ten Feizi[‡]

MRC Glycosciences Laboratory, Imperial College Faculty of Medicine, Northwick Park Hospital Campus, Watford Road, Harrow, Middlesex HA1 3UJ, U.K., and Department of Medical Biochemistry and Microbiology, Uppsala University, The Biomedical Center, P.O. Box 582, SE-75123 Uppsala, Sweden

Received December 16, 2003; Revised Manuscript Received May 4, 2004

ABSTRACT: Heparin lyases are valuable tools for generating oligosaccharide fragments and in sequence determination of heparan sulfate (HS). Heparin lyase III is known to cleave the linkages between *N*-acetylglucosamine (GlcNAc) or N-sulfated glucosamine (GlcNS) and glucuronic acid (GlcA) as the primary sites and the linkages between GlcNAc, GlcNAc(6S), or GlcNS and iduronic acid as secondary sites. N-Unsubstituted glucosamine (GlcN) occurs as a minor component in HS, and it has been associated with various bioactivities. Here we investigate the specificity of heparin lyase III toward the GlcN−GlcA linkage using a recombinant enzyme of high purity and as substrates the partially de-N-acetylated polysaccharide of *Escherichia coli* K5 strain and derived hexasaccharides. The specificity of lyase III toward the GlcN−GlcA linkage is deduced by sequencing of the oligosaccharide products using electrospray mass spectrometry with collision-induced dissociation and MS/MS scanning. The results demonstrate that under controlled conditions for partial digestion, lyase III does not act at the GlcN−GlcA linkage, whereas GlcNAc−GlcA is cleaved. Even under forced conditions for exhaustive digestion, the GlcN−GlcA linkage is only partly cleaved. It is this property of lyase III that has enabled the isolation of a unique, nonsulfated antigenic determinant ΔUA−GlcN−UA−GlcNAc from HS and from partially de-N-acetylated K5 polysaccharide. It was unexpected that pentasaccharide fragments were also detected among the digestion products of the K5 polysaccharide used. It is possible that these are products of an additional glycosidase activity of lyase III, although other mechanisms cannot be completely ruled out.

It has become apparent that heparan sulfates (HSs)[1] occurring as components of proteoglycans participate in a number of important biological processes and interact with a wide range of proteins, such as cytokines and chemokines, growth factors, and pathogenic agents in addition to the well-known interaction with the regulator of coagulation antithrombin III (*1*). HSs are also antigenic macromolecules (*2*−*4*). HS polysaccharides are complex carbohydrate chains. Alternating (1−4)-linked α-*N*-acetylglucosamine (GlcNAc) and *β*-glucuronic acid (GlcA) comprise 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 three sulfates per disaccharide unit, 6-O- and 2-N-disulfated glucosamine and 2-O-sulfated 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-sulfated or N-unsubstituted glucosamine (GlcN).

In the past, the presence of GlcN in HS chains was generally of little concern (*5*). Its importance has been recognized only recently (*4*, *6*−*11*). This unusual residue in HS or heparin is not an artifactual product during preparation and purification as originally thought but is presumably formed through regulated, incomplete action of an N-deacetylase/N-sulfotransferase enzyme (*12*), although endosulfamidase action cannot be excluded. GlcN amounts to 0.7−4% of total glucosamine in HS (*12*) or one to two residues in each HS chain (*13*) depending on the source. The location of the GlcN residue has been investigated, and evidence has been presented that it occurs largely clustered at the polysaccharide−protein linkage region of HS chains with less frequent peripheral location (*12*).

The antigens recognized by two monoclonal antibodies (mAb), 10E4 (*2*) and JM403 (*3*), have been shown to include a GlcN unit (*4*, *6*). The antigen recognized by 10E4 antibody is closely associated with prion lesions in the brain of mice infected with scrapie (*14*). In an earlier study (*4*), the HS sequence recognized by this antibody was investigated using

[†] This research was supported by a U.K. Medical Research Council program grant (G9601454) and a MRC strategic grant (G9631690) and in part by a grant from Polysackaridforskning AB (Uppsala, Sweden).

* To whom correspondence should be addressed. Phone: 44-20-8869 3252/55. Fax: 44-20-8869 3253. E-mail: w.chai@imperial.ac.uk.

[‡] MRC Glycosciences Laboratory, Imperial College Faculty of Medicine.

[§] Department of Medical Biochemistry and Microbiology, Uppsala University.

[1] Abbreviations: CID, collision-induced dissociation; ES-MS, electrospray mass spectrometry; GlcA, glucuronic acid; GlcN, N-unsubstituted glucosamine; GlcNAc, *N*-acetylglucosamine; GlcNS, N-sulfated glucosamine; GlcN(6S), 6-O-sulfated glucosamine; GlcNAc(6S), 6-O-sulfated *N*-acetylglucosamine; HexN, hexosamine; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; HA, hyaluronic acid; HS, heparan sulfate; IdoA, iduronic acid; NGL, neoglycolipid; PGC, porous graphitized carbon; SAX, strong-anion exchange; UA, hexuronic acid; ΔUA, 4,5-unsaturated hexuronic acid.

10.1021/bi036250k CCC: $27.50
Published on Web 06/08/2004

different preparations of HS with varying degrees of sulfation. The highest antigenicity was observed in a HS preparation with the lowest sulfate content. The HS polysaccharide was partially depolymerized with heparin lyase III, and the oligosaccharide fragments were examined for 10E4 antigen expression by the neoglycolipid technology (*15*). A nonsulfated antigen-positive tetrasaccharide was isolated and found to be an unusual sequence containing a GlcN residue: ΔUA−GlcN−UA−GlcNAc (*4*).

Heparin lyases have been widely used as degradative enzymes to deplete biological extracts from HS, for example, to establish whether HS is involved as a host component in the infectivity of prion protein (*16*). Heparin lyases are also valuable tools in sequence determination of HS and in generating oligosaccharide fragments for assigning biological roles to specific HS sequences. Three members of this family, lyases I, II, and III, have been widely used for sequencing (*17*, *18*) and generating HS oligosaccharide fragments (*19*, *20*) for defining structure−function relationships. However, the analysis of fine details of the substrate specificities of the heparin lyases has been difficult, and some published results have been contradictory. This is mainly due to the difficulties in obtaining enzymes and oligosaccharide substrates of high purity and also due to the intrinsic broad specificities of the enzymes. Generally, it is considered that lyases have primary and secondary cleavage sites, the former being the most readily cleaved. In the case of lyase III, the linkages between GlcNAc or GlcNS and GlcA are considered the primary sites, whereas the linkages between GlcNAc, GlcNAc(6S) or GlcNS, and IdoA are the secondary sites for cleavage (*21*).

Here, we investigate the specificity of heparin lyase III toward the GlcN−GlcA sequence in a HS-like polysaccharide using a recombinant lyase III of high purity. Due to the low abundance of GlcN in HS, we have used as substrates the partially de-N-acetylated polysaccharide of *Escherichia coli* K5 strain and the derived oligosaccharide fragments. The K5 polysaccharide consists of the repeating linear sequence −4GlcAβ1−4GlcNAcα1−. This backbone sequence is shared with the least sulfated region of HS. This polysaccharide has been used as a model for various studies of HS (*22*−*24*). In the present investigation, the K5 polysaccharide has been modified by limited alkali hydrolysis for partial removal of N-acetyl groups. The specificity of lyase III toward the linkage of GlcN−GlcA is deduced by sequencing of the oligosaccharide products by negative-ion electrospray mass spectrometry (ES-MS) with collision-induced dissociation (CID) and MS/MS scanning.

MATERIALS AND METHODS

Materials. K5 polysaccharide was kindly given by Dr. V. Cavazzoni (University of Milan, Italy). Recombinant heparin lyase III (E.C. 4.2.2.8), produced in *Flavobacterium heparinum*, was from IBEX Technologies (Montreal, Quebec, Canada). HS disaccharide standards IV-A (Di-A) and IV-H (Di-H) were from Sigma (Poole, U.K.).

Partial De-N-Acetylation of K5 Polysaccharide. K5 polysaccharide was partially de-N-acetylated in a random fashion by alkali hydrolysis (*25* and Benito Casu, Ronzoni Institute, Milan, personal communication). In brief, freshly prepared 2 M NaOH was added to the dry polysaccharide at a concentration of 10 mg/mL polysaccharide. Incubation was performed at 60 °C for 40 min. The reaction was stopped by acidification to pH 3 with 4 M HCl with the sample on ice, followed by neutralization with 2M NaOH to pH 7−8. The reaction mixture was dialyzed against water and lyophilized.

