Combined strong anion-exchange HPLC and PAGE approach for the purification of heparan sulphate oligosaccharides

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Heparan sulphates are highly sulphated linear polysaccharides involved in many cellular functions. Their biological properties stem from their ability to interact with a wide range of proteins. An increasing number of studies, using heparan sulphate-derived oligosaccharides, suggest that specific structural features within the polysaccharide are responsible for ligand recognition and regulation. In the present study, we show that strong anion-exchange HPLC alone, a commonly used technique for purification of heparan sulphate-derived oligosaccharides, may not permit the isolation of highly pure heparan sulphate oligosaccharide species. This was determined by PAGE analysis of hexa-, octa- and decasaccharide samples deemed to be pure by strong anion-exchange HPLC. In addition, subtle differences in the positioning of sulphate groups within heparan sulphate hexasaccharides were impossible to detect by strong anion-exchange HPLC. PAGE analysis on the other hand afforded excellent resolution of these structural isomers. The precise positioning of specific sulphate groups has been implicated in determining the specificity of heparan sulphate interactions and biological activities; hence, the purification of oligosaccharide species that differ in this way becomes an important issue. In this study, we have used strong anion-exchange HPLC and PAGE techniques to allow production of the homogeneous heparan sulphate oligosaccharide species that will be required for the detailed study of structure/activity relationships.

Key words: biological activity, SAX-HPLC, structure.

INTRODUCTION

Heparan sulphate (HS) proteoglycans are ubiquitous components of cell surfaces, extracellular matrices and basement membranes in vertebrates [1]. Because of their strategic positioning at the interface between the cells and their surrounding environment, they are involved in a multitude of biological functions. These range from the regulation of cell adhesion, migration and proliferation to lipid metabolism and anti-coagulant activities [2–4]. The HS chains carried by proteoglycans are responsible for most of these properties due to their ability to engage with a wide range of proteins through specific binding interactions. Essential compositional features of the HS chains, notably their sulphation pattern, governs the specificity of these interactions [5,6]. However, the striking level of structural variability displayed by the polysaccharide [7] complicates extensive studies of the relationships between HS molecular organization and its biological properties. This variability stems from a complex biosynthesis process involving a stepwise series of modifications of the polysaccharide precursor, initially composed of alternating GlcNAc and GlcA units. The first of these modifications consists of the N-deacetylation/N-sulphation of glucosamine clusters [8], producing regions within the chain that are particularly susceptible to further structural modification. These areas, known as sulphated domains or S-domains, are further transformed by C-5 epimerization of GlcA to L-iduronic acid, followed by O-sulphation, predominantly at positions C-2 of L-iduronic acid and C-6 of N-sulphated glucosamine and GlcNAc [9,10]. Other more infrequent O-sulphations occur at C-2 of GlcA [2] and C-3 of N-sulphated glucosamine [11–13]. The resulting mature HS chains are composed of hyper-variable highly sulphated S-domains, which alternate with sparsely modified regions. This biosynthetic route is clearly non-random with different cell types producing distinct HS structures in terms of their domain organization and sulphation content/pattern [5]. The S-domains of the HS chains are involved in mediating both recognition and activation of various HS-binding proteins, such as basic fibroblast growth factor [14–17], hepatocyte growth factor [18], platelet-derived growth factor [19], herpes simplex gC glycoprotein [20] and platelet factor 4 [21]. The binding of S-domains to proteins has been shown to be specific [6,7]. Moreover, subtle structural features of these S-domains have been implicated in their ability to inhibit or activate proteins [22]. To date, much of our understanding of HS biological activities has been determined using the structurally related molecule heparin. However, due to its less variable structure, heparin may not contain all the possible regulatory sequences found in HS.

Specific enzymes such as heparinase III have been used to excise S-domain oligosaccharides from HS chains in order to assess their biological properties and define structure/function relationships [14,18,22]. Sequencing of HS oligosaccharides is also now achievable [23–25]. However, due to the considerable heterogeneity of HS structure, purification of large sulphated oligosaccharides has been extremely difficult to achieve. Strong anion-exchange (SAX) HPLC is commonly used for the preparation of heparin oligosaccharides [26,27]. Oligosaccharide mixtures derived from heparin are much simpler than those derived from HS; even so, SAX-HPLC was used successfully for the purification of short HS oligosaccharides (di-, tetra- and hexasaccharides) [28]. Larger HS oligosaccharides (octasaccharides upwards), which are generally those promoting the activation of HS-dependent proteins, are more difficult to prepare and the level of purity achieved has never been assessed clearly. PAGE has been used for mapping the enzymic or chemical degradation patterns of HS chains from different sources [29,30] and for purification of short heparin-derived hexa- and octa-

Abbreviations used: dp, degree of polymerization; HS, heparan sulphate; S-domain, sulphated domain; SAX, strong anion-exchange.

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saccharides [31]. In the present study, we demonstrate that SAX-HPLC alone may not enable sufficient resolution of complex mixtures of HS oligosaccharides. In fact, subtle differences in positioning of sulphate groups within HS hexasaccharides were impossible to detect by SAX-HPLC. PAGE, on the other hand, showed excellent separations of samples with identical SAX-HPLC elution times. We therefore propose a combined approach based on the utilization of SAX-HPLC and PAGE, which can separate HS oligosaccharides with minor structural variations. Results obtained show that the coupling of both techniques enables the preparation of large amounts of highly pure HS oligosaccharide species. This new approach will facilitate the isolation of homogeneous HS oligosaccharides for the determination of precise structure/function relationships.

