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Strategy for the sequence analysis of heparin

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The versatile biological activities of proteoglycans are mainly mediated by their glycosaminoglycan (GAG) components. Unlike proteins and nucleic acids, no satisfactory method for sequencing GAGs has been developed. This paper describes a strategy to sequence the GAG chains of heparin. Heparin, prepared from animal tissue, and processed by proteinases and endoglucuronidases, is 90% GAG heparin and 10% peptidoglycan heparin (containing small remnants of core protein). Raw porcine mucosal heparin was labelled on the amino termini of these core protein remnants with a hydrophobic, fluorescent tag [*N*-4-(6-dimethylamino-2-benzofuranyl) phenyl (NDBP)-isothiocyanate]. Enrichment of the NDBP-heparin using phenyl-Sepharose chromatography, followed by treatment with a mixture of heparin lyase I and III, resulted in a single NDBP-linkage region tetrasaccharide, which was characterized as ΔUAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-Xylp-(1→*O*-Ser-NDBP (ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid). Several NDBP-octasaccharides were isolated when NDBP-heparin was treated with only heparin lyase I. The structure of one of these NDBP-octasaccharides, ΔUAp2S(1→4)-α-D-GlcNpAc(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-Xylp-(1→*O*-Ser NDBP (S is sulphate, Ac is acetate), was determined by ^{1}H-NMR and enzymatic methods. Enriched NDBP-heparin was treated with lithium hydroxide to release heparin, and the GAG chain was then labelled at xylose with 7-amino-1,3-naphthalene disulphonic acid (AGA). The resulting AGA-Xyl-heparin was sequenced on gradient PAGE using heparin lyase I and heparin lyase III. A predominant sequence in heparin at the protein core attachment site was deduced to be -D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S-(1→4)-α-D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(or6OH)(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpAc(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-Xyl-AGA.

Key words: heparin/fluorescent labelling/PAGE/sequence analysis

Introduction

Glycosaminoglycan (GAG) heparin is a highly sulphated, linear polysaccharide comprised of repeating 1→4 linked uronic acid and glucosamine residues (Linhardt and Loganathan, 1990). Heparin is biosynthesized as proteoglycan and processed into raw GAG heparin by proteinases and endoglucuronidases during mast cell degranulation (Lindahl, 1989; Linhardt and Loganathan, 1990). Approximately 10% of raw GAG heparin has core peptides attached to it and is called peptidoglycan heparin (Horner and Young, 1982). Pharmaceutical heparin is prepared by bleaching raw heparin, which destroys the peptide residues of peptidoglycan heparin (Linhardt, 1991). Heparin has been used clinically as an anticoagulant for the past 50 years and it is also being investigated as an agent to regulate complement activity, angiogenesis, atherosclerosis, and viral activity, as well as to stabilize and activate growth factors (Linhardt and Loganathan, 1990). The anticoagulant activity of heparin relies on a specific pentasaccharide sequence in heparin, remote from the core protein (Rosenfeld and Danishefsky, 1988), with which antithrombin interacts (Linhardt and Loganathan, 1990). Similarly, fibroblast growth factor binds at specific sites located proximal to the protein linkage region in heparan sulphate (Turnbull and Gallagher, 1991). It is likely that the other biological activities of heparin may originate from specific sequences within the polysaccharide structure making the sequencing of heparin an important undertaking (Marcum and Rosenberg, 1989; Conrad, 1993).

A promising new approach to sequence polysaccharides involves the use of gel electrophoresis of fluorescently labelled sugars (Linhardt *et al.*, 1993). The sequence of small tagged, linear oligosaccharides has been determined by treating the labelled carbohydrate with specific glycosidases resulting in assignable fluorescent band shifts on polyacrylamide gel electrophoresis (PAGE) (Lee *et al.*, 1991). Cheng *et al.* (1992) have also used PAGE-based approaches in attempts to sequence iodinated chondroitin sulphate. In a similar approach Lyon *et al.* (1994) immobilized heparan sulphate to beads, through its peptide component, and used enzymes to study its sequence.

Heparin shares a common carbohydrate–protein linkage region →4)-β-D-GlcAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-D-Xylp(1→*O*-Ser with other GAGs (Fransson, 1989; Sugahara *et al.*, 1991, 1992, 1994). Sugahara *et al.* (1992) isolated a tetrasaccharide and hexasaccharide containing a serine residue from the linkage region of raw heparin. The present paper describes a general approach utilizing fluorescent labelling and PAGE-based sequencing that extends the known sequence of heparin from its protein core attachment site.

Results

Preparation of enriched NDBP-heparin and NDBP-linkage region oligosaccharides

Fluorescent labelling of the peptide chain of raw heparin was achieved by reaction with NDBP in anhydrous formamide. Phenyl-Sepharose chromatography of the product led to good (>50%) recovery of the hydrophobic NDBP-heparin. The

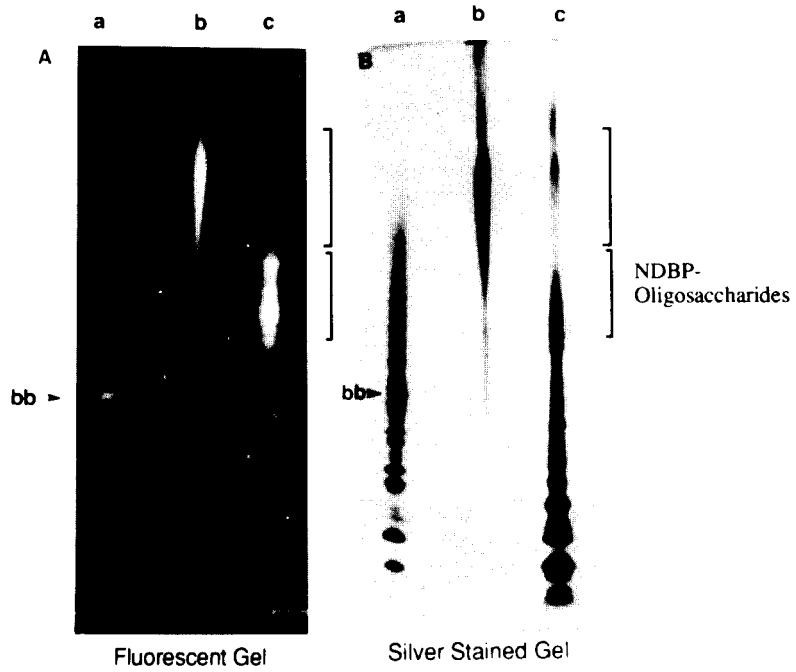

Fig. 1. Analysis of NDBP-heparin on gradient PAGE. (**A**) is the fluorescent gel and (**B**) is the same gel after silver staining. Lane a (in both panels) contains bovine lung heparin (not fluorescently labelled) partially depolymerized with heparin lyase I as molecular weight markers and bromophenol blue (bb) as electrophoresis indicator standard. Lane b (in both panels) contains undigested NDBP-heparin. Identical patterns are seen for lane b in both fluorescent and silver stained gel suggesting that the peptidoglycan heparin has been completely labelled. Lane c (in both panels) contains NDBP-heparin that has been treated with heparin lyase I. The positions of the NDBP-heparin and NDBP-oligosaccharides are indicated in both (A) and (B).

^{1}H-NMR of the purified product (data not shown) gave an *N*-acetate/anomeric proton ratio of 0.5, consistent with heparin but substantially different from the ratio of 1.5 that was observed for heparan sulphate (Griffin *et al.*, 1995). In addition, treatment of the purified product with heparin lyase III gave no reaction as measured by gradient PAGE, RP-HPLC or capillary electrophoresis (CE) (data not shown) confirming that the labelled chains were not heparan sulphate (Linhardt, 1994). Gradient PAGE analysis of the purified product gave a fluorescent banding pattern that was identical with the banding pattern obtained on silver staining (Figure 1). It also demonstrated that NDBP-heparin had the same average molecular weight as raw heparin (Figure 1, lane b). The ^{1}H-NMR spectrum of NDBP-heparin (data not shown) gave a peak integral ratio of 1:32 for an aromatic proton of NDBP to total anomeric protons. Thus, the purity of NDBP-heparin could be estimated at >95%.