Heparin Lyase III Digestion. The partially de-N-acetylated K5 (deAc-K5) was partially depolymerized by limited digestion with heparin lyase III essentially as described (*4*), and the reaction was stopped at around 50% completion as judged by UV absorption at 232 nm of aliquots after dilution with 0.03 M HCl (*26*). Briefly, the deAc-K5 (30 mg) was incubated with the lyase (60 mU) in 2 mL of 50 mM sodium phosphate buffer (pH 7.0, containing 0.1 M NaCl) at 30 °C for 5 h. The high molecular mass fraction, Fx (see below), obtained after gel filtration chromatography was redigested under the same conditions.

Further digestion of HPLC-purified hexasaccharides derived from lyase III-digested deAc-K5 was carried out under two conditions: controlled and forced. Under controlled conditions for partial digestion of hexasaccharides containing GlcN, a lower amount of lyase was used for 10 μg of substrates. Initially, 40 μU and 200 μU of enzyme were added in 30 μL of phosphate buffer at 30 °C, but no digestion took place. Digestion started when 1 mU of lyase III was added. The reaction was not complete within 17 h, even after the addition of 1 mU of enzyme and further incubation of 24 h, as described under Results. Under forced conditions for exhaustive digestion, 10 μg of hexasaccharides and 10 mU of lyase III were incubated in 30 μL of phosphate buffer at 30 °C. The digestion was complete within 17 h as assessed by HPLC of the digestion products. HPLC analysis was used to monitor the progress of the digestions.

Fractionation by Gel Filtration Chromatography. The digestion mixture of deAc-K5 was desalted on a short Sephadex G-10 column (1.6 cm × 36 cm) before fractionation on a Bio-Gel P-4 column (1.6 cm × 90 cm). Elution was carried out with 0.2 M NH_4OAc (pH 6.7) at a flow rate of 15 mL/h and monitored on-line by refractive index and UV at 232 nm. The pooled fractions, F1 to Fx (Figure 1A), were lyophilized and coevaporated with water to remove NH_4OAc. When Fx was similarly redigested with lyase III, subfractions f1 to f8 were obtained (Figure 1B). Quantitation was by carbazole assay as described (*27*).

HPLC. For analysis of oligosaccharide fractions and for separation of fraction F3, strong-anion exchange (SAX) HPLC on a Spherisorb S5-SAX column (4.6 mm × 250 mm, Waters, Milford, MA) was used with a titanium-lined Gilson liquid chromatograph system fitted with a variable wavelength UV detector monitored at 232 nm. Elution was carried out at 30 °C with a linear gradient of NaCl (solvent A, 0.2 M NaCl, and solvent B, 1.5 M NaCl, pH 3.5; 0−10% B in 20 min). The subfractions of F3 were collected, desalted on a G-10 column, and lyophilized.

For HPLC analysis of digestion products of the purified hexasaccharides, a porous graphitized carbon (PGC) column (Hypercarb 5μ, 30 mm × 4.6 mm, from Hypersil, Runcorn, U.K.) was used with UV detection at 232 nm. A gradient of acetonitrile was used for elution (solvent A, H_2O containing

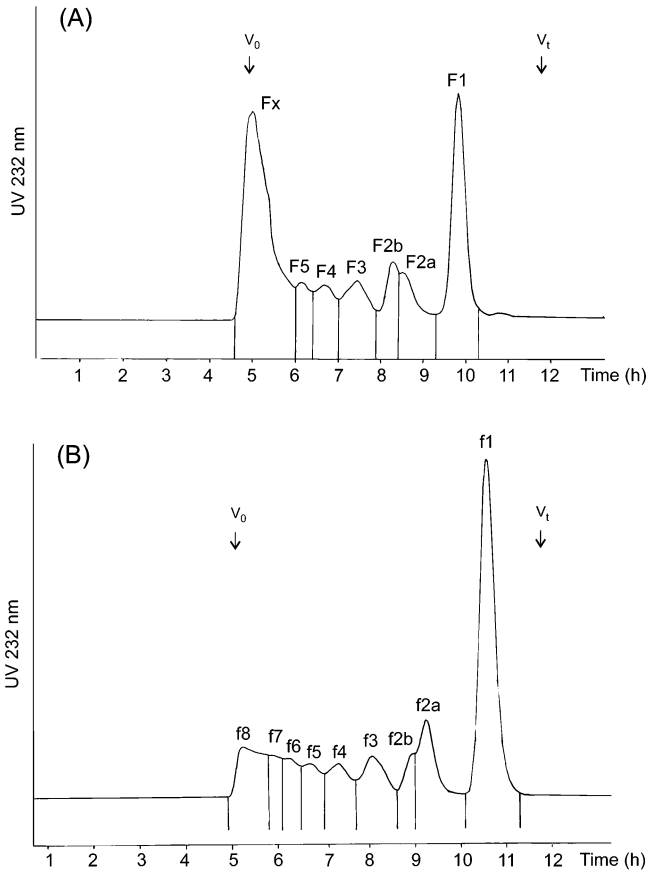


FIGURE 1: Gel filtration profiles of partially de-N-acetylated K5 (deAc-K5) digested by lyase III under controlled conditions. Polysaccharide deAc-K5 was partially digested by heparin lyase III and fractionated on Bio-Gel P4 as described under Materials and Methods (panel A). The high oligomeric fraction Fx was redigested with lyase III under the same conditions and fractionated on Bio-Gel P4 (panel B).

0.05% TFA, and solvent B, H_2O/acetonitrile 20:80 containing 0.05% TFA; 0−50% B in 40 min).

Electrospray Mass Spectrometry. ES-MS was carried out on a Micromass Q-Tof instrument (Micromass, Manchester, U.K.) in negative-ion mode. Nitrogen was used as the desolvation and nebulizer gas at a flow rate of 250 L/h and 15 L/h, respectively. Source temperature was 80 °C, and the desolvation temperature was 150 °C. For high sensitivity ES-MS analysis, sodium cations of oligosaccharide fractions were removed and converted into their ammonium salts with a minicolumn of cation exchange (AG50W-X8, H form) as described previously (*20*) and dissolved in acetonitrile/1 mM aq NH_4HCO_3 1:1 (v/v), typically at a concentration of ∼10 pmol/μL, of which 5 μL was loop-injected. Mobile phase (acetonitrile/1 mM aq NH_4HCO_3 1:1, v/v) was delivered by a syringe pump at a flow rate of 10 μL/min. Cone voltage was maintained at 50 eV, while capillary voltage was at 3 kV.

For collision-induced dissociation (CID) MS/MS product ion scanning, a cone voltage of 80 eV was employed to maximize the intensity of singly charged molecular ion. Argon was used as the collision gas at a pressure of 1.7 bar (measured in the gas line prior to the gas cell), and the collision energy was adjusted between 25 and 42 eV for optimal sequence information (25 eV for tetrasaccharides, 38 eV for pentasaccharides, and 42 eV for hexasaccharides).

Table 1: ESMS of Bio-Gel P4 Fractions of deAc-K5 after Partial Digestion with Heparin Lyase III

	$[M - H]^-$ [a]	assignment				
fraction	*m/z* (rel abun)	chain length	ΔUA	UA	HexN	HexNAc
F1	378.1 (100)	di	1	0	0	1
F2a	715.2 (100)	tetra	1	1	1	1
	757.2 (15)	tetra	1	1	0	2
F2b	757.2 (100)	tetra	1	1	0	2
F3	1094.3 (100)	hexa	1	2	1	2
	1136.3 (70)	hexa	1	2	0	3
F4	1474.4 (100)	octa	1	3	1	3
	1432.4 (75)	octa	1	3	2	2
F5	1811.4 (100)	deca	1	4	2	3
	1853.4 (75)	deca	1	4	1	4

[a] Molecular ions of the main components together with the relative abundances (in parentheses).

