Glycoconjugate Journal (1992) **9**:45–55

Sequence analysis of *p*-hydroxyphenyl-*O*-*β*-D-xyloside initiated and radio-iodinated dermatan sulfate from skin fibroblasts

LARS-ÅKE FRANSSON[1], BIRGITTA HAVSMARK[1], KATSUKIYO SAKURAI[2] and SAKARU SUZUKI[3]

[1] *Department of Medical and Physiological Chemistry, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden*
[2] *Tokyo Research Institute, Seikagaku Corporation, Tokyo 189, Japan*
[3] *Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-11, Japan*

Received 29 April 1991, revised 20 August 1991

To generate xyloside-primed dermatan sulfate suitable for sequence analysis, skin fibroblasts were incubated with *p*-hydroxyphenyl-*β*-D-xylopyranoside and [^{3}H]galactose, and free [^{3}H]glycosaminoglycan chains were isolated from the culture medium by ion exchange and gel chromatography. After ^{125}I labelling of their reducing-terminal hydroxyphenyl groups, chains were subjected to various chemical and enzymatic degradations, both partial and complete, followed by gradient polyacrylamide gel electrophoresis and autoradiographic identification of fragments extending from the labelled reducing-end to the point of cleavage. Results of periodate oxidation–alkaline scission indicated that the xylose moiety remained unsubstituted at C-2/C-3; exhaustive treatment with chondroitin AC-I lyase afforded the fragment ΔHexA-Gal-Gal-Xyl-R (R = radio-iodinated hydroxyphenyl group), and complete degradations with chondroitin ABC lyase as well as testicular hyaluronidase yielded the fragments ΔHexA/HexA-GalNAc-GlcA-Gal-Gal-Xyl-R with or without sulfate on the *N*-acetylgalactosamine. Partial digestions with testicular hyaluronidase or chondroitin B lyase indicated that glucuronic acid was common in the first three repeats after the linkage region and that iduronic acid could occupy any position thereafter. Hence, there were no indications of a repeated, periodic appearance of the clustered GlcA-GalNAc repeats which was previously observed in proteoglycan derived dermatan sulfate [Fransson L-Å, Havsmark B, Silverberg I (1990) *Biochem J* **269**:381–8], suggesting a role for the protein part in controlling the formation of particular copolymeric features during glycosaminoglycan assembly.

Keywords: glycosaminoglycan, dermatan sulfate, xylosides, sequencing

Abbreviations: GAG, glycosaminoglycans; CS, chondroitin sulfate; DS, dermatan sulfate; Ser, serine; Xyl, D-xylose; Gal, D-galactose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; ΔHexA, 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid; HO-Phe, *p*-hydroxyphenyl group; HO-Phe-Xyl, *p*-hydroxyphenyl-*O*-*β*-D-xylopyranoside; O_2N-Phe-Xyl, *p*-nitrophenyl-*β*-D-xylopyranoside; -OSO_3, ester sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FPLC, fast performance liquid chromatography; LC, standard liquid chromatography.

Glycosaminoglycans (GAG, i.e., glucosamino- or galactosaminoglycans) are linear, often sulfated carbohydrate polymers which usually are covalently linked to proteins (glycoproteins) forming proteoglycans. They are found in extracellular connective tissue matrices, both interstitially, sometimes associated with collagen fibrils, and in basement membranes, as well as at cell surfaces and intracellularly [1–4]. Proteoglycan synthesis takes place in two separate cellular compartments: the rough endoplasmic reticulum where the polypeptide is formed and the Golgi where glycosylation is completed. The first step in GAG assembly is the xylosylation of specific serine (Ser) residues [2]. A common feature of the GAG attachment site is the presence of adjacent glycines and, further away, acidic amino acids. The xylose (Xyl) is extended at the nonreducing end with the sequence GlcA-Gal-Gal- followed by polymerization of the main chain by repeated, alternating additions of hexosamine and uronic acid to form a $(GlcA\text{-}GalNAc)_n$-chain (as in chondroitin) or a $(GlcA\text{-}GlcNAc)_n$-chain (as in heparan, also termed *N*-acetylheparosan; for reviews see [4–6] and references therein).