MATERIALS AND METHODS

Materials

Porcine mucosal HS was obtained from Organon (Oss, The Netherlands). Heparinase III (*Flavobacterium heparinum*; EC 4.2.2.8) was purchased from Grampian Enzymes (Orkney, U.K.). Bio-Gel P-6 was from Bio-Rad (Hemel Hempstead, Herts., U.K.). PD-10 desalting columns were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). ProPac PA1 SAX-HPLC columns were purchased from Dionex (Camberley, Surrey, U.K.). Acrylamide and bis-acrylamide was supplied by Sigma-Aldrich (Poole, Dorset, U.K.). Azure A was from Fluka (Gillingham, Dorset, U.K.). Bromophenol Blue, Phenol Red, Xylene Cyanole and all other reagents were obtained from BDH-Merck (Lutterworth, Leics., U.K.) and were of AnalaR grade.

Preparation of HS oligosaccharides

HS hexa-, octa- and decasaccharides were prepared and purified by heparinase III treatment of porcine mucosal HS. Porcine mucosal HS (200 mg) was digested with 50 m-units of heparinase III in 1 ml of 100 mM sodium acetate/0.5 mM calcium acetate buffer, pH 7.0, for 24 h at 37 °C. The extent of the degradation was followed by monitoring the increase in UV absorbance at 232 nm; production of heparinase III-resistant oligosaccharides was achieved by further additions of enzyme until there was no further increase in absorbance. The oligosaccharides generated were size-separated by gel-filtration chromatography, using a Bio-Gel P-6 column (170×1.5 cm) run at 6 ml/h in 0.5 M ammonium hydrogen carbonate. Peaks were detected by measuring absorbance at 232 nm and 1 ml fractions were collected. Fractions containing size-defined oligosaccharide were pooled and freeze-dried several times to remove excess ammonium hydrogen carbonate.

The size-fractionated hexa-, octa- and decasaccharide mixtures were then resolved by SAX-HPLC, by application to a ProPac PA1 column (4×250 mm) equilibrated in Milli Q water, pH 3.0, at a flow rate of 1 ml/min. Samples were eluted using a 0–0.9 M NaCl gradient over 2 h and a 0.5–1.0 M NaCl gradient over 2 h for the hexa- and octa-/decasaccharides respectively in Milli Q water, pH 3.0, at a flow rate of 1 ml/min. Oligosaccharides were detected by absorbance at 232 nm; fractions (1 ml) containing peaks of interest were pooled, freeze-dried and desalted on a PD-10 column. Octa- and decasaccharide samples were purified further by SAX-HPLC using narrower NaCl gradients of 0.8–1.0 M NaCl and 0.64–0.74 M NaCl respectively over 2 h in Milli Q water, pH 3.0, at a flow rate of 1 ml/min. Fractions containing oligosaccharides were again pooled carefully to avoid cross-contamination, desalted and freeze-dried. Final sample purity for the octa- and decasaccharides was determined by SAX-HPLC, using the previous conditions above.

PAGE separation of HS oligosaccharides

Oligosaccharide samples (10–60 μg/lane, up to approx. 900 μg/gel) in 20 % glycerol were loaded into wells in a volume of 10–50 μl. A mixture of Xylene Cyanole, Bromophenol Blue and Phenol Red in 20 % glycerol was applied to a separate lane as electrophoresis markers. Samples were run initially through a 1.5 cm stacking gel (5 % acrylamide/2 % cross-linker) at a constant voltage of 150 V for about 1 h, then through a $16 \text{ cm} \times 12 \text{ cm} \times 0.75$ mm resolving gel (30 % acrylamide/5 % cross-linker) at a constant current of 25 mA for 3–4 h (maximum voltage of 1000 V) until the Phenol Red marker had reached the bottom of the gel. The discontinuous buffer system of Laemmli [32] was used, which comprised 0.375 M Tris/HCl, pH 8.8, in the resolving gel and 0.125 M Tris/HCl, pH 6.8, in the stacking gel, using 25 mM Tris/0.192 M glycine, pH 8.3, as tank buffer. After electrophoresis, oligosaccharide bands were visualized by staining with 0.08 % aqueous Azure A for 10 min under constant agitation. The excess of dye was removed by washing the gel twice in water for 5 min.

Recovery of HS oligosaccharides from polyacrylamide gels

Bands corresponding to each oligosaccharide species were excised from the gel, crushed and agitated overnight in 1 ml of PBS. After centrifugation, the supernatant was removed and stored at 4 °C, the pellet was again resuspended in 1 ml of PBS and left to agitate overnight. The operation was repeated once more and the three supernatant fractions pooled. The combined supernatants were then applied to a PD-10 desalting column, eluted with water, and oligosaccharide-containing fractions were pooled and freeze-dried. Next, the gel-extracted species were again purified by SAX-HPLC using a ProPac PA1 column (4×250 mm) with a gradient of 0.4–0.9 M NaCl over 1 h for the octa- and decasaccharides. Fractions containing oligosaccharides were pooled, desalted on a PD-10 column and freeze-dried. Final purity was then determined by re-analysing each individually extracted species by PAGE followed by staining with Azure A.

RESULTS

Analysis of HS oligosaccharides by SAX-HPLC and PAGE

The depolymerization of HS by heparinase III yields very complex mixtures of oligosaccharides. These were first fractionated according to their size by gel-filtration chromatography, which separated fragments ranging from di- to dodecasaccharides [22]. Size-defined oligosaccharides were then resolved by SAX-HPLC. Figure 1(A) shows a SAX elution profile for heparinase III-derived HS decasaccharides (denoted dp10, where dp is degree of polymerization or number of saccharide units, i.e. decasaccharide = dp10), with the level of complexity being characteristic of HS-derived oligosaccharides as opposed to those obtained by enzymic treatment of heparin. An expanded region of the profile (Figure 1B) was then analysed further by PAGE and the results clearly showed the mixture to be even more complex than that suggested by SAX-HPLC alone (Figure 1C). The gel in Figure 1(C) also shows that bands A and B, C and D, and E–G had similar mobilities in the gel but different SAX-HPLC elution times. Therefore, a two-stage