RESULTS

Oligosaccharide Fragments of Partially De-N-Acetylated K5 Obtained after Limited Digestion with Lyase III. The fractions obtained by gel filtration chromatography (Figure 1A) after limited digestion (enzyme-to-substrate ratio, 2 μU/1 μg) of deAc-K5 with lyase III were analyzed by negative-ion ES-MS (Table 1). The chain length and compositions of the main components in each fraction, in terms of hexuronic acid (UA), 4,5-unsaturated hexuronic acid (ΔUA), *N*-acetylhexosamine (HexNAc), and hexosamine (HexN) residues, can be readily deduced from the masses of $[M - H]^-$ ions detected. Among the fractions analyzed, about half of the tetra- and hexasaccharide fragments (F2a, F2b, and F3) contained one GlcN residue, whereas octa- and decasaccharide fragments (F4 and F5) contained one or two GlcN, indicating the success of alkali treatment for partial de-N-acetylation. Although there were fully N-acetylated sequences detected in the di- to hexasaccharides, no fully N-acetylated sequences were detected in the octa- and decasaccharides. It was interesting that two tetrasaccharide fractions were obtained and the earlier eluting peak F2b contained the fully N-acetylated tetrasaccharide whereas the later eluting peak F2a contained predominantly a tetrasaccharide with a GlcN residue. Although there is an overlap, the fully N-acetylated tetrasaccharide has a larger hydrodynamic volume and eluted earlier, in agreement with our previous findings (*4*).

The gel filtration profile (Figure 1B) of Fx (i.e., the highest molecular mass fraction from initial digestion) after redigestion with lyase III was very similar to that observed after the first digestion. By ES-MS analyses (data not shown) f1 to f5 were found to have similar compositions to F1 to F5 obtained after the first digestion, although the fully N-acetylated components were less abundant than previous respective fractions. This was apparent for the tetrasaccharide fractions as shown in Figure 1B; the fully N-acetylated f2b was less abundant than f2a. Fractions f6 and f7, which are likely to contain 12- and 14-mers, respectively, were not analyzed.

Sequence Determination of Tetra- to Hexasaccharides Obtained after Lyase III Digestion. Strong-anion exchange HPLC was used to further fractionate and purify di- to hexasaccharide fractions F1 to F3. Only one disaccharide (F1-1) was detected in F1 (Figure 2A), and based on comparison with the retention time of standard disaccharides, F1-1 was assigned as the GlcNAc-containing disaccharide

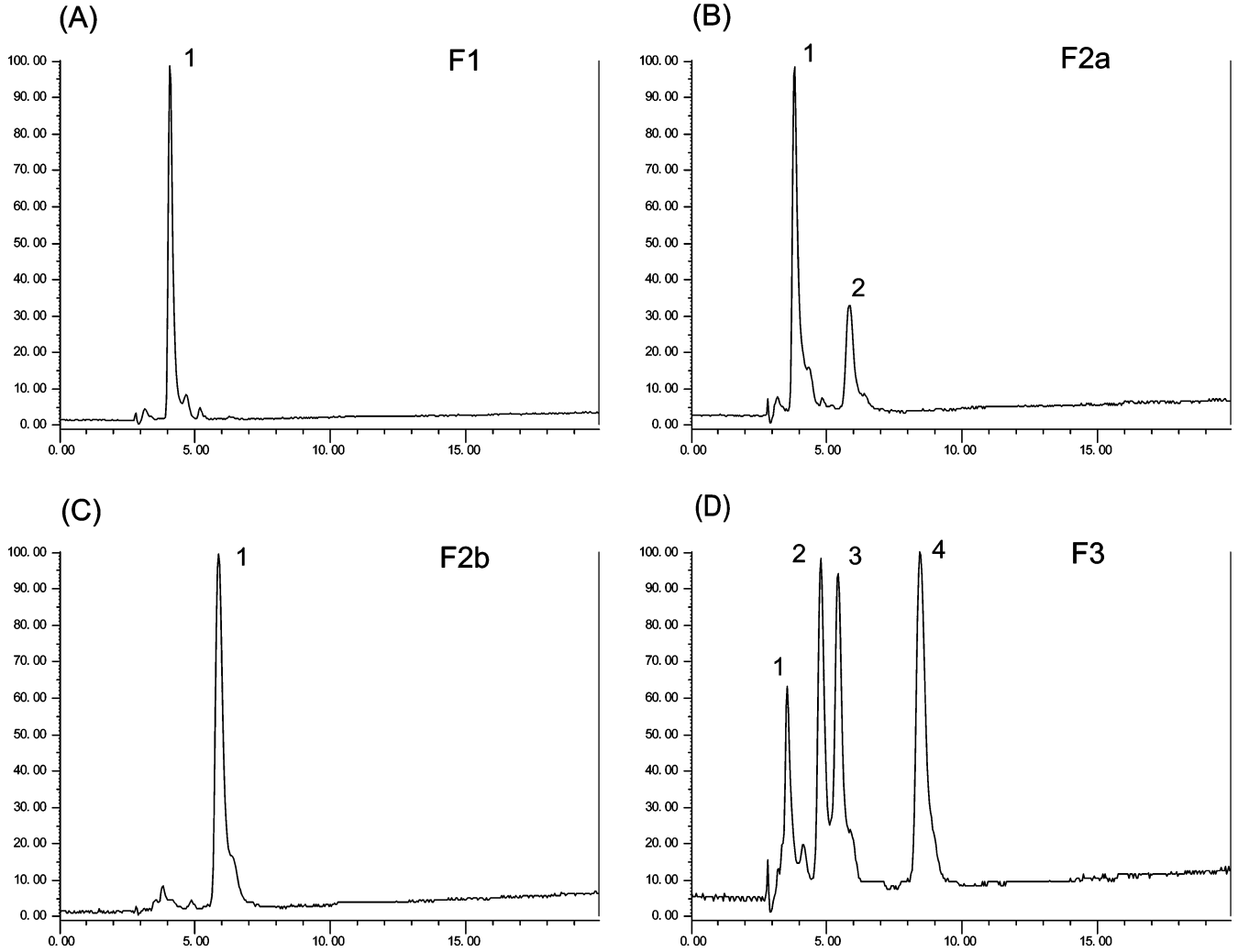


FIGURE 2: HPLC fractionation (with SAX column) of di- to hexasaccharide fractions F1 (panel A), F2a (panel B), F2b (panel C), and F3 (panel D).

Table 2: Sequence Determination by ES-MS/MS of HPLC Fractions of deAc-K5 after Partial Digestion with Heparin Lyase III

fractions	$[M - H]^-$	sequence assignment	designation
F1-1	378.1	ΔUA−GlcNAc	di-A
F2a-1	715.2	ΔUA−GlcN−GlcA−GlcNAc	tetra-H
F2a-2	757.2	ΔUA−GlcNAc−GlcA−GlcNAc	tetra-A
F2b-1	757.2	ΔUA−GlcNAc−GlcA−GlcNAc	tetra-A
F3-1	936.3	(a)[a] GlcN−GlcA−GlcNAc−GlcA−GlcNAc	penta-H1
		(b)[a] GlcNAc−GlcA−GlcN−GlcA−GlcNAc	penta-H2
F3-2	1094.3	ΔUA−GlcN−GlcA−GlcNAc−GlcA−GlcNAc	hexa-H1
F3-3	1094.3	ΔUA−GlcNAc−GlcA−GlcN−GlcA−GlcNAc	hexa-H2
F3-4	1136.3	ΔUA−GlcNAc−GlcA−GlcNAc−GlcA−GlcNAc	hexa-A

[a] The ratio of pentasaccharide sequence a to b in F3-1 is 2:1 based on MS/MS analysis.

di-A (Table 2). The disaccharide containing GlcN (ΔUA−GlcN, di-H) was not detected. As indicated by the masses of $[M - H]^-$ detected by ES-MS (Table 2), there were two tetrasaccharides in F2a (Figure 2B). The main component F2a-1 was deduced to be a mono-de-N-acetylated tetrasaccharide (tetra-H), and the minor component F2a-2 the fully N-acetylated tetrasaccharide, tetra-A (Figure 2B). Fraction F2b was deduced to contain mainly the fully N-acetylated tetrasaccharide tetra-A (Figure 2C) with a retention time identical to that of F2a-2. There were four subfractions in F3 (Figure 2D). F3-1 contained unusual pentasaccharides with the composition of $GlcN_1{\cdot}GlcNAc_2{\cdot}GlcA_2$ and F3-4 contained the fully acetylated hexasaccharide hexa-A. Fractions F3-2 and F3-3 were both hexasaccharides with one GlcN residue as indicated by ES-MS analysis (Table 2).