Treatment of NDBP-heparin with heparin lyase I resulted in a product mixture containing both NDBP-linkage region oligosaccharides and non-fluorescent heparin oligosaccharides (Figure 1, lane c). The non-fluorescent bands, visualized with silver staining, gave a banding pattern identical to that observed when pharmaceutical heparin was similarly analysed (Rice *et al.*, 1987). The purified NDBP-linkage region oligosaccharides observed by fluorescence were of higher molecular weight than the unlabelled, non-fluorescent oligosaccharides. This product mixture was purified using phenyl-Sepharose chromatography. The non-fluorescent heparin oligosaccharides eluted at high ammonium sulphate concentrations while the hydrophobic NDBP-linkage region oligosaccharides were only eluted when the column was washed with water. The purified NDBP-linkage region oligosaccharides resulted in a pattern of fluorescent bands (data not shown) that was identical to that observed for the heparin lyase I treated NDBP-heparin (Figure 1A, lane c). PAGE analysis of purified NDBP-linkage region oligosaccharides using silver staining (data not shown) gave the same cluster of high molecular weight bands observed by fluorescence but the low molecular weight non-fluorescent products were absent.

Isolation and characterization of NDBP-tetrasaccharide

The purified NDBP-linkage region oligosaccharides were further fractionated by RP-HPLC into their major fluorescent components (Figure 2A). The four fluorescent peaks, individually or collectively, collapsed into a single fluorescent peak following treatment with heparin lyase III (Figure 2B). This suggested that the heterogeneity of these NDBP-linkage region oligosaccharides is the result of differences in structure at their non-reducing ends. Exhaustive treatment of this sample with heparin lyase I or III gave no additional change. The fluorescent peak present in the greatest amount (Figure 2B) was purified by semi-preparative RP-HPLC and analysed using high-field NMR spectroscopy.

The ^{1}H-NMR spectrum of NDBP-tetrasaccharide clearly shows a single NDBP tag attached to serine (Figure 3). The NDBP-tetrasaccharide showed the expected number of signals confirming its high level of purity. Two-dimensional NMR spectroscopy (COSY and HOHAHA) was performed to elucidate the spin systems (Figure 4). Chemical shifts and coupling constants are presented in Table I. The single residue of uronic acid can be easily identified as ΔUAp by cross-peaks between protons at δ 5.758 (H4), 5.161 (H1), 4.012 (H3) and 3.812 (H2). The presence of xylose can be ascertained by connectivity between protons at δ 4.247 (H1) and 3.196 (H2) (Figure 4A). The spin system of two galactose residues can be traced starting from the anomeric protons at δ 4.209 and 4.012 (Figure 4A).

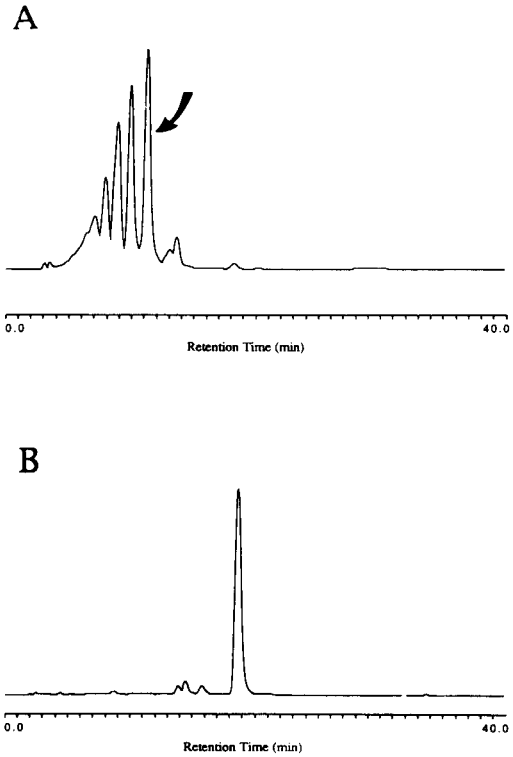

Fig. 2. Analysis of NDBP-oligosaccharides by RP-HPLC. (**A**) Separation of NDBP-oligosaccharides, prepared by treating NDBP-heparin with heparin lyase I, using RP-HPLC on a phenyl column (0.46 cm × 25 cm). The eluates were monitored with a flow cell fluorescence detector (Ex. 310 nm/Em. 450 nm). The arrow indicates the peak corresponding to the major NDBP-octasaccharide. (**B**) The RP-HPLC profile of NDBP-oligosaccharides treated with heparin lyase III. Treatment of the major NDBP-octasaccharide with heparin lyase III gives an identical chromatogram (not shown).

A cross-peak between protons at δ 4.187 and 3.929 (2H) suggests the presence of serine, while the singlet at δ 3.4 (Figure 3) showing no cross-peaks, can be assigned to the methylene protons of the glycine residue of the peptide chain. This serine residue is attached to xylose and the β and β′ protons are similar to the previously reported chemical shift (Sugahara *et al.*, 1994). Amino acid compositional analysis shows serine, glycine and alanine present in a ratio of 1:1.8:0.7. Glycine and alanine are found in higher than expected amounts (Ser:Gly,1:1) suggesting that some peptide contaminants are also present. Thus, the structure of the linkage region NDBP-tetrasaccharide is ΔUAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-D-Xylp(1→ (Figure 3).

Isolation and characterization of an NDBP-octasaccharide

Migration position on gradient PAGE suggested that each of the NDBP-oligosaccharides (Figure 2A) were of intermediate chain length, probably corresponding to octasaccharides and higher oligosaccharides. The most abundant oligosaccharide component of this mixture was prepared by semi-preparative RP-HPLC and characterized by NMR and enzymatic analysis. This major NDBP-oligosaccharide showed a single prominent peak at 10.3 min on CE with fluorescence detection. However, with UV detection at 232 nm additional minor peaks were observed, suggesting that the major NDBP-oligosaccharide had a purity of ~85%. On treatment with heparin lyase III, a single new fluorescent peak, corresponding to the NDBP-tetrasaccharide with retention time of 8.16 min, was observed. Analysis using detection at 232 nm showed two new peaks at 8.6 and 8.7 min. These peaks were identified as disaccharides by comigration with authentic standards (Ampofo *et al.*, 1992) having the structure ΔUAp2S(1→4)-α-D-GlcNpAc and ΔUAp(1→4)-α-D-GlcNpAc6S. This suggested that the NDBP-oligosaccharide was an NDBP-octasaccharide composed of the NDBP-tetrasaccharide and these two disaccharides.

The NDBP-octasaccharide was prepared on a larger scale by RP-HPLC to determine the sequence of saccharide residues. The one-dimensional ^{1}H-NMR spectrum of the NDBP-octasaccharide shows eight anomeric protons and suggests a purity of >85% (Figure 5). The most important feature of this spectrum is the presence of an unsulphated iduronic acid residue giving a signal at 4.956 p.p.m. No signals corresponding to a sulphated iduronic acid residue are observed between δ 5.2 and 5.3. The presence of ΔUAp2S was confirmed by the signals at δ 5.493 (H1) and δ 5.984 (H4). The H1 signals of residues G and E (Figure 5) can be differentiated by the expected higher resolution observed in the upfield signal due to the greater mobility of residue G located at the end of the oligosaccharide chain.

Two-dimensional homonuclear COSY of NDBP-octasaccharide provided the necessary connectivities for structure elucidation (Figure 6). The most deshielded protons at δ 5.984 and 5.493 correspond to the H4 and H1 of ΔUAp2S and show full connectivities to the other ring protons of this residue confirming the identity of the residue at the non-reducing end. The H1/H2 cross-peaks at δ 5.347 and 5.392 correspond to the H1 signals of the two GlcNAc residues. No H1/H2 cross-peak corresponding to GlcNpS is observed at δ 5.3/δ 3.1–3.2. The H1/H2 cross-peak at δ 4.956 and δ 4.636 correspond to IdoAp and GlcAp, respectively. Their position in the NDBP-octasaccharide, with IdoAp attached to the 4-position of GlcNAc and GlcAp attached to the 3-position of Galp, was confirmed by a NOESY experiment (data not shown). The two Galp residues show H1/H2 cross-peaks at δ 4.636 and 4.491. These cross-peaks are again distinguished by NOESY with the signal at δ 4.636 showing a strong cross-peak to the H3 of Galp and the signal at δ 4.491 showing a strong cross-peak to the H4 of Xylp. Xylose shows a H1/H2 cross-peak upfield at δ 4.408. The structure obtained using NMR (Figure 5) is fully consistent with the results of CE analysis.