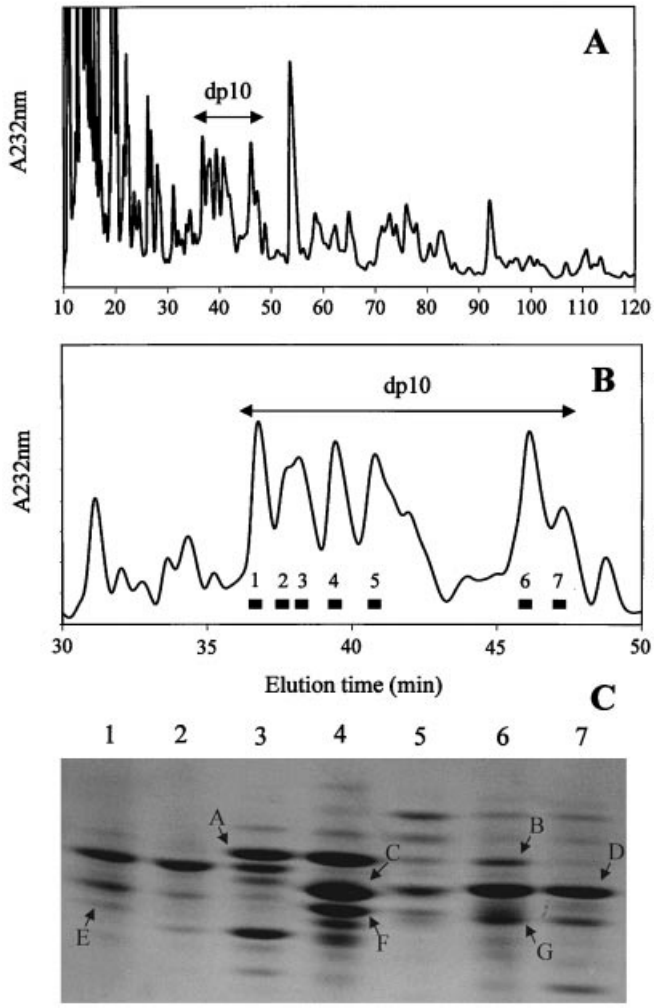

Figure 1 Separation of heparinase III-generated HS decasaccharides by SAX-HPLC and PAGE

HS oligosaccharides were isolated following heparinase III treatment of porcine mucosal HS as described in the Materials and methods section. Size-defined HS decasaccharides were separated by SAX-HPLC (**A**), using a ProPac PA1 column equilibrated with Milli Q water, pH 3.0, and eluted with a linear gradient of 0.5–1.0 M NaCl over 2 h at a flow rate of 1 ml/min. Decasaccharides (dp10) were detected by absorbance at 232 nm and 0.5 ml fractions collected. (**B**) Expansion of the above SAX-HPLC profile showing the regions from which fractions (1–7) were pooled (black bars), prior to PAGE analysis. Decasaccharide samples were then resolved on a 16 cm × 12 cm × 0.75 mm gel consisting of 30% acrylamide/5% cross-linker at a constant current of 25 mA for 3–4 h (maximum voltage of 1000 V) (**C**). A mixture of Xylene Cyanole, Bromophenol Blue and Phenol Red in 20% glycerol was used in a separate lane as visual markers for each run. Decasaccharide bands were visualized by staining the gel with 0.08% aqueous Azure A for 10 min under constant agitation. The excess of dye was removed by washing the gel several times in water.

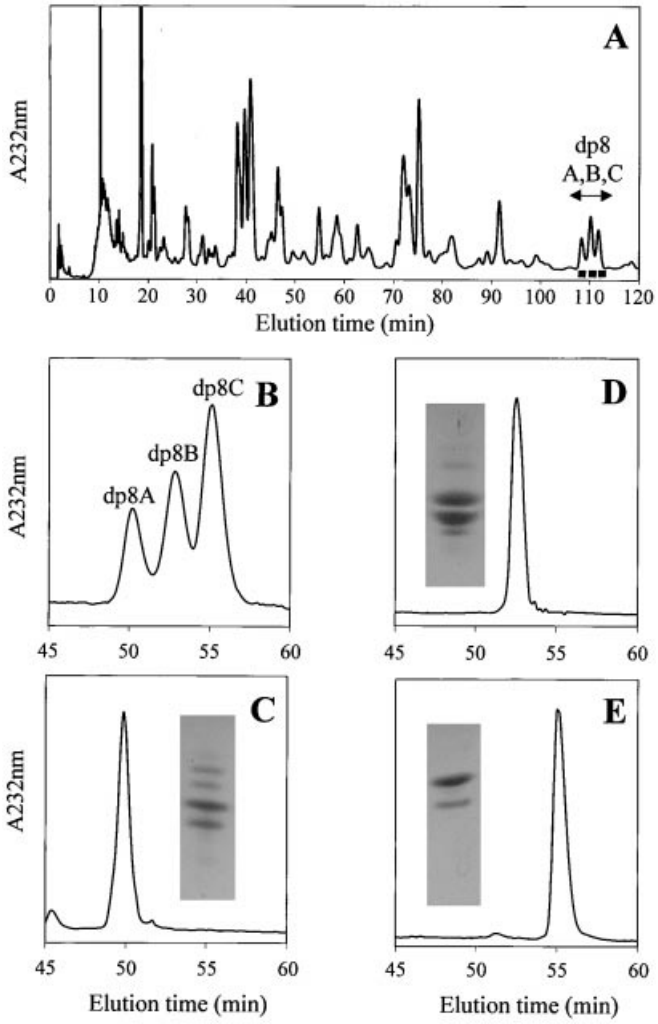

Figure 2 Separation of heparinase III-resistant HS octasaccharides by SAX-HPLC and PAGE