The sequences of the GlcN-containing oligosaccharides were determined directly by ES-MS/MS. For reference, the fully N-acetylated tetra-A and hexa-A were first used to establish the fragmentation patterns. The CID MS/MS product ion spectra were dominated by the nonreducing terminal A-, B-, and C-type of cleavages (*28*), as illustrated by the spectra of tetrasaccharides F2a-1 and F2b-1 (Figure 3). In the spectrum of F2b-1 (Figure 3A), B_1 and C_1 ions (*m*/*z* 157 and *m*/*z* 175, respectively) served to identify the nonreducing terminus as a ΔUA residue and C_2 at *m*/*z* 378 identified a GlcNAc linked to ΔUA. The doublet at *m*/*z* 277/

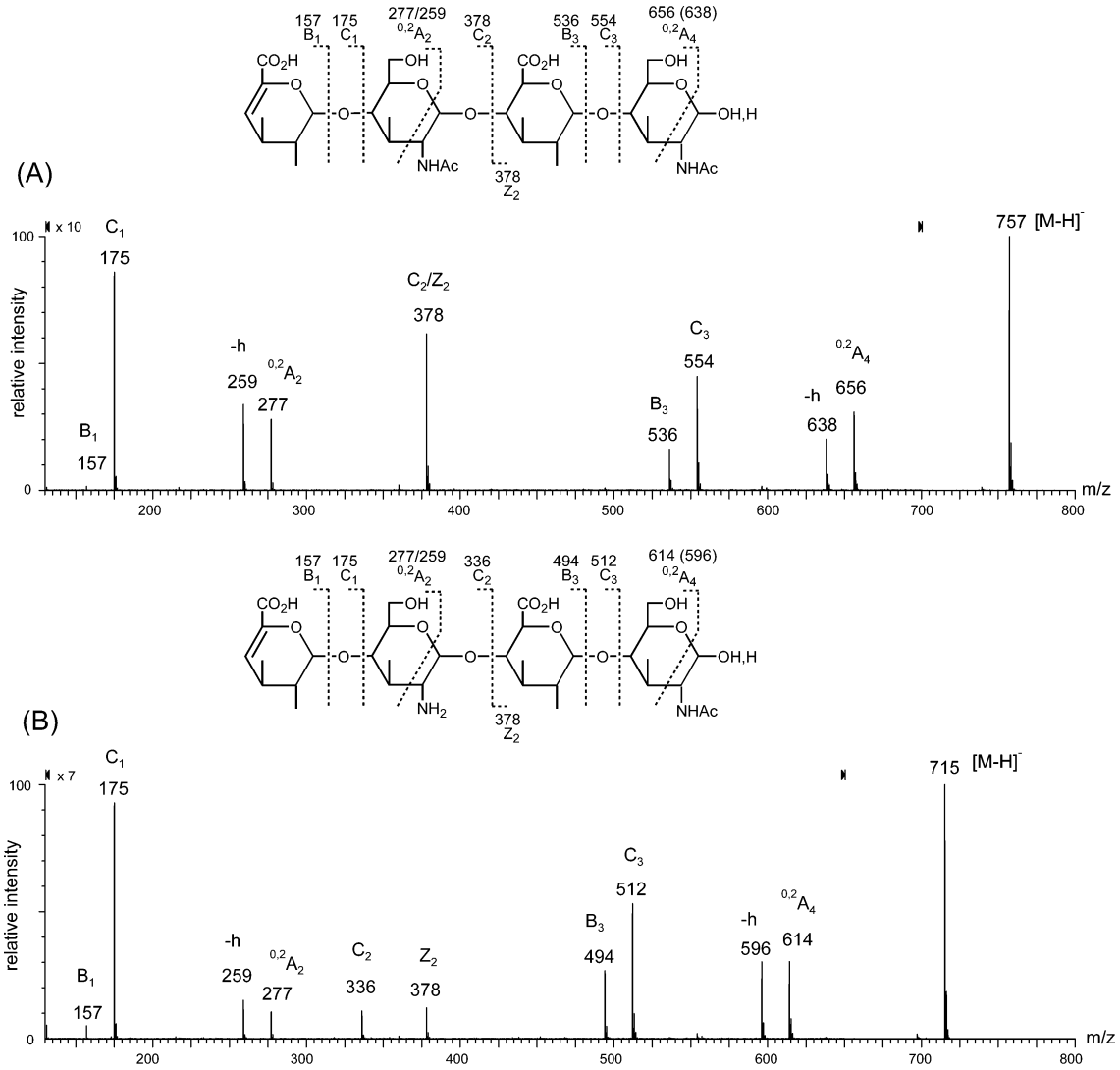


FIGURE 3: ES-MS/MS product ion spectra of fully N-acetylated tetrasaccharide F2b-1 (panel A) and the GlcN-containing tetrasaccharide F2a-1 (panel B).

259 resulting from $^{0,2}A_2$ cleavage and its dehydrated form was typical of a 4-linked GlcNAc (*29*, *30*). B_3 and C_3 ions (*m*/*z* 536 and 554, respectively) indicated a GlcA. The molecular ion at *m*/*z* 757 and a $^{0,2}A_4$ doublet (*m*/*z* 656 and 638) serve to clearly define a −4GlcNAc at the reducing terminal. Z_2 was the only reducing terminal fragment ion, and its *m*/*z* value at 378 was identical to the nonreducing terminal C_2 ion. The assignment of *m*/*z* 378 as representing two different fragment ions was corroborated by the MS/MS product ion spectrum (not shown) of the reduced tetrasaccharide F2b-1. After reduction, the terminal GlcNAc became an open chain, and there was an increment of 2 Da, and therefore, the C_2/Z_2 ion split into two ions: C_2 (*m*/*z* 378), which was not affected by the reduction, and Z_2, which was shifted to *m*/*z* 380. By comparison with the fragmentation pattern obtained for F2b-1, the sequence of F2a-1 can be readily deduced and designated tetra-H (Table 2). As shown in Figure 3B, the fragment ions B_1, C_1, and $^{0,2}A_2$ were identical to those of F2b-1, whereas C_2 was at *m*/*z* 336. The difference (42 Da lower than the corresponding fragment ion at *m*/*z* 378 of F2b-1) indicated a GlcN. The location of the GlcN residue at the internal position is corroborated by the subsequent shift of B_3, C_3, and $^{0,2}A_4$ ions and the unchanged Z_2 ion at *m*/*z* 378.

In a similar fashion, the sequences of three hexasaccharides F3-2, F3-3, and F3-4 were deduced (Figure 4 and Table 2). Fractions F3-2 and F3-3 each contained a single GlcN. In F3-2 (hexa-H1, Figure 4A), the GlcN was located next to the terminal ΔUA, whereas in F3-3 (hexa-H2, Figure 4B), it was at an internal position between two GlcA residues. F3-4 was identified as the fully N-acetylated hexasaccharide (hexa-A, Table 2, spectrum not shown). F3-1 showed a single molecular ion at *m*/*z* 936.3 consistent with a pentasaccharide with a GlcN but without the ΔUA residue that would be expected to be generated by the lyase. The MS/MS sequence analysis (spectrum not shown) indicated that there were two isomeric pentasaccharide sequences (Table 2) with the GlcN residues, in one at the nonreducing terminal (penta-H1) and in the other at an internal position (penta-H2).