Preparation, purification and characterization of AGA-Xyl-heparin

The analysis of NDBP-heparin on gradient PAGE showed that the NDBP-oligosaccharides migrated a very short distance into the gel and were poorly resolved (Figure 1, lane c). The reasons for the poor resolution and reduced migration were the absence of sufficient sulphate groups (carrying a negative charge) to drive separation by electrophoresis and the hydrophobic interaction of the NDBP label with polyacrylamide. This interaction was confirmed by the failure of NDBP-oligosaccharides to elute at the appropriate position on a polyacrylamide-based Biogel P-2 column. We reasoned that a hydrophilic, negatively charged fluorescent label would improve the PAGE-based separation of these oligosaccharides. 7-Amino-1,3-naphthalene disulphonic acid (AGA) has been used as a fluorescent label of neutral oligosaccharides at their reducing terminus (Lee *et al.*, 1991). To use this label it was first necessary to cleave the linkage between serine and xylose leaving the reducing end of xylose intact for reaction with AGA. Mild β-elimination

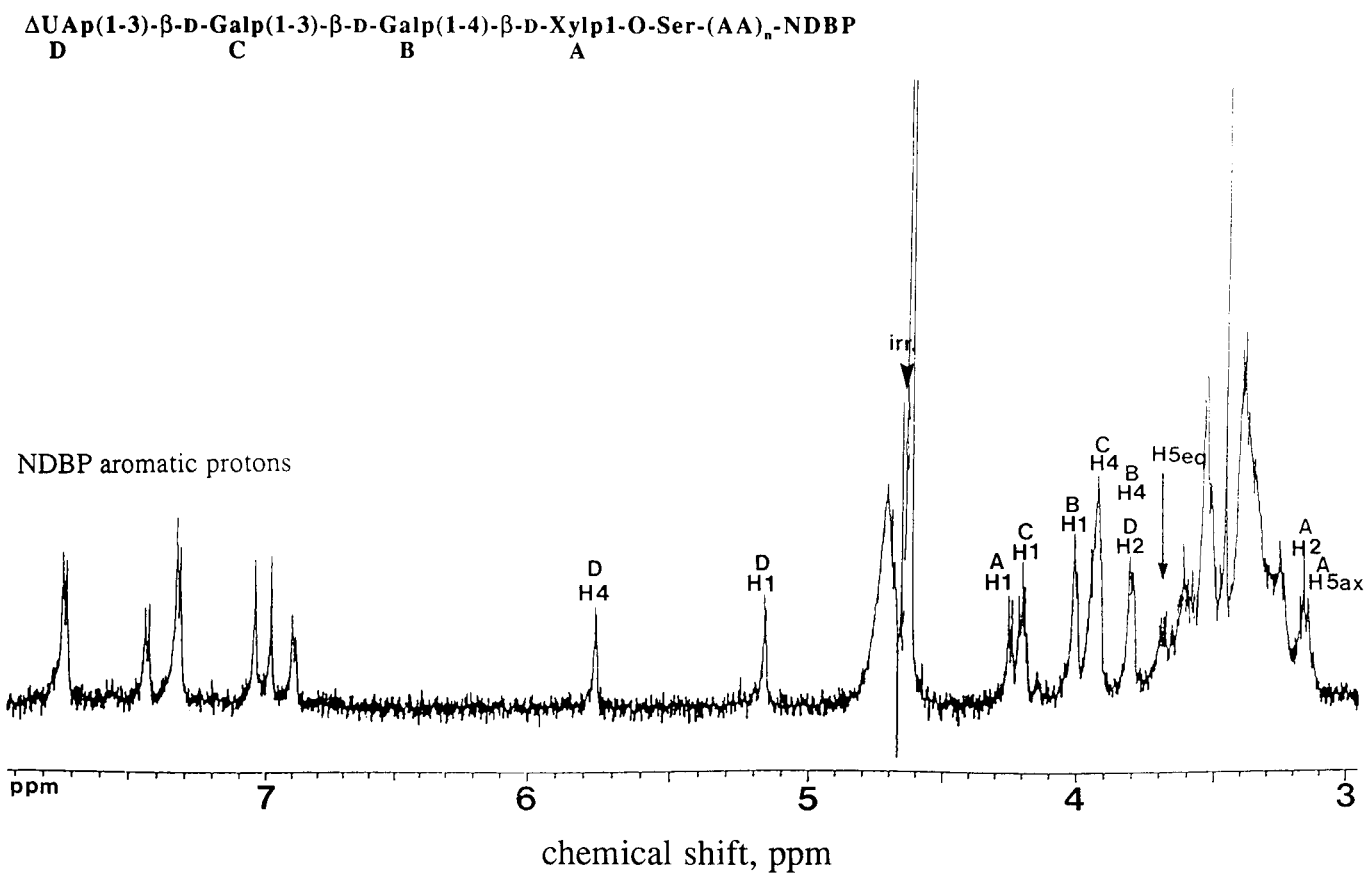

Fig. 3. The structure of NDBP-tetrasaccharide and its analysis by one-dimensional NMR. Important signals in the partial one-dimensional ^{1}H-NMR spectrum of NDBP tetrasaccharide are labelled (saccharide residues are labelled A–D from Xyl). AA represents additional amino acid residues that may be present and n = 0–2.

with lithium hydroxide at 4°C (Heinegård, 1972) was performed to prevent pealing reactions (Whistler and BeMiller, 1958) and the GAG heparin was precipitated using methanol. The heparin precipitate was recovered and labelled with AGA by reductive amination. Excess AGA, used to force the reaction to completion, was removed by dialysis (3500 molecular weight cut-off (MWCO)] and methanol precipitation of the AGA-Xyl-heparin conjugate.

The AGA-Xyl-heparin was purified by phenyl-Sepharose chromatography. Three fluorescent fractions were obtained and each was analysed by depolymerization with heparin lyase I followed by gradient PAGE. The 3.8 M ammonium sulphate fraction contained primarily free AGA, the 2.0 M ammonium sulphate fraction contained only AGA-Xyl-heparin, and the water fraction contained only unreacted NDBP-heparin. AGA-Xyl-heparin was recovered by dialysis and freeze-dried. The attachment site of AGA to the xylose of the heparin chain was confirmed by hydrolysis of AGA-Xyl-heparin with trifluoroacetic acid (TFA) followed by analysis on RPIP-HPLC and gradient PAGE (data not shown). Both analyses showed that AGA-Xyl was the only monosaccharide conjugate present.

Determination of heparin sequence by gradient PAGE analysis

AGA-Xyl-heparin was first depolymerized with excess heparin lyase I and further depolymerized using a mixture of heparin lyase I and lyase III. The depolymerized products were analysed on a gradient PAGE gel. The degree of polymerization of heparin oligosaccharides was estimated as <18 based on their migration distance in comparison to bromophenol blue (Rice *et al.*, 1987; Edens *et al.*, 1992). Gradient PAGE analysis of AGA-Xyl-heparin is shown in Figure 7. AGA-Xyl-heparin not treated with the lyase shows a smear near the top of the gel (well above bromophenol blue) consistent with an expected average MW ~12 000 (Figure 7, lane A). AGA-Xyl-heparin depolymerized with heparin lyase I showed a cluster of 3–4 bands (b–e) in the middle of the gel (Figure 7, lane B), while additional treatment with a mixture of heparin lyase I and heparin lyase III showed an intense, rapidly migrating band (a) and two lower intensity bands (b and c) corresponding to oligosaccharides of larger molecular size (Figure 7, lane C).