HS octasaccharide mixtures were isolated from exhaustive heparinase III digests of porcine mucosal HS as described in the Materials and methods section. Heparinase III-resistant HS octasaccharides were separated initially by SAX-HPLC (**A**) using a ProPac PA1 column equilibrated with Milli Q water, pH 3.0. Octasaccharide mixtures were applied to the column and eluted with a linear gradient of 0.5–1.0 M NaCl in Milli Q water, pH 3.0, over 2 h at a flow rate of 1 ml/min. Octasaccharides were detected by absorbance at 232 nm and 0.5 ml fractions collected. Three peaks were chosen for further study, pooled as indicated (black bars) and designated dp8A, dp8B and dp8C. These octasaccharide pools were then reapplied separately to the SAX-HPLC column and eluted using a narrower gradient of 0.8–1.0 M NaCl in Milli Q water, pH 3.0, over 2 h; 0.5 ml fractions were collected and fractions corresponding to individual peaks pooled. Sample purity was determined by re-running each sample on SAX-HPLC using the narrower gradient (**B**) mixture of the three octasaccharides: (**C**) dp8A; (**D**) dp8B; (**E**) dp8C. Insets show the corresponding PAGE separation of each SAX-HPLC-pure sample; conditions for PAGE were as described for Figure 1(B).

technique utilizing both SAX-HPLC and PAGE seemed attractive as a method for the purification of single HS oligosaccharide species.

Analysis of SAX-HPLC-purified HS octa- and decasaccharides by PAGE

In order to try to simplify the SAX elution profiles the study was continued with more extensively degraded HS oligosaccharides, which showed little if any susceptibility to further heparinase III treatment. This gave a similar but slightly less complex profile for the decasaccharides than seen previously in Figure 1(A). HS-derived octasaccharides were purified initially using a 0.5–1.0 M NaCl gradient over 2 h (Figure 2A) and three peaks (A, B and C) were chosen for further study. The middle portion of each peak of interest was pooled and reapplied to the SAX column and eluted with a narrower gradient of 0.8–1.0 M NaCl over 2 h. This gave adequate resolution of the three peaks of interest (Figure 2B), and fractions corresponding to single peaks were pooled (additional elongation of the NaCl gradient failed to improve the

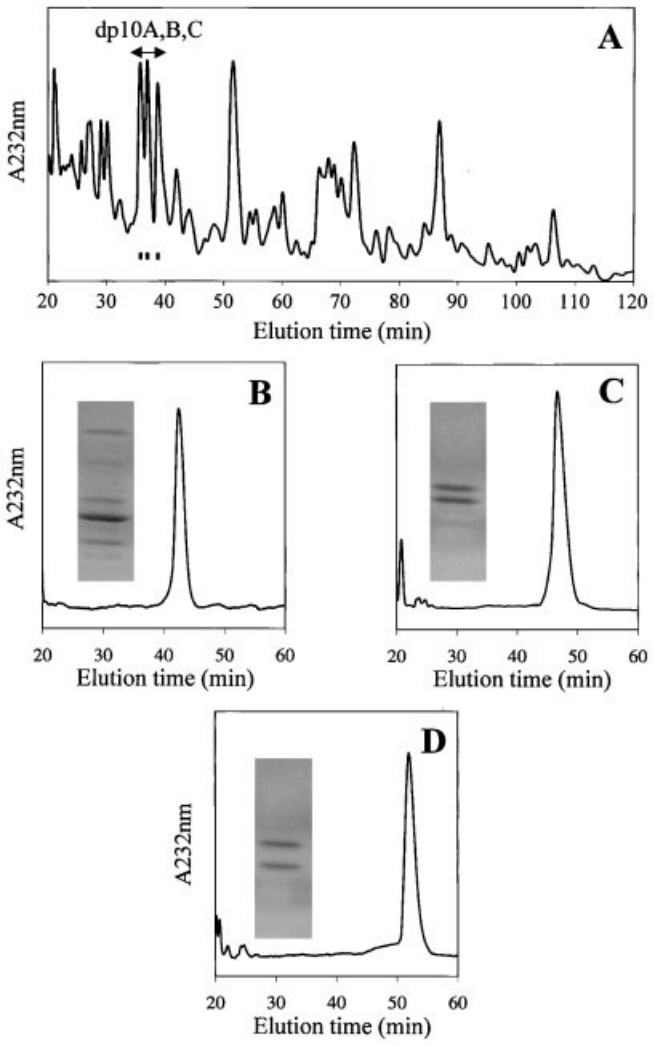

Figure 3 Separation of heparinase III-resistant HS decasaccharides by SAX-HPLC and PAGE

Heparinase III-resistant HS decasaccharide mixtures were isolated from exhaustive heparinase III digests of porcine mucosal HS as described in the Materials and methods section. Decasaccharides were initially separated by SAX-HPLC (**A**) using a ProPac PA1 column equilibrated with Milli Q water, pH 3.0. Decasaccharide mixtures were applied to the column and eluted with a linear gradient of 0.5–1.0 M NaCl in Milli Q water, pH 3.0, over 2 h at a flow rate of 1 ml/min. Decasaccharides were detected by absorbance at 232 nm and 0.5 ml fractions collected. Three peaks were chosen for further study, pooled as indicated (black bars) and designated dp10A, dp10B and dp10C. These decasaccharide pools were then reapplied to the SAX-HPLC column and eluted using a narrower gradient of 0.64–0.74 M NaCl in Milli Q water, pH 3.0, over 2 h; 0.5 ml fractions were collected and the peaks of interest pooled. Sample purity was determined by re-running each sample on SAX-HPLC using the previous gradient: (**B**) dp10A; (**C**) dp10B; (**D**) dp10C. Insets show the corresponding PAGE separation of each SAX-HPLC-pure sample; conditions for PAGE were as described for Figure 1(B).

separation of the peaks). In order to confirm their SAX-HPLC purity, samples were run for a second time using the narrower gradient (Figures 2C, 2D and 2E). Analysis by PAGE showed several bands for each SAX-HPLC-pure sample (see Figures 2C, 2D, and 2E, insets), and confirmed the complex nature of heparinase III-generated HS oligosaccharides even after prolonged treatment with the enzyme.