Sequence analysis of the di- to hexasaccharides in f1−f3, obtained after repeated digestion, was carried out by PGC-HPLC profiling rather than MS/MS. The resolution of PGC-HPLC is higher (*31*) than SAX-HPLC, and thus an unambiguous comparison can be made of the HPLC profile with those of the sequence-defined di- to hexasaccharides obtained after the first digestion. The PGC-HPLC chromatograms of disaccharide standards, di-H and di-A, can be used to demonstrate its resolving power (Figure 5A). The single peak

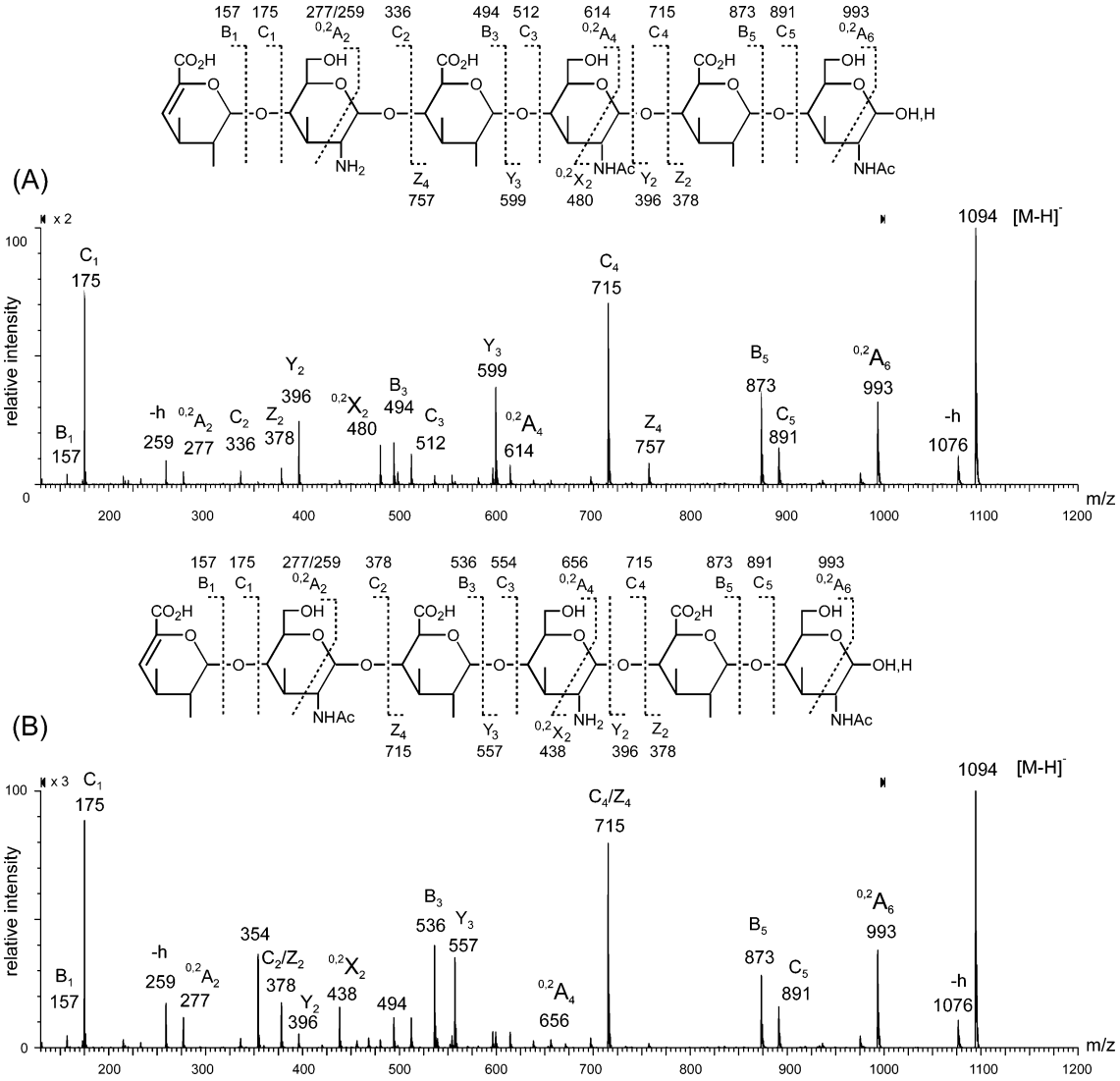


FIGURE 4: ES-MS/MS product ion spectra of two isomeric GlcN-containing hexasaccharides, F3-2 (panel A) and F3-3 (panel B).

at retention time 9.7 min was from di-H. The doublet at 14.0 and 17.3 min was from di-A. The split peaks of di-A were assigned to α- and *β*-anomers of di-A that were separable due to the high resolution power. The assignment of the α,*β*-isomers was tentative and based on knowledge that in aqueous solution at a neutral pH, the α-isoform is more abundant (*32*). However, in di-H, the α-anomeric form was the dominant configuration, and only a single peak appeared. This is consistent with previous NMR spectroscopic studies, which have shown that HexNAc-terminating glycosaminoglycan oligosaccharides have α,*β*-isoforms, whereas HexNS- and HexN-terminating oligosaccharides are dominated by α-isoforms (*32*, *33*).

The PGC-HPLC profiles of the respective di- to hexasaccharide fractions obtained from the first (F1, F2a, F2b, and F3) and repeated digestion (f1, f2a, f2b, and f3) were very similar. As examples, the chromatograms of f2a and f3 are shown in Figure 5. Apart from some differences in intensity, the retention times of eluted peaks of these were identical to those of F2a and F3, respectively (data not shown). Chromatograms of f1 and f2b each showed only a single component (not shown). Fraction f1 gave two peaks the retention times of which were identical to those of α- and *β*-peaks of di-A (Figure 5A), whereas the positions of the two peaks in fraction f2b were the same as those of 2α and 2*β* of fraction f2a (Figure 5B). As with F3, fraction f3 contained four components, and each showed α,*β*-separation (Figure 5C). Although the peak splitting due to α,*β*-separation is not ideal for preparative work, the higher resolving power provides more reliable comparison.

It is interesting to note that a GlcN residue was not found at a reducing terminal position in any of the tetra-, penta-, and hexasaccharides identified, even those obtained after repeated digestion. This indicated that GlcN−GlcA is not a primary site for cleavage by lyase III and that GlcNAc−GlcA is the preferred site.

The GlcN-containing tetrasaccharide F2a-1 (tetra-H) has a sequence that is similar to that of the 10E4 antigen-positive tetrasaccharide isolated after heparin lyase digestion of a heparan sulfate preparation that is bound by the monoclonal antibody 10E4 (*4*), the difference being that in tetra-H the internal hexuronic acid is GlcA, whereas in the HS-derived antigen-positive tetrasaccharide, this had not been identified. In experiments to be described elsewhere (C. Leteux, W. Chai, C. Westling, U. Lindahl, A. M. Lawson, and T. Feizi, unpublished observations), the GlcN-containing tetrasaccharide F2a-1 (tetra-H), after conversion into a neoglycolipid (NGL), behaves identically to the tetrasaccharide isolated