NDBP-tetrasaccharide was the smallest oligosaccharide isolated from NDBP-heparin after treatment with a mixture of heparin lyase I and heparin lyase III. It is reasonable to assume that the replacement of NDBP-core peptide with AGA will not alter the substrate specificities of the heparin lyases, since the first lyase cleavage site is at least four sugar residues away from the core peptide. Previous studies with AGA-labelled oligosaccharides, using a wide range of different glycosidases, showed no changes in reactivity or specificity associated with the use of this label (Lee *et al.*, 1991). The fastest migrating, and major band (Figure 7, lane C, band a) was assigned to the AGA-tetrasaccharide, ΔUAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-D-Xyl-AGA. Band b in lanes B and C was tentatively assigned as AGA-labelled hexasaccharide based on its reduced migration. When the major NDBP-octasaccharide was treated with heparin lyases I and III and analysed on

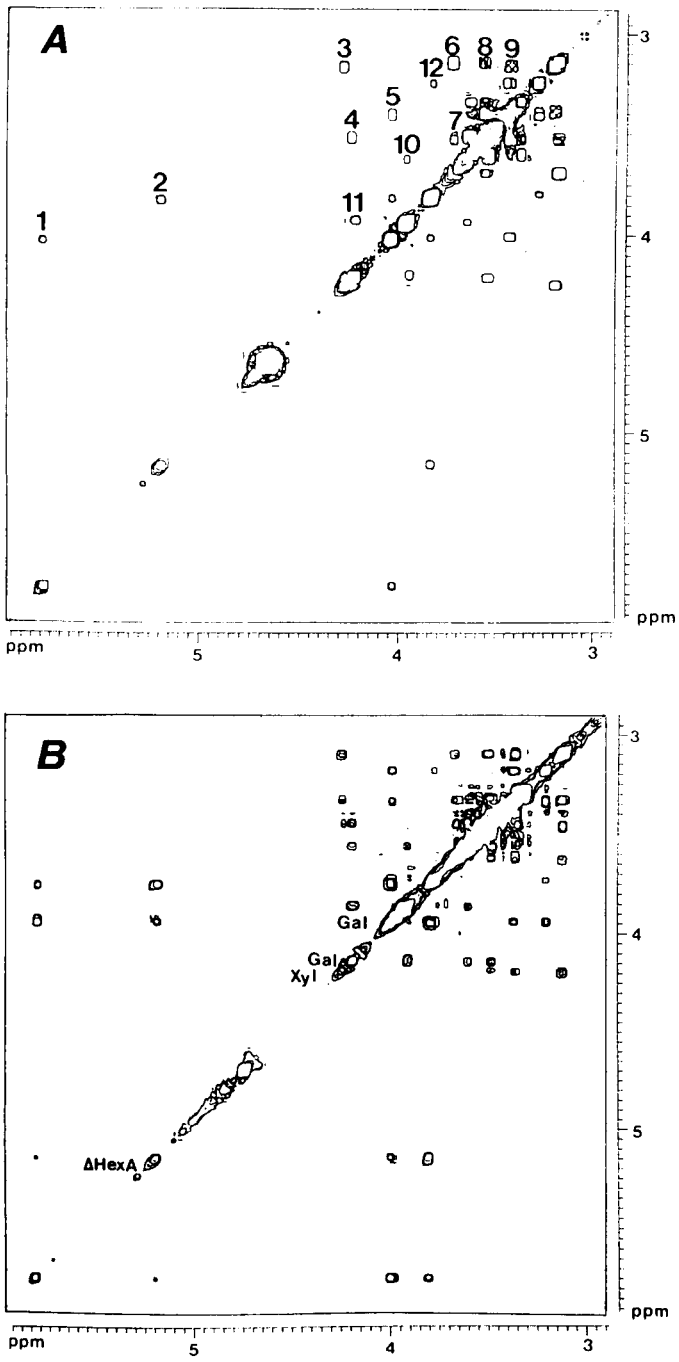

Fig. 4. Two-dimensional NMR spectroscopy of the NDBP-tetrasaccharide. (**A**) COSY spectrum. Cross-peaks: 1, H3/H4 of D (see Figure 3 for structure of saccharide residues A–D); 2, H1/H2 of D; 3, H1/H2 of A; 4, H1/H2 of C; 5, H1/H2 of B; 6, H5ax/H5eq of A; 7, H4/H5eq of A; 8, H4/H5ax of A; 9, H2/H3 of A; 10, H3/H4 of B; 11, Hα/Hβ,Hβ′ of Ser; 12, H3/H4 of B. (**B**) HOHAHA confirms the cross-peaks observed in the COSY spectrum.

RP-HPLC, the transient appearance of a peak, corresponding to an NDBP-hexasaccharide was observed eluting between NDBP-octasaccharide and NDBP-tetrasaccharide.

A major AGA-labelled octasaccharide was expected on the heparin lyase I treatment of AGA-Xyl-heparin by analogy to the isolation of NDBP-octasaccharide from NDBP-heparin treated with heparin lyase I. Band c (Figure 7, lane B) is the most intense fluorescent band, and is tentatively assigned as corresponding to the AGA-labelled octasaccharide mixture

Table I. Chemical shifts (p.p.m.) for coupling constants (Hz) for the saccharide of the NDBP tetrasaccharide

	Residue				
Proton	D	C	B	A	Ser
H1	5.161	4.209	4.012	4.247	
$(J_{1,2})$[a]	4.2	8.1	8.3	7.8	
H2	3.812	3.504	3.401	3.196	
$(J_{2,3})$	3.5	8.3	7.9	8.1	
H3	4.012	3.621	3.243	3.387	
$(J_{3,4})$	4.5	2.4	2.1	n.d.[b]	
H4	5.758	3.953	3.794	3.526	
$(J_{4,5})$		<1.5	<1.5	n.d.	
H5	–[d]	n.d.	n.d.	3.609 (eq)[c] 3.142 (ax)	
Hα					4.187
Hβ					3.929
Hβ′					3.929

[a]Coupling constants.
[b]Not determined.
[c]Axial and equatorial protons of xylose residue.
[d]Not present.

including a major octasaccharide with the structure of ΔUAp2S(1→4)-α-D-GlcNp2Ac(1→4)-α-L-IdoAp(1→4)-α-D-GlcNp2Ac6S(1→4)-β-D-GlcAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-D-Xyl-AGA. The reduced migration and the relative spacing of bands c, d and e permit their tentative assignment as AGA-labelled octasaccharides, decasaccharides and dodecasaccharides, respectively.

All of the oligosaccharides, formed on the treatment of AGA-Xyl-heparin with heparin lyase I, contain heparin lyase III cleavable sites with a structure of -D-GlcNpAc or GlcNpAc6S(1→4)-β-D-GlcAp(1→3. The observation of band b in lanes B and C corresponding to hexasaccharide(s), suggests that this linkage site at the non-reducing end of the octasaccharide can be cleaved by both heparin lyase I and heparin lyase III. The NMR analysis of the major NDBP-octasaccharide demonstrates the presence of linkages within the structure -D-GlcNpAc or GlcNpAc6S(1→4)-α-L-IdoAp(1→4), susceptible to both heparin lyase I and heparin lyase III (Desai *et al.*, 1993a,b). By continuing to use the same analytical approach, tentative structures for band d and band e can be deduced. Band d corresponds to a decasaccharide containing one additional disaccharide unit at its non-reducing end, which is cleavable by heparin lyase I. Band e corresponds to a dodecasaccharide that has two disaccharide units at its non-reducing terminus, which are both cleavable by heparin lyase I. Only heparin lyase I cleaves the linkage between →4)-α-D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→ (Linhardt *et al.*, 1986; Linhardt, 1994). To confirm these assignments a sequence for heparin extending 13 saccharide units from the core protein can be proposed (Figure 8, legend). Using this sequence the molecular weight of each AGA-labelled oligosaccharide was calculated, plotted as a function of migration distance and the linear regression analysis afforded an r^2 of 0.997 (Figure 8).

Discussion

The linkage regions of heparin and related proteoglycans (Lindahl, 1966a,b; Horner, 1971; Robinson *et al.*, 1978; Cheng *et al.*, 1992) have been under extensive investigation (Rosenfeld and Danishefsky, 1988; Fransson, 1989; Sugahara *et al.*, 1991,