The glycan chains can also be subjected to various modifications including C-2 phosphorylation of xylose and C-5 uronosyl epimerization of glucuronic acid, as well as

different forms of sulfation [4–6]. In the case of galactosaminoglycans, chondroitin sulfate (CS) is formed after 4-*O*- and/or 6-*O*-sulfation of *N*-acetylgalactosamine, whereas C-5 uronosyl epimerization (conversion of glucuronic acid to iduronic acid) coupled with *O*-sulfation at C-4 of *N*-acetylgalactosamine [7] produces dermatan sulfate (DS). *O*-Sulfation of iduronic acid or glucuronic acid can also occur to a limited degree. All of these modifications can take place in the same chain, giving rise to complex copolymeric sequences [8]. A method for sequence analysis of core protein-bound DS was recently developed by Fransson *et al.* [9]. After introduction of ^{125}I labelled *p*-hydroxyphenyl groups (HO-Phe) at the residual serine of proteolytically released DS, sequence analysis was performed by partial degradation, separation of fragments using gradient PAGE, and autoradiographic detection.

The role of the core protein in regulating the modification steps involved in DS formation is not fully understood. To study this issue uncoupling of glycan assembly from core protein synthesis is desirable. Exogenous β-D-xylosides can compete with xylosylated core protein and serve as alternative acceptors for the polymerization of GAG chains [5]. Structural analysis can then reveal whether GAG chains primed on free xyloside are different from chains assembled on the xylosylated protein core. The nature of the aglycone portion of the xyloside is critical for its ability to penetrate cells and act as an artificial initiator [10]. The aglycone must be sufficiently hydrophobic and devoid of charged groups. The various aryl and alkyl xylosides tested so far can readily initiate galactosaminoglycan synthesis (CS and DS), whereas they are usually weak or inactive as initiators of heparan/heparin synthesis, even in the presence of inhibitors of core protein synthesis [5, 10].

Xyloside-primed DS chains have recently been analysed for disaccharide composition [11] and by oligosaccharide mapping [12]. DS chains initiated at low xyloside concentration (0.05 mM and below) yield oligosaccharide maps similar to natural DS in the small proteoglycans biglycan and decorin. However, at high concentrations of xyloside (1 mM or higher) effects on the rate of epimerization of glucuronic acid to iduronic acid and on the extent of *N*-acetylgalactosamine 4-sulfation become noticeable. Here we have investigated the capacity of *p*-hydroxyphenyl-*O*-β-D-xylopyranoside (HO-Phe-Xyl) to initiate CS/DS synthesis in human skin fibroblasts both at low and high concentrations. The xyloside-initiated GAGs isolated from the culture medium were then ^{125}I labelled in the HO-Phe group and subjected to various degradations followed by gradient PAGE and autoradiography. By this approach more detailed sequence analysis can be performed and core protein-bound DS chains [9] can be more closely compared with those primed on free xyloside. The major difference observed is that the repeated, periodic appearance of clustered GlcA-GalNAc repeats found in proteoglycan-derived DS was not seen in chains initiated onto free xyloside.

Materials and methods

Materials

Dermatan sulfate (DS) preparation DS-36 from pig skin and oligosaccharides derived from DS-36 by digestion with testicular hyaluronidase were the same as used elsewhere [9]. Chondroitin sulfate (CS, mainly 4-sulfated) from bovine nasal cartilage was a gift from Professor D. Heinegård of the Department of Medical and Physiological Chemistry, Lund. Unsaturated disaccharides were obtained after exhaustive digestion of CS with chondroitin ABC lyase [9]. HO-Phe-Xyl was synthesized as follows (see also [10]). *p*-Hydroxyphenyl-2,3,4-tri-*O*-acetyl-α,β-D-xylopyranoside was prepared from tetra-*O*-acetyl-D-xyloside (2.5 g) by reaction with *p*-hydroquinone (3.45 g) and $ZnCl_2$ (0.1 g) in toluene (50 ml) chloroform (50 ml) at 110°C for 2 h; yield, 3 g. The β-anomer in the resulting tri-*O*-acetylated α,β-D-xyloside was isolated by chromatography on a silica gel column (1 cm × 16 cm) eluted with chloroform methanol (20/1 by vol) and then deacetylated by treating with LiOH (10 mg) in methanol (50 ml) at room temperature for 1 h; yield, 1.2 g. The isolated material had R_F 0.38 on TLC (Silica $60F_{254}$ in $CHCl_3$:CH_3OH, 7:2 by vol), $[\alpha]_D^{20}$ −62° (about 1 in water), and IR (cm^{-1}) 3450 (OH), 1600 (Phe). *p*-Nitrophenyl-β-D-xylopyranoside (O_2N-Phe-Xyl) was purchased from Sigma Chemical Co, St. Louis, MO, USA.