Single-peak SAX-HPLC samples, from the even more complex mixture of decasaccharides (Figure 3), were also composed of several oligosaccharide species (see Figures 3B, 3C and 3D, insets). These samples had again been purified using a narrower gradient of 0.64–0.74 M NaCl over 2 h, following an initial separation using a gradient of 0.5–1.0 M NaCl over 2 h. To date, we have not found a single SAX-HPLC-pure sample of HS-derived octa- and decasaccharides to be homogeneous by PAGE analysis.

Differences in HS oligosaccharide structure and their effect on SAX-HPLC and PAGE separations

Heparinase III-derived HS hexasaccharide mixtures were resolved by SAX-HPLC and a single peak chosen and pooled as indicated in Figure 4(A). The presence of a single peak following re-chromatography of this pooled sample using the previous conditions (Figure 4A, inset) verified the sample's apparent purity. This SAX-HPLC-purified peak was subjected to PAGE analysis and two main species were resolved clearly (Figure 4B). These hexasaccharides, which have been sequenced [24], differ only in the positioning of a single 6-*O*-sulphate group, which is on either the internal or reducing-end disaccharide. The failure of SAX-HPLC to resolve even short HS oligosaccharides, which inherently separate more efficiently than the larger fragments, reinforces the difficulties that lie ahead in the purification of homogeneous HS oligosaccharide samples.

Purification of HS oligosaccharides by a combination of SAX-HPLC and PAGE

Once the ability of PAGE to resolve oligosaccharides with similar charge content (i.e. similar SAX-HPLC elution times) had been established, the possibility of its use as a preparative technique was investigated. Sample dp10A (Figure 3) was run on a polyacrylamide gel and bands corresponding to the four main species were cut from it (Figure 5). Samples were recovered from the gel and Azure A dye and other low-molecular-mass contaminants removed using a desalting column. Re-analysis of these samples by PAGE showed that single species could be obtained with no detectable cross-contamination (Figure 5B). Another example of gel extraction is shown in Figure 6, in which the two components of the dp8C octasaccharide sample (Figure 6A, inset), species 1 (Figure 6B, inset) and species 2 (Figure 6C, inset) were purified to homogeneity. Comparison of the band intensities of several samples before and after extraction from the gel showed recoveries of 70–80 % (results not shown). However, high-molecular-mass or oligosaccharide-binding contaminants were a problem when biological activities of these gel-extracted samples were assessed (results not shown). These contaminants were removed by SAX-HPLC using a short NaCl gradient as in Figures 6(B) and 6(C), following removal of Azure A and other low-molecular-mass material. The reapplication and elution from the SAX column of the dp8C sample, gel-purified species 1 and 2 and a co-injected mixture of species 1 and 2 also allowed the verification of their near-identical elution times (Figures 6B, 6C and 6D). The results show clearly that the purification protocol did not, at any stage, bring about partial chemical desulphation of oligosaccharides, which may have resulted in the complex banding pattern observed by PAGE.

DISCUSSION

The biological diversity of HS activities is related to the molecule's ability to interact with a wide range of proteins. Numerous studies have suggested that distinct S-domains within the HS chain are responsible for ligand recognition and that specific

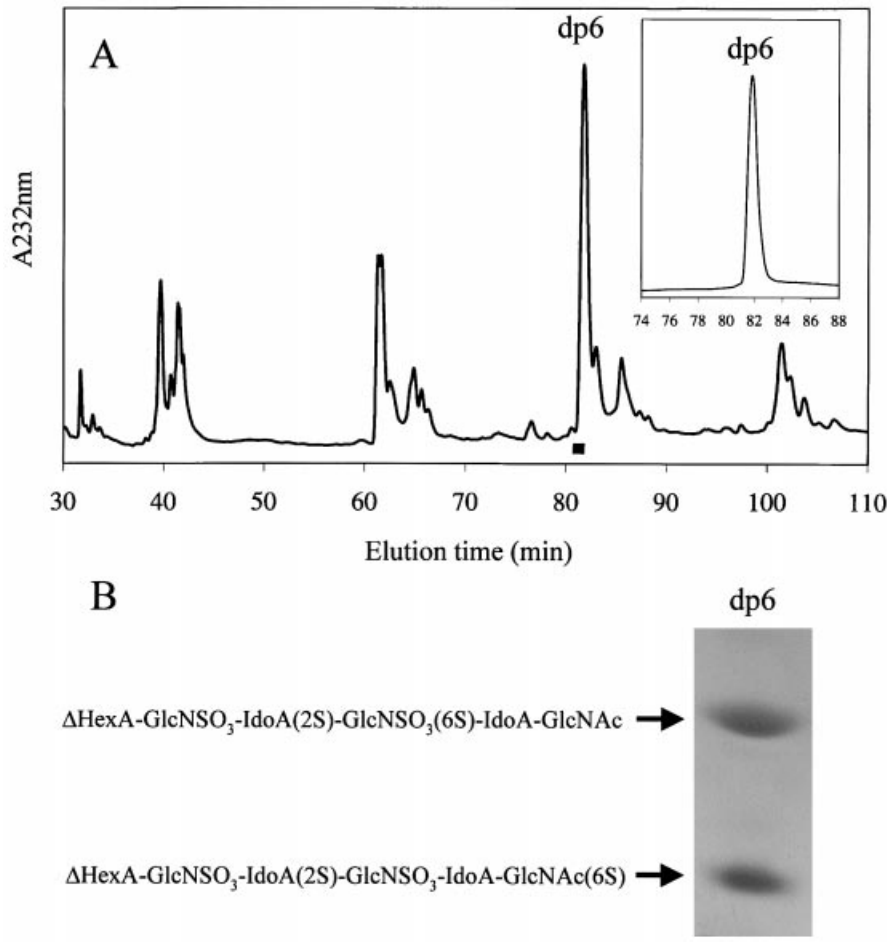

Figure 4 Analysis of two structurally defined HS hexasaccharides by PAGE and SAX-HPLC