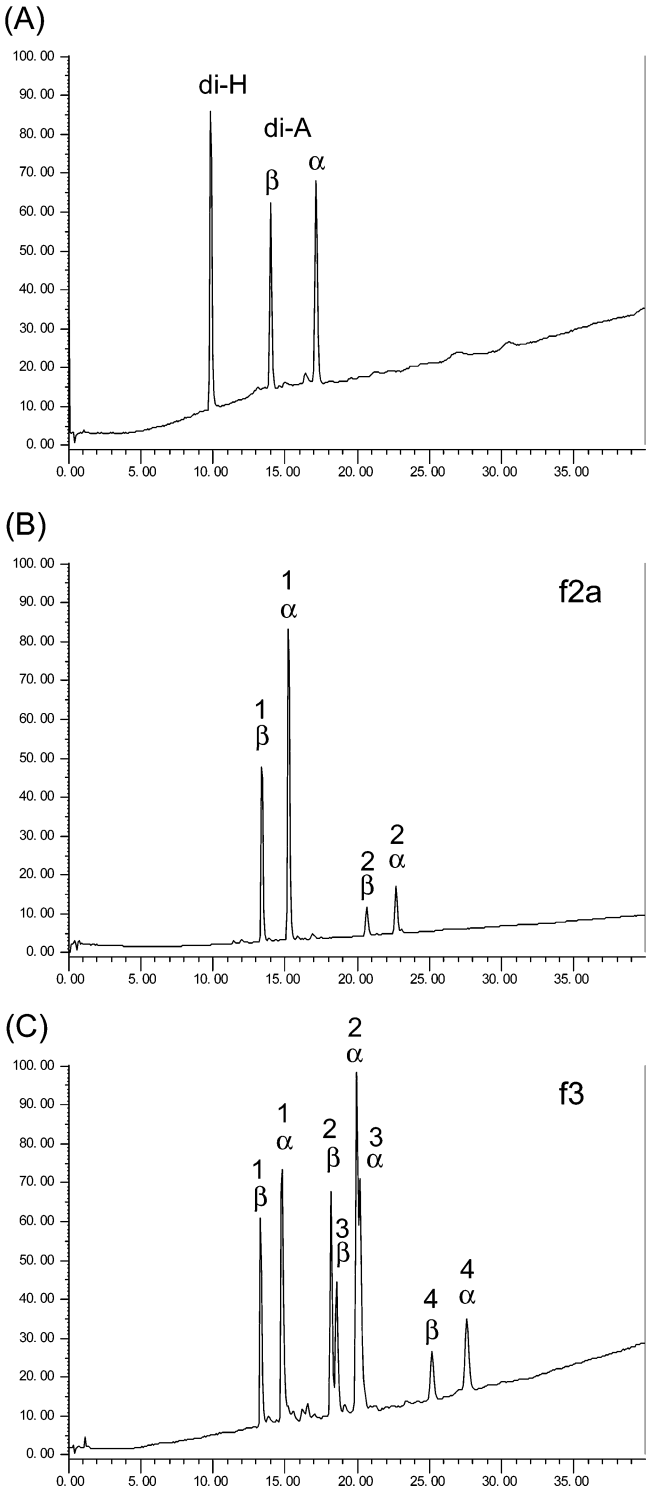


FIGURE 5: HPLC analysis (with PGC column) of di- to hexasaccharides: disaccharide standards di-H and di-A (panel A), tetrasaccharide fraction f2a (panel B), and hexasaccharide fraction f3 (panel C).

from a HS preparation in binding by the mAb 10E4 (*4*). In contrast, the NGL of the fully N-acetylated tetrasaccharide F2a-2 (tetra-A) is not bound. At the request of reviewers of our manuscript, the immunochemical data, corroborating the requirement of a GlcN for binding to the tetrasaccharide fragment, are illustrated here (Figure 6).

Lyase III Digestion of Sequence-Defined Hexasaccharides. The purified homogeneous hexasaccharides F3-2, F3-3, and F3-4 were used as substrates for action of lyase III under

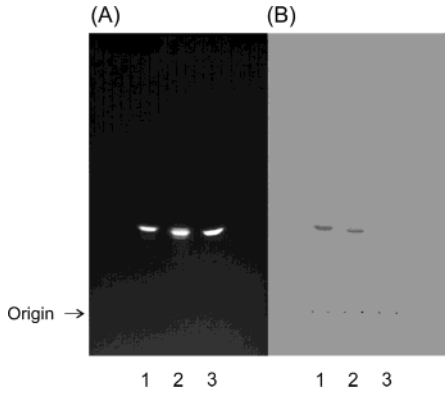


FIGURE 6: Antigenic analysis of the NGLs of tetrasaccharides. Oligosaccharides F2a (tetra-H) and F2a-2 (tetra-A) were converted to fluorescent NGLs as described (*4*), and together with the NGL of the 10E4 antigen-positive tetrasaccharide isolated earlier from HS, they were resolved by high-performance TLC using a solvent mixture of chloroform/methanol/water, 60:35:8 (by volume). The fluorescent NGLs were visualized under UV light at 366 nm in panel A (lanes 2, 3, and 1, respectively). Binding by 10E4 antibody was detected in panel B as described (*4*). In brief, after blocking nonspecific binding sites with 1% casein (Pierce, U.K.) in Tris-buffered saline, 10 mM Tris-HCl buffer, 150 mM NaCl, pH 8.0 (TBS), the TLC plate was overlaid with 10E4 antibody (10 μg/mL) in 1% casein; antibody-binding was detected using protein-LA-peroxidase (1/500 of stock) in 1% casein followed by FAST DAB peroxidase substrate.

Table 3: HPLC Analysis of Cleavage Products Obtained from Hexasaccharides under Controlled Conditions for Partial Digestion by Heparin Lyase III

fractions	digestion thttime (h)[b]	compositions (%)[a] hexa	tetra-H	tetra-A	di-H	di-A
F3-2	1	67.7	14.0	*c*	3.0	15.3
	17	47.7	22.0	*c*	5.3	25.0
	+2	46.0	21.5	*c*	5.6	26.9
	+24	39.4	23.9	*c*	6.6	30.1
F3-3[d]	2	33.4	35.5	*c*	0.5	30.6
	18	30.7	37.0	*c*	0.6	31.7
	+2	29.0	36.0	*c*	1.3	33.7
	+24	25.8	36.8	*c*	2.8	34.6

[a] Compositions determined by calculation of UV absorption at 232 nm. Hexa, the parent hexasaccharide; tetra-H, GlcN-containing tetrasaccharide F2a-1; tetra-A, fully *N*-acetylated tetrasaccharide F2b-1; di-H, ΔUA−GlcN; di-A, ΔUA−GlcNAc. [b] +2 and +24 denote 2 and 24 h after addition of a further aliquot of lyase III (1 mU). [c] Not detected. [d] Contained ~15% F3-2 as impurity due to overlapping peaks. See Figure 2D.

controlled conditions for partial digestion (enzyme-to-substrate ratio 0.1 mU/1 μg) and under forced conditions for exhaustive digestion (enzyme-to-substrate ratio 1 mU/1 μg). The substrate concentration was more dilute (0.3 μg/μL) than in the large-scale digestion of the polysaccharide (15 μg/μL). Due to the potential complexity of the digestion products derived from complete and incomplete digestion by lyase III, high-resolution PGC-HPLC was used for analysis of the digestion products. These products are presented in Tables 3 and 4 and used to deduce the specificity of the enzyme.

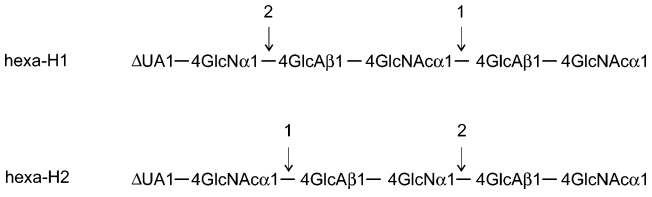
Under the controlled conditions for partial digestion, the parent hexasaccharides were gradually cleaved at the GlcNAc−GlcA linkage, and tetra-H and di-A were produced (Table 3 and Scheme 1). Cleavage at site 2 (Scheme 1) is very limited as can be judged from the limited amount of di-H detected. It is deduced that the absence of tetra-A is

Table 4: HPLC Analysis of Cleavage Products Obtained from Hexasaccharides under Forced Conditions for Exhaustive Digestion by Heparin Lyase III

fractions	digestion time (h)	compositions (%)[a] hexa	tetra-H	tetra-A	di-H	di-A
F3-2	2	*c*	22.4	*c*	19.6	58.0
	17	*c*	8.3	*c*	29.7	61.0
F3-3	2	*c*	24.8	*c*	29.1	46.1
	17	*c*	2.0	*c*	33.0	65.0
F3-4	2	*c*	*c*	*c*	2.5[b]	97.5
	17	*c*	*c*	*c*	5.3[b]	94.7

[a] Compositions determined by calculation of UV absorption at 232 nm. Hexa, the parent hexasaccharide; tetra-H, GlcN-containing tetrasaccharide F2a-1; tetra-A, fully *N*-acetylated tetrasaccharide F2b-1; di-H, ΔUA−GlcN; di-A, ΔUA−GlcNAc. [b] From an impurity. See comment in Results section. [c] Not detected.

Scheme 1



due to the slow action at site 2 (Scheme 1) and subsequent cleavage at site 1 of any produced tetra-A resulting in di-A. Even after 24 h incubation and further addition of lyase, the cleavage at GlcN−GlcA was limited. This was indicated by the small amounts of di-H produced and high abundances of tetra-H (Table 3). However, di-A generated was about 5 times more abundant than di-H in F3-2 and 10−60 times more abundant in F3-3, although not all the GlcNAc−GlcA bonds were completely cleaved under these conditions.

Under the conditions of exhaustive digestion, the fully N-acetylated hexasaccharide F3-4 produced almost exclusively di-A even after incubation for 2 h (Table 4). A small amount of di-H (Table 4) is likely to have arisen from coeluted unknown higher oligomeric impurities containing GlcN. However, for the GlcN-containing hexasaccharides F3-2 and F3-3, the preferred cleavage at the GlcNAc−GlcA site (site 1, Scheme 1) was apparent even under the exhaustive digestion conditions. After 2 h incubation, there were no hexasaccharides detected. In addition to the disaccharides, considerable amounts of tetra-H were detected for both F3-2 and F3-3. Tetra-A was not detected. After 17 h, there were still small amounts of tetra-H remaining and the resulting disaccharides di-H and di-A were in the approximate ratio of 1:2 as expected.