Cell culture media were obtained from NordVacc AB, Sweden. The enzymes used were chondroitin ABC lyase (EC 4.2.2.4), chondroitin AC-I lyase (EC 4.2.2.5) and chondroitin B lyase (EC 4.2.2.6; all products of Seikagaku but obtained through ICN Biomedicals), testicular hyaluronidase (EC 3.2.1.35; 15000 units mg^{-1}, from Leo, Helsingborg, Sweden), and β-D-galactosidase (EC 3.2.1.23; Sigma grade IX).

The following special chemicals and radiochemicals were used: acrylamide and *N*,*N*′-methylenebisacrylamide (Serva), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Sigma), Na^{125}I (1.7×10^4 Ci g^{-1}, Cintichem, Tuxedo, NY), [^{35}S]sulfate (1310 Ci $mmol^{-1}$, Amersham International, UK), D-[6-^{3}H]-galactose (20 Ci $mmol^{-1}$, American Radiolabelled Chemicals, St. Louis, MO, USA). Other chemicals were of analytical grade.

The prepacked columns, column media and membranes/films used were: fast-desalting (FD) Sephadex G-25 10/10, Superose 6 HR 10/30 and Mono Q HR 5/5 (Pharmacia-LKB), DE-53 DEAE-cellulose (Whatman), Bio-Gel P-6 and Zetaprobe nylon (Bio-Rad), Hyperfilm (Amersham), and X-omat AR5 (Kodak). Films were developed with a Gevamatic 60 (Agfa-Gaevert) and scanning was performed with a videodensitometric system (Cybernetic vision and Makab, Göteborg, Sweden). Electrophoresis equipment was from Pharmacia-LKB and the local workshop, and the semi-dry electroblotter (type B) was manufactured by Ancos A/S, Copenhagen, Denmark).

Isolation of metabolically labelled free GAGs

Fibroblasts from human embryonic skin were grown as monolayers in Earle's minimal essential medium supplemented

with 10% (by vol) donor calf serum, 2 mM L-glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). Confluent cultures between passages 5 and 15 were used in the experiments. Incorporation of radioactive precursors was performed in low-sulfate medium ($MgCl_2$ instead of $MgSO_4$) for 24 h with $Na_2{}^{35}SO_4$ (50 μCi ml^{-1}) or in regular medium with D-[6-^{3}H]galactose (20 μCi ml^{-1}) in the absence or presence of HO-Phe-Xyl (see appropriate Figs). The [^{3}H]galactose precursor is expected to introduce its radioactive tracer into GAGs from UDP-Gal, UDP-GlcA and UDP-Xyl (the latter two are formed via UDP-Glc). To inhibit sulfation of the GAGs in some of the experiments, 10 mM sodium chlorate ($NaClO_3$) was added to the cultures [13].