Size-defined HS hexasaccharides from a heparinase III digest of porcine mucosal HS were prepared as described in the Materials and methods section. (**A**) These samples were resolved by SAX-HPLC using a linear gradient of 0–0.9 M NaCl in Milli Q water, pH 3.0, over 2 h at a flow rate of 1 ml/min. Peaks were detected by absorbance at 232 nm and 1 ml fractions collected. One peak (dp6) was chosen for further study and fractions were pooled as indicated (black bar). The purity of the sample was determined by reapplication of the sample to the SAX column and elution under the previous conditions (inset). The hexasaccharide sample was analysed by PAGE (**B**), with conditions as described for Figure 1(B). The structures shown for the two hexasaccharide species were determined by sequence analysis [24]. $GlcNSO_3$, N-sulphated glucosamine; $GlcNSO_3$(6S), $GlcNSO_3$ 6-*O*-sulphate; GlcNAc(6S), *N*-acetylglucosamine 6-*O*-sulphate; ΔHexA, Δ4,5-unsaturated uronic acid; IdoA, iduronic acid; IdoA(2S), iduronic acid 2-*O*-sulphate.

structural features govern the specificity of the interaction/biological response [5,6]. For example, the high-affinity binding of HS/heparin to antithrombin is mediated by a particular sulphated pentasaccharide sequence [33]. Recent work using HS-derived oligosaccharides, which promote activity of basic fibroblast growth factor, suggested that specific positioning of one or two 6-*O*-sulphate groups is required for the promotion of biological activity [22]. Moreover, studies on other growth factors including acidic fibroblast growth factor [34,35] and hepatocyte growth factor [18] have also implicated precise sulphate-group positioning in conferring specificity in their interactions with HS. In order to determine the exact structural requirements for both binding and activation/inhibition of different HS-binding proteins it will be necessary to purify S-domains and study their structure/activity relationships. Enzymes such as heparinase III have been used to produce S-domain oligosaccharides from intact HS [17,18,22]. Suitable methods for screening biological activities also exist, as do methods for the sequencing of HS oligosaccharides [23–25]. However, studies aimed at determining the exact structural requirements for HS-mediated biological responses will be difficult without the ability to purify homogeneous HS oligosaccharide species.

The most prevalent technique used to date for the purification of HS oligosaccharides is SAX-HPLC [22,26–28]. However, the final purity following this step has only been assessed for samples up to hexasaccharide length [28]. The homogeneity of larger HS oligosaccharides, which generally contain the protein-activating sequences, has never been determined adequately. The separation limits of SAX-HPLC were investigated using heparinase III-generated HS hexa-, octa- and decasaccharides. Heparinase III treatment of HS has been shown previously by SAX-HPLC to generate complex mixtures of oligosaccharides [22]. However, PAGE analysis of a region of the SAX-HPLC profile for a decasaccharide mixture showed a further level of complexity, with fractions of similar SAX-HPLC elution times containing multiple bands. Also, some bands had similar mobilities in the gel but different SAX elution times. In all, approx. 17 different species seen on the gel corresponded to a group of six poorly resolved SAX-HPLC peaks. The results show that HS oligosaccharides of similar structure, in terms of length and net

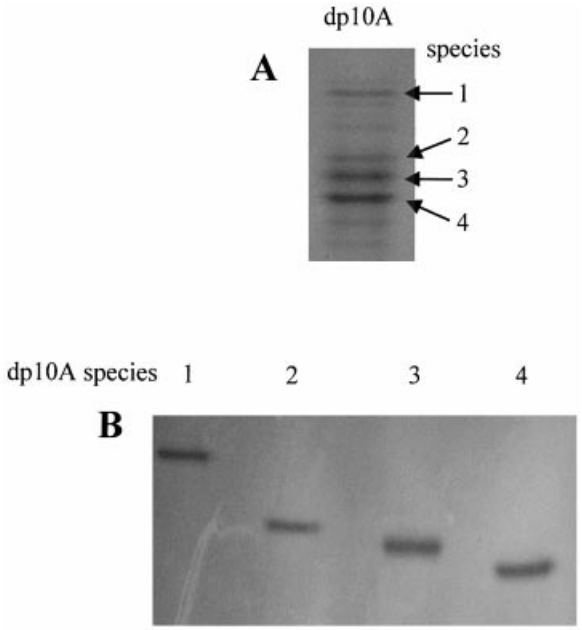

Figure 5 Isolation and recovery of HS decasaccharides by PAGE

The HS decasaccharide sample dp10A previously purified to apparent homogeneity by extensive SAX-HPLC (Figure 3B) was subjected to PAGE (**A**) as described in Figure 1(B). The bands corresponding to the dp10 species 1, 2, 3 and 4 were cut from the gel, crushed and left to agitate overnight in 1 ml of PBS in separate tubes. After centrifugation, the supernatants were recovered, stored at 4 °C and the washing step repeated twice. The pooled supernatants for each species were applied separately to a PD-10 desalting column, eluted with water and the oligosaccharide-containing fractions pooled and freeze-dried. Purity was then determined by re-analysing each individual extracted species by PAGE (**B**).

charge, are unlikely to be resolved to homogeneity by either of the above techniques alone.