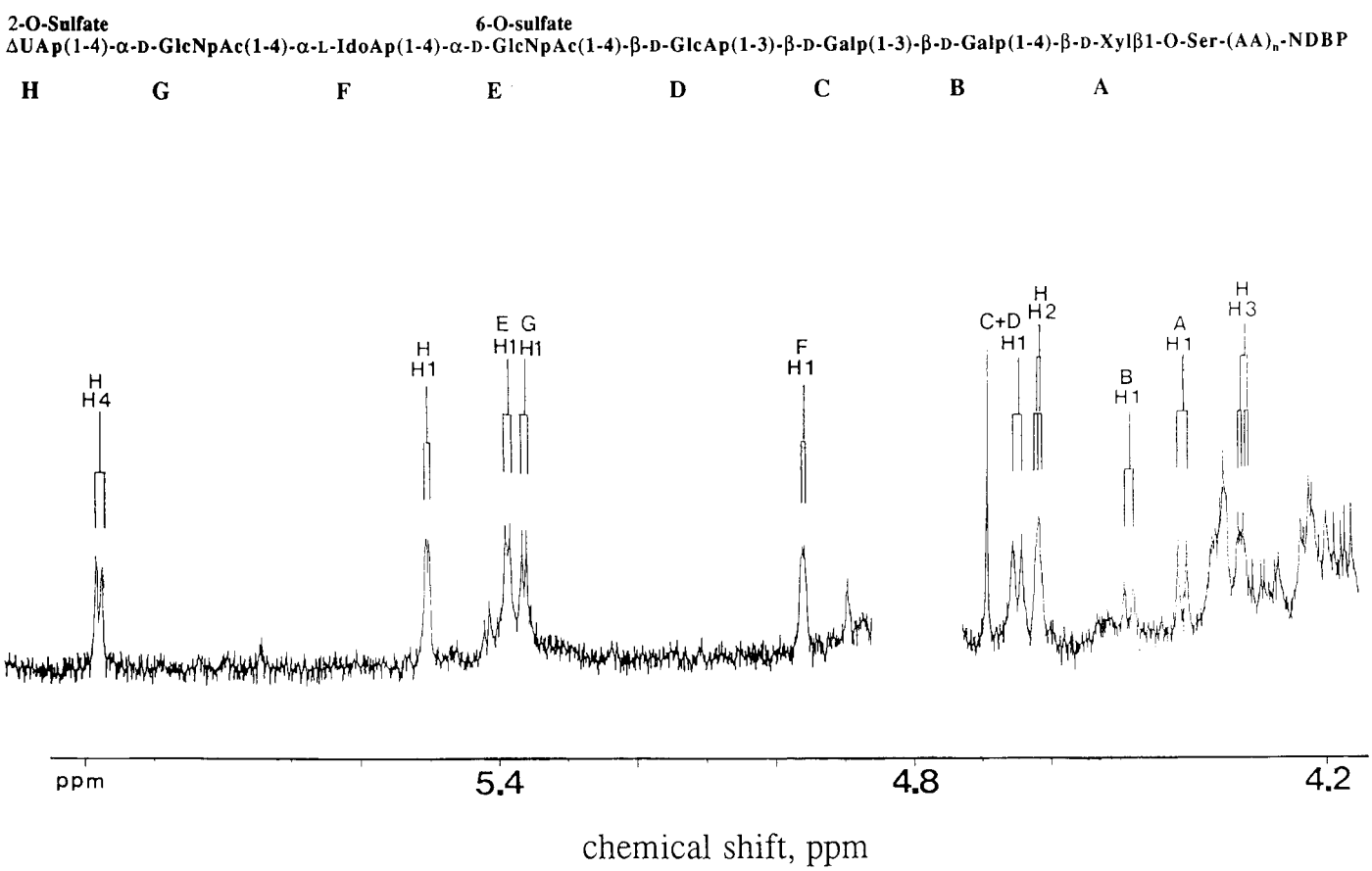

Fig. 5. Partial one-dimensional ^{1}H-NMR spectrum of the NDBP-octasaccharide (saccharide residues are labelled A–H from Xyl). Chemical shifts are: 5.984 p.p.m. (H4 of H), 5.493 p.p.m. (H1 of H), 5.392 p.p.m. (H1 of E), 5.347 p.p.m. (H1 of G), 4.956 p.p.m. (H1 of F), 4.636 p.p.m. (H1 of C and D), 4.611 p.p.m. (H2 of H), 4.491 p.p.m. (H1 of B), 4.408 p.p.m. (H1 of A), and 4.319 p.p.m. (H3 of H). The HOD peak has been removed. AA represents additional amino acid residues that may be present and n = 0–2.

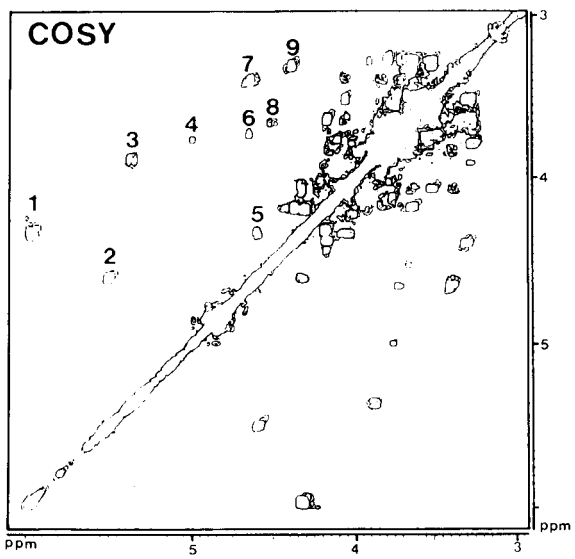

Fig. 6. Two-dimensional COSY spectrum of the NDBP-octasaccharide. Cross-peaks: 1, 2 and 5 are H4/H3, H1/H2 and H2/H3 of residue H; 3, H1/H2 of residues E and G; 4, H1/H2 of residue F; 6, H1/H2 of residue C; 7, H1/H2 of residue D; 8, H1/H2 of residue B; 9, H1/H2 of residue A.

1992, 1994). The structure of the heparin linkage region may have important implications on remote structures in the chain (Rosenfeld and Danishefsky, 1988). It is often these remote structures that are involved in the protein interaction associated with the biological activities of heparin and heparan sulphate. Sugahara *et al.* (1992, 1994) have reported the structure of the linkage region oligosaccharides from heparin and heparan sulphate. In these studies heparin and heparan sulphate were exhaustively depolymerized and oligosacchrides containing peptide components were isolated and their structures characterized. While this approach has yielded considerable information about the linkage region itself, no connection can be made between a given linkage region structure and the more remote structures present within the glycosaminoglycan chain. Cheng *et al.* (1992) suggested the use of a new gel electrophoresis-based sequencing method to study glycosaminoglycan sequences remote from the protein core. Glycosaminoglycan chains were released from a chondroitin sulphate proteoglycan through mild β-elimination and a radiolabelled tag was introduced into xylose. These labelled chains were then treated with chondroitin lyases and analysed using PAGE. While this approach is an improvement on oligosaccharide mapping (Rice *et al.*, 1987; Linhardt *et al.*, 1988, 1990), providing sequence information on the glycosaminoglycan chain, it requires the use of an intact proteoglycan. In the case of heparin, intact proteoglycan is generally unavailable with the exception of rat skin proteoglycan heparin (Horner, 1971; Robinson *et al.*, 1978). Even if the proteoglycan form of heparin could be easily prepared, substantial amounts would be required to facilitate definitive structural characterization of its linkage region structure using mass spectral and NMR analysis (Sugahara *et al.*, 1991, 1992, 1994). The approach described

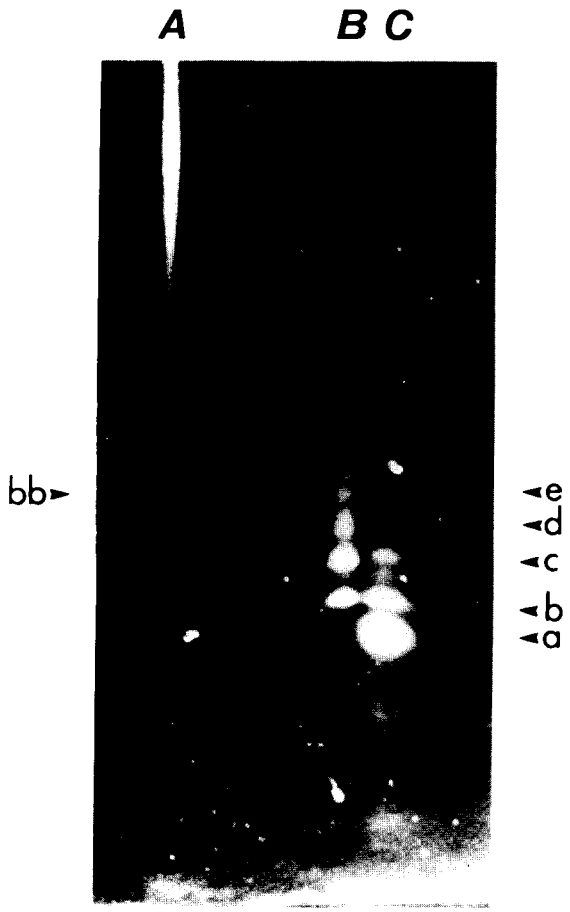

Fig. 7. Fluorescent gel pattern of AGA-Xyl-heparin for sequence analysis of heparin. Lane A contains undigested AGA-Xyl-heparin. The arrow shows the migration position observed for bromophenol blue (bb). Lane B contains AGA-Xyl-heparin treated with heparin lyase I. Lane C contains AGA-Xyl-heparin treated with a mixture of heparin lyase I and the heparin lyase III. Bands a–e in lanes B and C correspond to oligosaccharides ranging from tetrasaccharide to dodecasaccharide in size.