After incorporation of radioactive precursors the culture media were decanted and the cell layers were rinsed gently with phosphate-buffered saline (PBS; 0.137 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4: 3 ml × 5 ml/75 cm^2 dish). The media and the washing were combined, adjusted to pH 5.8 with 2 M HOAc and applied to columns (1 ml) of DE-53 DEAE-cellulose which were equilibrated with 6 M urea, 10 mM *N*-ethylmaleimide and 0.2 M NaOAc, pH 5.8. The columns were washed with five bed vols of the equilibrating buffer, followed by 6 M urea, 50 mM Tris-HOAc, pH 8.0, and 50 mM Tris-HOAc, pH 7.5. Polyanionic material was then displaced by elution with 5 × 1 bed vol. of 4 M guanidinium chloride containing 50 mM NaOAc, pH 5.8. The material was precipitated with 9 vols of ethanol, centrifuged, dissolved in 200 μl of 4 M guanidinium chloride, 0.2% by vol Triton X-100 and 50 mM NaOAc, pH 5.8, and subjected to gel chromatography (FPLC) on Superose 6 HR 10/30, which was eluted with the same solvent at a rate of 0.4 ml min^{-1} using a Pharmacia-LKB FPLC system. One-min fractions were collected and analysed for radioactivity in a scintillation counter (LKB-Wallach) using ReadySafe (Beckman) as a scintillator. In some experiments, free GAGs could be monitored by the dimethylmethylene blue (DMB) method [14]. Pooled material (Fig. 1) was diluted 10-fold with water and subjected to chromatography on columns (0.2 ml) of DEAE-cellulose as described above. The eluted material was precipitated with ethanol, converted to sodium salts by repeated treatment with NaOAc-saturated ethanol and finally lyophilized in a SpeedVac.

Preparation of ^{125}I labelled GAGs

Portions (1–10 μg) of HO-Phe-Xyl initiated GAGs in 100 μl of 50 mM Tris-HCl, pH 7.5, were mixed with 0.4 mCi of $Na^{125}I$ and 20 μl of chloramine-T solution (0.6 mg ml^{-1} in the same buffer). After 2 min at room temperature the reaction was terminated by the addition of 200 μl of $Na_2S_2O_5$ solution (1.2 mg per ml buffer) followed by the addition of 400 μl of KI solution (10 mg ml^{-1}). The reaction mixtures were applied to columns (0.2 ml) of DEAE-cellulose which were equilibrated and eluted as described above. The radio-iodinated GAGs were recovered by ethanol precipitation and further purified by ion exchange chromatography (HPLC) on Mono Q HR 5/5 as described previously [15]. Pooled material (Fig. 2) was diluted 10 times with 7 M urea and recovered by ion exchange chromatography on DEAE-cellulose (see above). Prior to analysis, GAGs were converted to sodium salts by precipitation with NaOAc-saturated ethanol, washed with absolute ethanol, dried and dissolved in 50 μl of water.

Degradation methods

Non-substituted iduronic and xylose were oxidized with periodate (low pH and temperature) and subsequently cleaved by alkaline elimination [8, 16]. Digestions with chondroitin eliminases were conducted as described by the manufacturer. Cleavage of GalNAc-HexA bonds was achieved by using chondroitin ABC lyase and cleavage of GalNAc-GlcA bonds by using chondroitin AC-I lyase both in 0.1 M Tris-HOAc, 10 mM EDTA, pH 7.3, at 37°C. Selective scission of linkages between *N*-acetylgalactosamine and iduronic acid (sulfated or nonsulfated) was obtained by using chondroitin B lyase in 50 mM Tris-HCl, pH 8.0, containing 0.05% (w/v) bovine serum albumin, at 30°C. Clustered GlcA-GalNAc repeats were cleaved (between *N*-acetylgalactosamine and glucuronic acid) by using testicular hyaluronidase in 0.15 M NaCl, 50 mM NaOAc, pH 5.5, at 37°C [8]. Non-reducing terminal unsaturated hexuronic acid (ΔHexA) was eroded by treatment with mercuric salt [17]. Nonreducing terminal galactose was removed with β-galactosidase as described [18].