Prolonged heparinase III treatment was performed on samples in an attempt to reduce the complexity of the oligosaccharide mixtures generated. This resulted in the simplification of the SAX elution profiles, although the mixtures were still incredibly diverse. Resolution by SAX-HPLC was improved further by using narrower ranges of NaCl gradients. Conditions were optimized for each oligosaccharide sample until no further improvement in resolution occurred. Analysis of these single peaks by PAGE enabled excellent separation of the HS oligosaccharides present and showed that all the samples, assessed to be pure by SAX-HPLC in this study, were in fact heterogeneous. The extent of contamination shows that SAX-HPLC alone may not be relied upon for the preparation of homogeneous HS oligosaccharide structures. The separation of HS oligosaccharides by both SAX-HPLC and PAGE is dependent on multiple complex parameters, such as net charge, length, uronate epimerization and sulphate positioning. We have exploited the different selectivities of SAX-HPLC/PAGE and a simple extraction procedure to produce homogeneous HS oligosaccharide species from SAX-purified samples in milligram quantities. Gradient PAGE and electrotransfer have been used previously for the isolation of small heparin oligosaccharides [31]. Mixtures of heparin-derived oligosaccharides are, however, much less complex in nature than those derived from HS. Also, our PAGE and extraction procedure does not require gradient gels and minimizes the risk of band broadening and sample loss as a result of electrotransfer.

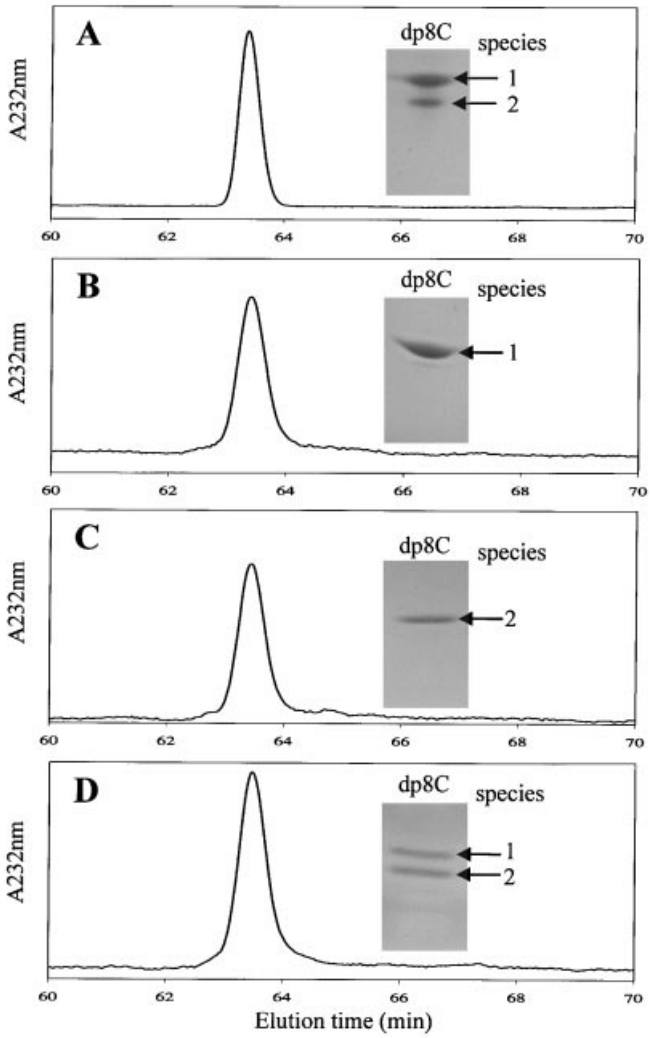

Figure 6 SAX-HPLC purification of gel-extracted HS octasaccharides

The HS octasaccharide sample dp8C previously purified to apparent homogeneity by extensive SAX-HPLC (Figure 2E) was subjected to PAGE separation and the two species extracted as described for Figure 5. The dp8C mixture prior to gel extraction (**A**), the main component; species 1 (**B**), the minor component; species 2 (**C**) and a re-mixed sample of species 1 and 2 (**D**) were applied to a ProPac PA1 column equilibrated with Milli Q water, pH 3.0, and eluted with a linear gradient of 0.4–0.9 M NaCl over 1 h at a flow rate of 1 ml/min. Fractions containing oligosaccharides were pooled, desalted on a PD-10 column then freeze-dried. Insets show PAGE separations of the oligosaccharides prior to injection, with conditions for PAGE as described for Figure 1(B).

Theoretically, many structural possibilities exist for HS-derived oligosaccharides as a result of differences in, for example, sulphation pattern and uronate epimerization. The combined application of both SAX-HPLC and PAGE methodology has allowed us to demonstrate the remarkable array of species actually present in these preparations. Specific positioning of sulphate groups has been implicated in the ability of HS oligosaccharides to modulate growth-factor activity [18,22,34,35]. It seems likely that other structural difference will also play a role in determining the biological activities of HS oligosaccharides. The failure of SAX-HPLC to resolve two structurally defined hexasaccharides [24], which differ only in the location of a single 6-*O*-sulphate group, and their subsequent dramatic separation by PAGE demonstrates the potential

difficulties in isolating closely related HS oligosaccharide structures. The isolation of homogeneous HS oligosaccharides will be essential for the accurate determination of HS structure/activity relationships. The combination of both SAX-HPLC and PAGE separation/extraction methods described here should therefore constitute an essential tool for investigating the mechanism by which HS modulates the activity of its ligands.