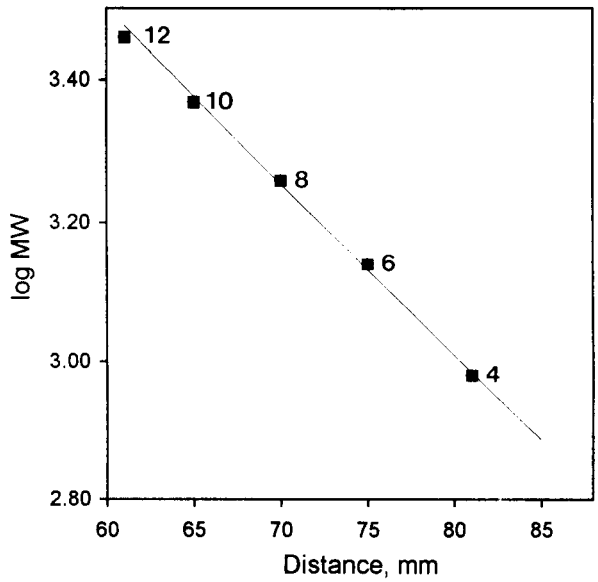

Fig. 8. The log MW of AGA-labelled oligosaccharides shown as a function of migration distance on PAGE analysis. The sequence for AGA-Xyl-heparin suggested by the banding observed in Figure 7 lanes B and C suggests a major sequence, -D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S (or 6OH)(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNpAc(1→4)-α-L-IdoAp (1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→3)-β-D-Galp(1→3)-β-D-Galp(1→4)-β-Xyl-AGA, based on the known specificity of heparin lyase I and II (Desai *et al.*, 1993 a,b). Distance was measured from the top of the gel to the center of the band. The tetrasaccharide (MW 917) in lane C, hexasaccharide (MW 1376) in lane C, octasaccharide (MW 1835) average position in lanes C and B, decasacccharide (MW 2329) in lane B and dodecasaccharide (MW 2823) in lane B were used to determine migration distance. The 6-position was assumed to be unsubstituted when it could be either 6-S or 6-OH and the non-reducing residue was ΔUAp (tetrasaccharide) or ΔUAp2S (higher oligosaccharides). Linear regression showed a curve fit of $r^2 = 0.997$.

here utilizes a hydrophobic label, coupled to the free amino group of the peptidoglycan component of raw heparin, to effectively capture the peptidoglycan heparin. The NDBP-labelled peptidoglycan is then purified by hydrophobic interaction chromatography using an approach similar to one previously reported by Ogamo *et al.* (1981) and Uchiyama *et al.* (1984). This approach ensures that the isolated linkage region containing chains being analysed arise entirely from heparin and not from heparan sulphate [often contaminating heparin preparations (Griffin *et al.*, 1995)] since the isolated NDBP-labelled chains are insensitive to treatment with heparin lyase III. Heparin lyase III does not act on heparin but it nearly completely degrades heparan sulphate (Linhardt *et al.*, 1986, 1990; Desai *et al.*, 1993a,b; Linhardt, 1994). The ^{1}H-NMR of NDBP-heparin also showed an *N*-acetate to anomeric proton ratio consistent with heparin but very different from that observed for heparan sulphate (Linhardt *et al.*, 1992; Griffin *et al.*, 1995).

Treatment of NDBP-heparin with heparin lyase I afforded several NDBP-oligosaccharides as observed by gel electrophoresis (Figure 1) and RP-HPLC (Figure 2). Subsequent treatment with heparin lyase III afforded a single NDBP-oligosaccharide and two disaccharides. NMR analysis (Figures 3 and 4) of this NDBP-oligosaccharide clearly demonstrated that it was the linkage region tetrasaccharide previously characterized by Sugahara *et al.* (1992). Purification and subsequent analysis of the major NDBP-oligosaccharide afforded using heparin lyase III showed it to be an octasaccharide containing, within its structure, a linkage region hexasaccharide also previously reported by Sugahara *et al.* (1992). Sequence analysis of NDBP-heparin by PAGE posed a problem. The same hydrophobicity that assisted in the isolation of these NDBP-derivatives, resulted in their interaction with the polyacrylamide gel matrix leading to poor separations. Thus, the hydrophobic NDBP tag was removed from NDBP-heparin by mild β-elimination and replaced with a hydrophilic AGA label. Four fluorescent oligosaccharides were observed when AGA-Xyl-heparin was treated with heparin lyase I and analysed using both PAGE and RP-HPLC. This result suggests substantial sequence variability beyond the tetrasaccharide linkage region. The variability in the linkage region may result because the peptidoglycan chains prepared from raw heparin arise from different sites in the precursor proteoglycan(s). Alternatively, the random endolytic action pattern of the heparin lyase I may be responsible for the multiple oligosaccharide products that are observed (Linhardt *et al.*, 1989). Once a linkage region, smaller than dodecasaccharide, is cleaved from the heparin polymer, it may no longer be a good substrate of heparin lyase I. These poor substrate properties (Linhardt *et al.*, 1986; Desai *et al.*, 1993a,b; Linhardt, 1994) may result from the low level of sulphation in the linkage

region. Oligosaccharides having low sulphation, however, are excellent substrates for heparin lyase III (Desai *et al.*, 1993a,b). Hence, heparin lyase III acts within the linkage region with high enzymatic activity reducing the oligosaccharide mixture to a single tetrasaccharide product. In addition to sulphation level, other factors influence oligosaccharide sensitivity towards heparin lyase I. Heparin lyase I acts best on substrates of a certain minimum size (Rice and Linhardt, 1989; Desai *et al.*, 1993b). When the oligosaccharide size is reduced to a tetrasaccharide or hexasaccharide, substrate resistance towards heparin lyase I dramatically increases. Thus, the enzyme's random action pattern might afford either resistant (poor substrates) or sensitive (good substrates) from a polysaccharide chain having a single sequence. This might explain why multiple fluorescent bands are observed in Figure 5, lane B. As sequencing efforts proceed further from the core peptide (for example, when AGA-Xyl-heparin is partially depolymerized by heparin lyase I), the peculiarities of enzyme action patterns and resistant substrates should become less of a problem. A putative sequence for heparin, extending 13 saccharide residues from the core peptide, is proposed. Using this sequence the molecular weights of the AGA-labelled oligosaccharides corresponding to the bands observed in Figure 7 (lanes B and C) were calculated. Migration distance of heparin oligosaccharides has previously been shown to be linearly related to log MW (Edens *et al.*, 1992). Figure 8 shows such a linear correlation confirming the size of the oligosaccharides corresponding to each band in Figure 7. This sequence suggested by the banding pattern in Figure 7 may represent a composite sequence of the major heparin chains, if sequence variability is responsible for the multiple bands observed in Figure 7, lane C. Alternatively, this sequence may represent the sequence of a single heparin chain, if the multiple bands arise from substrate resistance to heparin lyase I.

This paper describes an approach for sequencing heparin glycosaminoglycan chain. Our experimental results indicated that this approach, in principle, can be used to sequence any proteoglycan-derived GAG chains. The sequence determined by gradient PAGE strongly supports the hypothesis that GAG chain had defined non-random structure(s) near the core peptide. It is possible, however, that there is not a single sequence but rather a collection of a small number of closely related sequences that extend from the protein core. Studies involving the partial enzymatic depolymerization of fluorescent end-labelled GAG may result in obtaining sequences extending even further from the core protein attachment site. Improvements in the resolution of PAGE and in fluorescent labelling are also critical to separate oligosaccharides of the same size but differing only in fine structure. Successful exploitation of this new approach may lead to the sequencing of larger domains within a variety of GAG polymers.