Thus, the preference for the cleavage by lyase III at the linkage of GlcNAc−GlcA over GlcN−GlcA was apparent under both digestion conditions.

DISCUSSION

An understanding of the substrate specificities of heparin lyases is important for their application in fragmentation and analysis of the sequences that occur in glycosaminoglycans related to HS. However, the purities of the lyases and sequence-defined oligosaccharide substrates used for the investigation have often posed problems, and this has frequently led to ambiguous conclusions as discussed previously (*21*). In addition, the relatively broad specificities of the enzymes (*21*, *34*) give rise to further difficulties in interpretation. In the present study, a highly purified recombinant lyase III has been used. The specificity of heparin lyase III toward the GlcN−GlcA linkage has been deduced by analyses of the reaction products of polysaccharide and of sequence-defined oligosaccharides used as substrates.

Because the purities of the oligosaccharide substrates and analyses of the enzymatic reaction products are important, different HPLC methods have been employed. SAX-HPLC is sufficient for separation of isomeric structures with the same chain length. However, for a complex mixture containing oligosaccharides of different size, overlapping of peaks can be a problem. In this case, a PGC column is ideally suited for analysis, although α,*β*-anomeric isomers can complicate the HPLC profile. It is interesting that α,*β*-isoforms exist in oligosaccharides with a GlcNAc but not in those with a GlcN at the reducing end.

Mass spectrometry has been an important method for characterization of HS oligosaccharides with high sensitivity (*20*, *35*). The relatively regular, linear sequences of each class of glycosaminoglycans means that structural details, such as chain length and monosaccharide composition, together with the presence of sulfate and acetyl substitutions, can be deduced from the molecular mass information, which is sufficiently accurate with negative-ion ES-MS. CID and MS/MS scanning can provide sequence information (*4*, *36*, *37*). As illustrated in the present investigation, sequence analysis including determination of the location of GlcN residues can be readily carried out for the lyase digestion products, di-, tetra-, penta-, and hexasaccharides. The results have unambiguously shown that under the controlled digestion conditions, lyase III does not act at the linkage of GlcNα1−4GlcA, whereas cleavage occurs at the site GlcNAcα1−4GlcA (Scheme 1). Even under exhaustive digestion conditions GlcNα1−4GlcA is only partially cleaved.

In earlier studies, lyase III had been used to generate a range of oligosaccharide fragments from porcine intestine mucosal HS for testing for 10E4 antigenicity, and a tetrasaccharide fraction, after conversion to neoglycolipid, had been shown to be bound by mAb 10E4 (*4*). This antigen-positive tetrasaccharide consisted of a unique nonsulfated motif, ΔUA−GlcN−UA−GlcNAc. The tetrasaccharide with the sequence ΔUA−GlcN−GlcA−GlcNAc (tetra-H), isolated in the present study after heparin lyase III treatment of partially de-N-acetylated K5 polysaccharide, has also been shown to be bound by this antibody, whereas the analogue ΔUA−GlcNAc−GlcA−GlcNAc is not bound (Figure 6). Thus the requirement of an internal GlcN for 10E4 antibody binding to this particular tetrasaccharide sequence is corroborated, and it is clear that the antibody can accommodate an internal GlcA. It has emerged, moreover, that an important element of the 10E4 antigen-active tetrasaccharide, that is, an internal GlcN linked to a hexuronic acid (GlcN−GlcA in the K5 polysaccharide), is preserved due to its resistance to lyase III. It should be noted that the requirement for GlcN has so far been demonstrated only with the tetrasaccharide epitope containing a nonreducing-terminal unsaturated ΔUA residue; the relevance of this requirement to 10E4 binding to intact HS remains unclear. Previous work has demonstrated that 10E4 can recognize sulfated domains in HS (*2*, *38*). Further work is required to determine whether the nonsulfated, antigen-positive tetrasaccharide sequence identified repre-

sents part of a larger sulfated motif in the intact HS chain.

The pentasaccharide fragments detected without ΔUA residues were unexpected among the digestion products of the highly purified lyase III. Trisaccharide analogues were not observed. The pentasaccharides had GlcN or GlcNAc at the nonreducing and GlcNAc at the reducing terminus. Odd-numbered oligosaccharide fragments have also been observed for hyaluronic acid (HA) after HA lyase digestion (*39*). In this case, the odd-numbered oligomers were exclusively with ΔUA at the nonreducing terminus and GlcA at the reducing terminus but not with GlcNAc at both termini. This was attributed to either a secondary glycosidase activity of the lyase itself or to the action of a contaminating glycosidase. In another study (*40*) with HA from the same source as substrates, odd-numbered oligosaccharide fragments were detected as minor components with HA lyase only but not with HA hydrolase. This indicates that the odd-numbered oligomers are unlikely to be the simple cleavage products of the nonreducing end sequence of HA polysaccharide. The odd-numbered oligosaccharide fragments obtained from deAc-K5 after heparin lyase III digestion could be considered as the possible products of the following reactions: (a) cleavage by lyase III of the nonreducing terminal pentasaccharide sequences of the deAc-K5 polysaccharide, which terminate with GlcNAc or GlcN; (b) cleavage by a contaminating glycosidase that removes the terminal ΔUA of hexasaccharide fragments produced by lyase III; (c) cleavage by lyase III, which has an additional glycosidase activity. The relatively high abundances of the pentasaccharides compared with the hexasaccharides detected at UV 232 nm (see Figure 2D) suggest that they are unlikely to be the cleavage products derived from the nonreducing terminal sequence of the long deAc-K5 polysaccharide chains. The presence of a contaminating glycosidase that removes the ΔUA/GlcA is a possibility as an exohexuronidase that hydrolyzes the terminal ΔUA residues is produced by the soil bacterium *Flavobacterium heparinum* (*41*), in which the recombinant heparin lyase III is expressed and purified. However, an additional glycosidase activity of lyase III cannot be completely ruled out, and the origin of the pentasaccharides requires further investigation.

ACKNOWLEDGMENT

We thank IBEX Technologies for providing heparin lyase III used for the present study and Dr. Colin Herbert for assistance in oligosaccharide fractionation.