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REFERENCES

1 Gallagher, J. T. (1989) The extended family of proteoglycans: social residents of the pericellular zone. Curr. Opin. Cell Biol. **1**, 1201–1218
2 Lindahl, U., Lidholt, K., Spillmann, D. and Kjellen, L. (1994) More to ''heparin'' than anticoagulation. Thromb. Res. **75**, 1–32
3 Gallagher, J. T. (1996) Heparan sulphate proteoglycans: The control of cell growth. In Extracellular Matrix, vol. 2 (Comper, W. A., ed.), pp. 230–245, Harwood Academic, Amsterdam
4 Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J. and Zhang, L. (1997) Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? J. Clin. Invest. **99**, 2062–2070
5 Salmivirta, M., Lidholt, K. and Lindahl, U. (1996) Heparan sulfate: a piece of information. FASEB J. **10**, 1270–1279
6 Lyon, M. and Gallagher, J. T. (1998) Bio-specific sequences and domains in heparan sulphate and the regulation of cell growth and adhesion. Matrix Biol. **17**, 485–493
7 Lindahl, U., Kusche-Gullberg, M. and Kjellen, L. (1998) Regulated diversity of heparan sulfate. J. Biol. Chem. **273**, 24979–24982
8 Lindahl, U. (1989) Biosynthesis of heparin and related polysaccharides. In Heparin (Lane, D. A. and Lindahl, U., eds), pp. 159–189, Edward Arnold, London
9 Lindahl, U. and Kjellen, L. (1987) Biology of proteoglycans. In The Biology of the Extracellular Matrix (Wight, T. N. and Mecham, R. P., eds), pp. 59–104, Academic Press, New York
10 Gallagher, J. T., Lyon, M. and Steward, W. P. (1986) Structure and function of heparan sulphate proteoglycans. Biochem. J. **236**, 313–325
11 Marcum, J. A., Atha, D. H., Fritze, L. M., Nawroth, P., Stern, D. and Rosenberg, R. D. (1986) Cloned bovine aortic endothelial cells synthesize anticoagulantly active heparan sulfate proteoglycan. J. Biol. Chem. **261**, 7507–7517
12 Pejler, G., Backstrom, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S. and Timpl, R. (1987) Structure and affinity for antithrombin of heparan sulfate chains derived from basement membrane proteoglycans. J. Biol. Chem. **262**, 5036–5043
13 Edge, A. S. and Spiro, R. G. (1990) Characterization of novel sequences containing 3-O-sulfated glucosamine in glomerular basement membrane heparan sulfate and localization of sulfated disaccharides to a peripheral domain. J. Biol. Chem. **265**, 15874–15881
14 Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. and Gallagher, J. T. (1992) Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. J. Biol. Chem. **267**, 10337–10341
15 Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K. and Kimata, K. (1992) Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. Biochem. J. **285**, 805–813
16 Maccarana, M., Casu, B. and Lindahl, U. (1993) Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. J. Biol. Chem. **268**, 23898–23905
17 Walker, A., Turnbull, J. E. and Gallagher, J. T. (1994) Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. J. Biol. Chem. **269**, 931–935
18 Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T. and Gallagher, J. T. (1994) Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J. Biol. Chem. **269**, 11216–11223
19 Feyzi, E., Lustig, F., Fager, G., Spillmann, D., Lindahl, U. and Salmivirta, M. (1997) Characterization of heparin and heparan sulfate domains binding to the long splice variant of platelet-derived growth factor A chain. J. Biol. Chem. **272**, 5518–5524
20 Feyzi, E., Trybala, E., Bergstrom, T., Lindahl, U. and Spillmann, D. (1997) Structural requirement of heparan sulfate for interaction with herpes simplex virus type 1 virions and isolated glycoprotein C. J. Biol. Chem. **272**, 24850–24857
21 Stringer, S. E. and Gallagher, J. T. (1997) Specific binding of the chemokine platelet factor 4 to heparan sulfate. J. Biol. Chem. **272**, 20508–20514
22 Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P. and Gallagher, J. T. (1998) Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. J. Biol. Chem. **273**, 22936–22942
23 Turnbull, J. E., Hopwood, J. J. and Gallagher, J. T. (1999) A strategy for rapid sequencing of heparan sulfate and heparin saccharides. Proc. Natl. Acad. Sci. U.S.A. **96**, 2698–2703
24 Vivès, R. R., Pye, D. A., Salmivirta, M., Hopwood, J. J., Lindahl, U. and Gallagher, J. T. (1999) Sequence analysis of heparan sulphate and heparin oligosaccharides. Biochem. J. **339**, 767–773
25 Merry, C. L., 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
26 Rice, K. G., Kim, Y. S., Grant, A. C., Merchant, Z. M. and Linhardt, R. J. (1985) High-performance liquid chromatographic separation of heparin-derived oligosaccharides. Anal. Biochem. **150**, 325–331
27 Pervin, A., Gallo, C., Jandik, K. A., Han, X. J. and Linhardt, R. J. (1995) Preparation and structural characterization of large heparin-derived oligosaccharides. Glycobiology **5**, 83–95
28 Hileman, R. E., Smith, A. E., Toida, T. and Linhardt, R. J. (1997) Preparation and structure of heparin lyase-derived heparan sulfate oligosaccharides. Glycobiology **7**, 231–239
29 Turnbull, J. E. and Gallagher, J. T. (1988) Oligosaccharide mapping of heparan sulphate by polyacrylamide-gradient-gel electrophoresis and electrotransfer to nylon membrane. Biochem. J. **251**, 597–608
30 Lyon, M. and Gallagher, J. T. (1990) A general method for the detection and mapping of submicrogram quantities of glycosaminoglycan oligosaccharides on polyacrylamide gels by sequential staining with azure A and ammoniacal silver. Anal. Biochem. **185**, 63–70
31 al-Hakim, A. and Linhardt, R. J. (1990) Isolation and recovery of acidic oligosaccharides from polyacrylamide gels by semi-dry electrotransfer. Electrophoresis **11**, 23–28
32 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
33 Lindahl, U., Thunberg, L., Backstrom, G., Riesenfeld, J., Nordling, K. and Bjork, I. (1984) Extension and structural variability of the antithrombin-binding sequence in heparin. J. Biol. Chem. **259**, 12368–12376
34 Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U. and Rapraeger, A. C. (1993) Activating and inhibitory heparin sequences for FGF-2 (basic FGF) Distinct requirements for FGF-1, FGF-2, and FGF-4. J. Biol. Chem. **268**, 23906–23914
35 Ishihara, M., Takano, R., Kanda, T., Hayashi, K., Hara, S., Kikuchi, H. and Yoshida, K. (1995) Importance of 6-O-sulfate groups of glucosamine residues in heparin for activation of FGF-1 and FGF-2. J. Biochem. (Tokyo) **118**, 1255–1260

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