Materials and methods

Materials

Raw (unbleached Stage 12) porcine mucosal heparin (145 U/mg) was from Celsus Laboratories, Cincinnati, OH. Heparin lyase I (heparinase I, EC 4.2.2.7) [130 units (μmol/min)/mg] (Lohse and Linhardt, 1992) and heparin lyase III (heparan sulphate lyase, heparitinase, EC 4.2.2.8) [63.5 units (μmol/min)/mg] were purified from *Flavobacterium heparinum* in our laboratory (Lohse and Linhardt, 1992). These enzymes were also obtained from Seikagaku America, Inc., Rockville, MD, and IBEX Technologies, Montreal, Quebec, Canada. Bovine kidney heparan sulphate and heparin/heparan sulphate disaccharide standards were from Grampian Enzymes (Aberdeen, UK). Alcian blue 8 GX, sodium borohydride and sodium cyanoborohydride, phenyl-Sepharose Cl-4B, and sulphopropyl (SP)-Sephadex were from Sigma Chemical Co., St Louis, MO. Deuterium oxide (^2H_2O) (99.99 atom % ^2H), 3-(trimethylsilyl)-propionic acid-2,2,3,3-d_4-sodium salt (TSP-d_4), (R)-(+)-*N,N*-dimethyl-1-phenethylamine, and 7-amino-1,3-naphthalene disulphonic acid, mono potassium salt (AGA) were from Aldrich Chemical Co., Milwaukee, WI. Molecular sieves (4 Å) were from MCB Manufacturing Chemists, Cincinnati, OH. Acrylamide, bromophenol blue, ammonium persulphate, and sodium borate were from Boehringer Mannheim, Indianapolis, IN. *N,N'*-Methylenebisacrylamide (electrophoresis grade), formamide (ultrapure, electrophoresis grade), *N,N*-dimethyl formamide, ethylenediaminetetraacetate disodium, triethylamine, silver nitrate, ammonium hydroxide, acetic acid, glycine, boric acid and *N,N,N',N'*-tetramethylethylenediamine were from Fisher Chemical Co., Fairlawn, NJ. Bio-Gel P2 (fine) was from BioRad, Richmond, CA. *N*-4-(6-dimethylamino-2-benzofuranyl) phenyl-isothiocyanate hydrochloride (NDBP) was from Molecular Probes, Inc., Eugene, OR. Dialysis membrane for desalting was from Spectrum Medical, Houston, TX, and pressure filtration apparatus and membranes were from Amicon, Danvers, MA. HPLC-grade water and acetonitrile (EM Science, Gibbstown, NJ) were used throughout the HPLC separation. Ultrapure sodium bicarbonate (>99.998%) was obtained from Alfa, Ward Hill, MA. HPLC separation was performed on dual LC7A face programmable pumps (Shimadzu, Tokyo, Japan) and a variable-wavelength 2141 UV detector (LKB, Piscataway, NJ) and a fluorescence detector RF-535 (Shimadzu, Tokyo, Japan). A Macintosh LC-II personal computer was attached to the detectors to collect and process data using Dynamax software from Rainin, Woburn, MA. RP and RPIP column were a phenyl column (4.6 mm × 25 cm), or a semipreparative phenyl column (1 cm × 25 cm) having 5 μm particle size from Vydac, Hesperia, CA. A Microfuge E centrifuge was from Beckman Instruments (Palo Alto, CA). A BioRad Protean II (20 cm × 22 cm) (Richmond, CA) vertical-slab-gel unit equipped with a BioRad model 1420B power source were used for electrophoresis. The transilluminator was from Spectronic Co., Westbury, NY. CE analysis was performed with a Dionex Capillary Electrophoresis System (Sunnyvale, CA) equipped with a variable wavelength UV and fluorescence detector. Bruker AMX 600 NMR spectrometer with ASPECT X32 computer was used to perform the NMR experiments. Amino acid analysis was performed by the Protein Structure Laboratory at the University of California, Davis.

Gradient PAGE

Discontinuous, linear gradient, polyacrylamide gels (22 cm × 20 cm × 0.75 cm, 12–22% total acrylamide) were prepared and run as previously described (Al-Hakim and Linhardt, 1990). Oligosaccharide samples (10–20 μg) were mixed with equal volume of 50% (w/w) sucrose. The migration pattern of fluorescently labelled compounds was visualized using a transilluminator with the excitation wavelength at 365 nm. Oligosaccharides were also visualized by staining with alcian blue or with alcian blue followed by silver staining (Al-Hakim and Linhardt, 1991).

Preparation of NDBP-heparin

Raw heparin (500 mg) was dissolved in 50 ml of ultrapure formamide by mixing on an orbital shaker at 250 r.p.m. for 10 h at 37°C. NDBP-isothiocyanate (12.5 mg) dissolved in 1.5 ml of *N,N*-dimethyl formamide was added to preincubated heparin formamide solution and shaken for an additional 12 h at 37°C. The reaction mixture was then diluted 10-fold with water and kept at 4°C for 1–2 h to allow the unreacted NDBP-isothiocyanate to precipitate from the solution. The precipitate was removed using a 0.45 μm disposable filter. The volume of the filtered supernatant was then reduced to 50 ml by pressure filtration using a 3000 MWCO membrane. The concentrated solution was dialysed for 3 days against water using a 3500 MWCO membrane and the solution was freeze-dried to recover NDBP-heparin.

Enrichment of NDBP-heparin by phenyl-Sepharose chromatography and its characterization

A 300 ml phenyl-Sepharose column (5 cm × 25 cm) was used to purify up to 0.5–1 g of fluorescently labelled heparin. The elution conditions followed the methods of Ogamo *et al.* (1981) and Uchiyama *et al.* (1984) with minor modifications. The phenyl-Sepharose column was first washed with 10 volumes of water and then equilibrated with 3 volumes of 3.8 M ammonium sulphate at pH 3.3 at a flow rate ~80 ml/h. NDBP-heparin (1 g) dissolved in 50 ml of 3.8 M ammonium sulphate at pH 3.3, was loaded on the column. The column was washed at 4°C with 1000 ml of the same solution, and then with 1000 ml each of 3.4 M (pH 3.35), 3.0 M (pH 3.4), 2.0 M ammonium sulphate (pH 3.5), and water. The column was regenerated with equal volume of 95% ethanol. Fractions (6 ml) were collected and elution was monitored by spectrofluorimeter (Ex. 310 nm/Em. 450 nm after diluting aliquots 10-fold) and by carbazole reaction (Bitter and Muir, 1962). The fractions, eluting with water, having

fluorescence and containing heparin by carbazole assay, were combined and concentrated to 30 ml by pressure filtration using a 3000 MWCO membrane. The concentrated solution was dialysed for 3 days against water using a 3500 MWCO membrane and freeze-dried. The NDBP-heparin was then loaded onto a P-2 column (2.5 cm × 40 cm) and eluted with water and the fluorescent peak (Ex. 310 nm/Em. 450 nm) in the void volume was collected and freeze-dried. The total isolated yield of NDBP-heparin is ~5–6% (based on the weight of raw heparin starting material). NDBP-heparin was characterized by 600 MHz ^{1}H-NMR spectroscopy and by gradient PAGE.

Preparation and enrichment of NDBP-linkage region oligosaccharides

Enriched NDBP-heparin was dissolved (15 mg/3 ml) in 5 mM sodium phosphate buffer containing 200 mM sodium chloride, pH 7.15. The solution was preincubated at 30°C for about 1 h, 125 mIU of heparin lyase I were added (in 100 µl 50 mM phosphate buffer, containing 2 mg/ml of bovine serum albumin as a stabilizer, pH 7.0) and incubated for 6 h. Another 125 mIU of heparin lyase I were added and the sample was incubated for additional 6 h. The NDBP-linkage region oligosaccharides were dialysed for 1 day against water using a 1000 MWCO membrane and freeze-dried.

The NDBP-linkage region oligosaccharides were purified on a 10 ml phenyl-Sepharose column (1 cm × 8 cm) using the same procedure described for the enrichment of NDBP-heparin. The elution was monitored by both absorbance (232 nm) and fluorescence (Ex. 310 nm/Em. 450 nm). The enriched NDBP-linkage region oligosaccharides were eluted with water, dialysed exhaustively against water using a 1000 MWCO membrane and freeze-dried.