REFERENCES

1. Conrad, H. E. (1998) in *Heparin-Binding Proteins*, Academic Press, San Diego, CA.
2. David, G., Bai, X. M., Van Der Schueren, B., Cassiman, J.-J., and Van Der Berghe, H. (1992) Developmental changes in heparan sulfate expression: in situ detection with mAbs, *J. Cell Biol. 119*, 961−975.
3. van den Born, J., van den Heuvel, L. P. W. J., Bakker, M. A. H., Veerkamp, J. H., Assmann, K. J. M., and Berden, J. H. M. (1992) A monoclonal antibody against BM heparan sulfate induces an acute selective proteinuria in rats, *Kidney Int. 41*, 115−123.
4. Leteux, C., Chai, W., Nagai, K., Herbert, C. G., Lawson, A. M., and Feizi, T. (2001) 10E4 antigen of scrapie lesions contains an unusual nonsulfated heparan motif, *J. Biol. Chem. 276*, 12539−12545.
5. Riesenfeld, J., and Rodén, L. (1990) Quantitative analysis of *N*-sulfated, *N*-acetylated, and unsubstituted glucosamine amino groups in heparin and related polysaccharides, *Anal. Biochem. 188*, 383−389.
6. van den Born, J., Gunnarsson, K., Bakker, M. A. H., Kjellén, Kusche-Gullberg, M., Maccarana, M., Berden, J. H. M., and Lindahl, U. (1995) Presence of N-unsubstituted glucosamine units in native heparan sulfate revealed by a monoclonal antibody, *J. Biol. Chem. 270*, 31303−31309.
7. Norgard-Sumnicht, K., and Varki, A. (1995) Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted aminogroups, *J. Biol. Chem. 270*, 12012−12024.
8. Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-3A sulfates *N*-unsubstituted glucosamine residues, *J. Biol. Chem. 274*, 38155−38162.
9. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. M., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) A novel role for 3-*O*-sulfated heparan sulfate in herpes simplex virus 1 entry, *Cell 99*, 13−22.
10. Mani, K., Jönsson, M., Edgren, G., Belting, M., and Fransson, L.-Å. (2000) A novel role for nitric oxide in the endogenous degradation of heparin sulfate during recycling of glypican-1 in vascular endothelial cells, *Glycobiology 10*, 577−586.
11. Ding, K., Jönsson, M., Mani, K., Sandgren, S., Beltong, M., and Fransson, L.-Å. (2001) *N*-Unsubstituted glucosamine in heparan sulfate of recycling glypican-1 from suramin-treated and nitrite-deprived endothelial cells, *J. Biol. Chem. 276*, 3885−3894.
12. Westling, C., and Lindahl, U. (2002) Location of N-unsubstituted glucosamine residues in heparan sulfate, *J. Biol. Chem. 277*, 49247−49255.
13. Toida, T., Yoshida, H., Toyoda, H., Koshiishi, I., Imanari, T., Hileman, R., Fromm, J. R., and Linhardt, R. J. (1997) Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissue and species, *Biochem. J. 322*, 499−506.
14. McBridge, P. A., Wilson, M. I., Eikelenboom, P., Tunstall, A., and Bruce, M. E. (1998) Heparan sulfate proteoglycan is associated with amyloid plaques and neuroanatomically targeted PrP pathology throughout the incubation period of scrapie-infected mice, *Exp. Neurol. 149*, 447−454.
15. Feizi, T., Stoll, M. S., Yuen, C.-T., Chai, W., and Lawson, A. M. (1994) Neoglycolipids: probes of oligosaccharide structure, antigenicity and function, *Methods Enzymol. 230*, 484−519.
16. Deleault, N. R., Lucassen, R. W., and Supattapone, S. (2003) RNA molecules stimulate prion protein conversion, *Nature 425*, 717−720.
17. Merry, C. L. R., Lyon, M., Deakin, J. A., Hopwood, J. J., and Gallagher, J. T. (1999) Highly sensitive sequencing of the sulfated domains of heparan sulfate, *J. Biol. Chem. 274*, 18455−18462.
18. Venkataraman, G., Shriver, Z., Raman, R., and Sasisekharan, R. (1999) Sequencing complex polysaccharides, *Science 286*, 537−542.
19. Merchant, Z. M., Kim, Y. S., Rice, K. G., and Linhardt, R. J. (1985) Structure of heparin-derived tetraasaccharides, *Biochem. J.* 229, 369−377.
20. Chai, W., Luo, J., Lim, C. K., and Lawson, A. M. (1998) Characterization of heparin oligosaccharide mixtures as ammonium salts using electrospray mass spectrometry, *Anal. Chem. 70*, 2060−2066.
21. Desai, U. R., Wang, H.-M., and Linhardt, R. J. (1993) Specificity studies on the heparin lyases from *Flavobacterium heparinum*, *Biochemistry 32*, 8140−8145.
22. Kusche, M., Hannesson, H. H., and Lindahl, U. (1991) Biosynthesis of heparin. Use of *Escherichia coli* K5 capsular polysaccharide as a model substrate in enzymic polymer-modification reactions, *Biochem. J. 275*, 151−158.
23. Nader, H. B., Kobayashi, E. Y., Chavante, S. F., Tersariol, I. L. S., Castro, R. A. B., Shinjo, S. K., Naggi, A., Torri, G., Casu, B., and Dietrich, C. P. (1999) New insights on the specificity of heparin and heparan sulfate lyases from *Flavobacterium heparinum* revealed by the use of synthetic derivatives of K5 polysaccharide from *E. coli* and 2-O-desulfated heparin, *Glycoconjugate J. 16*, 265−270.
24. Kuberan, B., Lech, M., Zhang, L., Wu, Z. L., Beeler, D. L., and Rosenberg, R. D. (2002) Analysis of heparan sulfate oligosaccharides with ion pair-reverse phase capillary high performance liquid chromatography-microelectrospray ionization time-of-flight mass spectrometry, *J. Am. Chem. Soc. 124*, 8707−8718.
25. Kuberan, B., Beeler, D. L., Lech, M., Wu, Z. L., and Rosenberg, R. D. (2003) Chemoenzymatic synthesis of classical and nonclas-

sical anticoagulant heparan sulfate polysaccharides, *J. Biol. Chem. 278*, 52613−52621.
26. Chai, W., Kogelberg, H., and Lawson, A. M. (1996) Generation and structural characterization of a range of unmodified chondroitin sulfate oligosaccharide fragments, *Anal. Biochem. 237*, 88−102.
27. Chai, W., Beeson, J. G., and Lawson, A. M. (2002) The structural motif in chondroitin sulfate for adhesion of *Plasmodium falciparum*-infected erythrocytes comprises disaccharide units of 4-*O*-sulfated and nonsulfated *N*-acetylgalactosamine linked to glucuronic acid, *J. Biol. Chem. 277*, 22438−22446.
28. Domon, B., and Costello, C. E. A. (1988) A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra f glycoconjugates, *Glycoconjugate J. 5*, 397−409.
29. Chai, W., Piskarev, V., and Lawson, A. M. (2001) Negative-ion electrospray mass spectrometry of neutral underivatized oligosacharides, *Anal. Chem. 73*, 651−657.
30. Chai, W., Piskarev, V., and Lawson, A. M. (2002) Branching pattern and sequence analysis of underivatized oligosaccharides by combined MS/MS of singly and doubly charged molecular ions in negative-ion electrospray mass spectrometry, *J. Am. Soc. Mass Spectrom. 13*, 670−679.
31. Davies, M. J., and Hounsell, E. F. (1996) Comparison of separation modes of high-performance liquid chromatography for the analysis of glycoproptein- and proteoglycan-derived oligosaccharides, *J. Chromatogr. A 720*, 227−233.
32. Yamada, S., Yoshida, K., Sugiura, M., and Sugahara, K. (1992) One- and two-dimensional ^{1}H NMR characterization of two series of sulfated disaccharides prepared from chondroitin sulfate and heparan sulfate/heparin by bacterial eliminase digestion, *J. Biochem. (Tokyo) 112*, 440−447.
33. Chai, W., Hounsell, E. F., Bauer, C. J., and Lawson, A. M. (1995) Characterisation by LSI-MS and ^{1}H NMR spectroscopy of tetra-, hexa, and octa-saccharides of porcine intestinal heparin, *Carbohydr. Res. 269*, 139−156.
34. Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., and Gallagher, J. T. (1990) Examination of the substrate specificity of heparin and heparan sulfate lyases, *Biochemistry 29*, 2611−2617.
35. Juhasz, P., and Biemann, K. (1994) Mass spectrometric molecular-weight determination of highly acidic compounds of biological significance via their complexes with basic polypeptides, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4333−4337.
36. Chai, W., Green, B. N., and Lawson, A. M. (1993) Electrospray mass spectrometry of heparin oligosaccharides, *Proc. 41st ASMS Conf. Mass Spectrom. Allied Top.*, 85.
37. McClellan, J. E., Costello, C. E., O'Connor, P. B., and Zaia, J. (2002) Inlfuence of charge state on product ion mass spectra and the determination of 4S/6S sulfation sequence of chondroitin sulfate oligosaccharides, *Anal. Chem. 74*, 3760−3771.
38. Ai, X., Do, A.-T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., and Emerson, C. P., Jr. (2004) Osulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling, *J. Cell Biol. 162*, 341−351.
39. Price, K. N., Tuinman, A., Baker, D. C., Chisena, C., and Cysyk, R. L. (1997) Isolation and characterization by electrospray-ionization mass spectrometry and high-performance anion-exchange chromatography of oligosaccharides derived from hyaluronic acid by hyaluronate lyase digestion: Observation of some heretofore unobserved oligosaccharides that contain an odd number of units, *Carbohydr. Res. 303*, 303−311.
40. Chai, W., Beeson, J. G., Kogelberg, H., Brown, G. V., and Lawson, A. M. (2001) Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes by structurally defined hyaluronic acid dodecasaccharides, *Infect. Immun. 69*, 420−425.
41. Myette, J. R., Shriver, Z., Kiziltepe, T., McLean, M. W., Venkataraman, G., and Sasisekharan, R. (2002) Molecular cloning of the heparin/heparan sulfate Δ4,5 unsaturated glycuronidase from flavobacterium heparinum, its recombinant expression in *Escherichia coli*, and biochemical determination of its unique substrate specificity, *Biochemistry, 41*, 7424−7434.

BI036250K