Isolation of NDBP-octasaccharide by reversed-phase (RP) HPLC

Enriched NDBP-oligosaccharide mixture (20 and 200 µg were injected on analytical and semi-preparative columns, respectively), were subjected to RP-HPLC on a phenyl column with fluorescence (Ex. 310 nm/Em. 450 nm) and dual wavelength absorbance (232 nm and 310 nm) detection. The samples were eluted by a linear gradient of 20–40% of B over 60 min at a flow rate of 1 ml/min (4 ml/min, semi-preparative). Solvent A contained 0.05% (v/v) aqueous trifluoroacetic acid and solvent B contained 0.05% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile in water. Peaks were collected, neutralized with sodium bicarbonate, dialysed exhaustively against water using a 1000 MWCO membrane and freeze-dried.

Heparin lyase III treatment of NDBP-octasaccharide

NDBP-octasaccharide (10 µg) was dissolved in 10 µl of 50 mM sodium phosphate buffer, pH 7.0, 100 mIU of heparin lyase III were added and the solution incubated at 43°C for 20 h. The resulting solution was loaded onto a SP-Sephadex column (1 cm × 5 cm), equilibrated with 30 mM hydrochloric acid, to remove the protein. The column was then washed with 30 mM hydrochloric acid. The eluent was collected, neutralized with dilute sodium hydroxide, freeze-dried and reconstituted in 10 µl of distilled water. The resulting product solution was analysed by CE.

Heparin lyase III treatment of NDBP-oligosaccharide mixture

Enriched NDBP-oligosaccharide mixture (15 mg, prepared by heparin lyase I treatment of NDBP-heparin and phenyl-Sepharose chromatography) was dissolved in 50 mM sodium phosphate buffer, pH 7.0, 200 mIU of heparin lyase III were added and the solution incubated at 43°C for 20 h. An additional 200 mIU of heparin lyase III were added and the reaction incubated for 24 h. The sample was then desalted using a Biogel P-2 column monitored by fluorescence (Ex. 310 nm/Em. 450 nm). The desalted sample was subjected to RP-HPLC (as previously described) to obtain the pure NDBP-tetrasaccharide.

Two-dimensional (2D) NMR analysis of NDBP-tetrasaccharide and NDBP-octasaccharide

The spectra were obtained on a Brucker AMX 600 NMR spectrometer at ambient temperature (298 K). The sample (0.1–1 mM) was dissolved in ^2H_2O (99.9%), filtered through a 0.45 µm membrane and freeze-dried. After exchanging three times by freeze-drying from ^2H_2O, the sample was dissolved in 500 µl of ^2H_2O (99.996%). The 2D experiments including COSY, NOESY and HOHAHA were performed using standard Brucker software. 2D NOESY experiments were recorded with a mixing time of 300 ms. 2D HOHAHA spectra were performed using MLEV-17 spin-lock pulse sequence of 100 ms. All 2D spectra were performed with 256 or 512 t_1 experiments, and 80 scans of 1024 (COSY and HOHAHA) or 2048 (NOESY) data points were collected in each t_1 increment.

Capillary electrophoresis analysis of NDBP-octasaccharide and NDBP-tetrasaccharide

CE was used to identify the disaccharides (absorbance detection at 232 nm) formed by the heparin lyase III treatment of NDBP-octasaccharide. CE was also used to identify and establish the purity of the NDBP-tetrasaccharide (fluorescence detection at Ex. 310 nm/ Em. 450 nm) produced by the heparin lyase III treated NDBP-oligosaccharides. A new capillary (69 cm total length, 75 µm internal diameter) was activated by sequentially washing with phosphoric acid (0.1 M, 500 µl), sodium hydroxide (0.5 M, 1 ml), distilled, deionized water (37°C, 0.5 ml) and run buffer (1 ml). The buffer consisted of 10 mM sodium borate containing 50 mM SDS at pH 8.8. The samples (1 mg/ml), prepared in deionized water, were injected (~14 nl) into the capillary using hydrostatic pressure. Electrophoresis was performed at a constant voltage of 20 kV for a period of 30 min and data points were sampled at a rate of 10 Hz.

RP-ion-pairing (IP) HPLC analysis of NDBP-oligosaccharides and AGA-monosaccharides

RPIP-HPLC separation used a phenyl column with the chiral, (R)-(+)-*N*, *N*-dimethyl-1-phenethylamine, as an ion-pairing agent (Kim *et al.*, 1995). Solvent A contained an aqueous solution of 0.2% (w/v) chiral amine, pH 3.5 (adjusted by acetic acid) and solvent B contained an equal volume mixture of solvent A and acetonitrile. A linear gradient, from 80% to 50% solvent A over 60 min at a flow rate of 1 ml/min, was used to purify NDBP-octasaccharide. A linear gradient, from 99% to 80% solvent A over 60 min at a flow rate of 1 ml/min, was used to analyse AGA-monosaccharide conjugates prepared by reductive amination as previously described (Lee *et al.*, 1991).

Preparation of AGA-Xyl-heparin

The procedure to remove the labelled core peptide was analogous to the mild β-elimination conditions for proteoglycans reported by Heinegård (1972). NDBP-heparin (10 mg) was dissolved in 5 ml of 0.5 M lithium hydroxide (nitrogen saturated) and the solution was kept at 4°C for 24 h under nitrogen atmosphere. The reaction was stopped by addition of 5 ml of 0.5 N acetic acid and immediately freeze-dried. The sample was dissolved in 5 ml of 0.2 M sodium phosphate buffer and dialysed against water using 1000 MWCO membrane under nitrogen atmosphere and freeze-dried. The sample was then reconstituted in 1 ml of 16% (w/v) sodium chloride and precipitated by adding 6 ml of methanol. The GAG precipitate was dialysed against water using a 1000 MWCO membrane and freeze-dried. Released heparin (5–8 mg) was dissolved in 1.6 ml of formamide containing 1 mg of dried molecular sieves (4 Å) by shaking at 250 r.p.m. at 37°C for 1 h. A solution of AGA in formamide [400 µl of 10% (w/v) AGA] was added and shaken for 12 h. Sodium cyanoborohydride (6 mg) was then added. After 6 h additional sodium cyanoborohydride (6 mg) was added. After shaking for another 12 h, the reaction mixture was diluted with 10 volumes of water, dialysed using a 1000 MWCO membrane and freeze-dried. To remove the unreacted AGA tag, the mixture was reconstituted in 200 µl of 16% (w/w) sodium chloride solution. The AGA-Xyl-heparin was precipitated by adding anhydrous methanol to the final concentration of 80% (v/v) and recovered by centrifuging for 5 min. The precipitate was washed with cold methanol. A phenyl-Sepharose column (10 ml) was used to remove residual hydrophobic NDBP-heparin from AGA-Xyl-heparin. AGA-Xyl-heparin was reconstituted in 1 ml of 3.8 M (pH 3.3) ammonium sulphate solution and loaded on the column, equilibrated by 3.8 M (pH 3.3) ammonium sulphate, at 4°C. The column was then washed successively with 50 ml portions of 3.8 M (pH 3.3) and 2.0 M (pH 3.5) ammonium sulphate solutions and water, at a flow rate of 20 ml/h. The fluorescent fractions (Ex. 310 nm/Em. 450 nm) eluting with 2 M ammonium sulphate were combined, dialysed against water using a 1000 MWCO membrane and freeze-dried.

Analysis of AGA-Xyl-heparin

The contamination of NDBP-heparin in the AGA-Xyl-heparin was estimated by gradient PAGE following heparin lyase I depolymerization since NDBP-heparin and AGA-Xyl-heparin gave fluorescent bands with different migration distance.

AGA-Xyl-heparin was hydrolysed to monosaccharides using trifluoroacetic acid (TFA) to confirm that only Xyl had been labelled. AGA-Xyl-heparin (100 µg) was dissolved in 200 µl of 2 M TFA in a 1 ml ampoule, sealed under argon, and heated at 100°C for 6 h. The hydrolysed AGA-Xyl-heparin was diluted with 5 ml of water and freeze-dried. One portion of the freeze-dried sample was analysed by RPIP-HPLC (Kim *et al.*, 1995), another portion was analysed by gradient PAGE gel. Appropriate standard monosaccharide-AGA conjugates were prepared (Lee *et al.*, 1991; Kim *et al.*, 1995) and used in control experiments and as HPLC and electrophoresis standards.

Acknowledgements

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Abbreviations

AGA, 7-amino-1,3-naphthalene disulphonic acid; CE, capillary electrophoresis; GAG, glycosaminoglycan; MWCO, molecular weight cut-off; NDBP, *N*-4-(6-dimethylamino-2-benzofuranyl) phenyl; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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