Separation methods

Fragments of GAGs were separated either by gel chromatography or by gradient polyacrylamide gel electrophoresis (PAGE). For chromatography we used a pre-packed column of fast-desalting (FD) Sephadex G-25 (Pharmacia-LKB) or a column (18 mm × 1000 mm) of Bio-Gel P-6 (Bio-Rad). The former was eluted with 20% (by vol) ethanol in water at a rate of 1 ml min^{-1} (FPLC mode) and the latter with 0.5 M NH_4HCO_3 at a rate of 5 ml h^{-1} (LC mode). Fractions (0.5 and 2.5 ml, respectively) were assayed for ^{3}H and ^{35}S radioactivity by liquid scintillation (see above) or for ^{125}I radioactivity by using an LKB 1271 Ria-Gamma Counter.

Gradient PAGE was conducted as described in detail elsewhere [9]. In brief, the gels were 18 cm wide, 40 cm high and 0.75 mm thick and consisted of a 20–30% gradient resolving gel and a 5% stacking gel with application wells. The buffers used were the same as described by Turnbull and Gallagher [19]. Samples were freeze-dried, dissolved in 10 μl stacking gel buffer containing 25% (w/v) sucrose and layered onto the bottom of the wells. Electrophoresis was carried out for approx. 20 h with 600 V (constant voltage) and 8–10 mA. The gels were cut and standards were stained separately with Azure A.

The degradation products separated by gradient PAGE were blotted onto Zetaprobe nylon by semi-dry electroblotting

[20]. The trans-unit consisted of (a) six layers of filter paper soaked in 0.3 M Tris-NaOH, pH 10.3 (first anode buffer), (b) three layers of filter paper soaked in 25 mM Tris-NaOH, pH 10.4 (second anode buffer), (c) the Zetaprobe membrane wetted in distilled water, (d) the gel which had been equilibrated for 15 min in the second anode buffer and (e) three filter papers soaked in 26 mM Tris-NaOH, 40 mM 6-amino-n-hexanoic acid, pH 9.4 (cathode buffer). Transfer was achieved at 0.8 mA cm^{-2} for 40 min. The membranes were dried and exposed to x-ray films for 24–72 h at −60°C. After development of the films, tracks were scanned by videodensitometry in the transmittance mode. In some experiments the wet gels were directly subjected to autoradiography [9].

Results and discussion

HO-Phe-Xyl as an initiator of GAG synthesis

To determine if HO-Phe-Xyl could serve as a primer for GAG synthesis, fibroblasts were incubated for 24 h with various concentrations of HO-Phe-Xyl in the presence of [^{35}S]sulfate. Polyanionic material was recovered from the medium by chromatography on DEAE-cellulose and then size-separated into proteoglycans and free GAG chains on Superose 6. As shown in Fig. 1a–c addition of xyloside inhibited proteoglycan synthesis/secretion and resulted in the formation of free GAG chains. We also made a comparison between HO-Phe-Xyl and the more commonly used O_2N-Phe-Xyl (Table 1). Both xylosides gave similar results and optimal stimulation by HO-Phe-Xyl was achieved at 0.5–1.0 mM xyloside. Pre-incubation with xyloside for 30 min before the 24 h metabolic labelling gave the same results. By using the dimethylmethylene blue method, we estimated the maximum yield to be 150–250 μg GAG per 75 cm^2 dish.

The ^{35}S labelled GAG formed in the presence of 0.5 mM HO-Phe-Xyl was characterized by ion exchange HPLC on Mono Q before and after treatment with chondroitin ABC lyase (Fig. 2a, b). Before digestion, the GAG had the same elution position as standard CS or DS (Fig. 2a). It was also completely degraded by the ABC lyase (Fig. 2b), indicating that the HO-Phe-Xyl-initiated GAG was a galactosaminoglycan (CS/DS).

^{125}I labelling of HO-Phe-Xyl-initiated GAG

For these experiments we used GAGs metabolically labelled with [^{3}H]galactose to minimize interference by β-emission in the final autoradiography. These GAGs were produced in the presence of 0.05 or 0.5 mM HO-Phe-Xyl and isolated after chromatography on Superose 6 (Fig. 1d–f). In one case (Fig. 1f), $NaClO_3$ was added to inhibit sulfation of the xyloside-initiated GAG [11]. This GAG was produced in reduced quantities compared to the regular one (Fig. 1e). We also prepared ^{3}H labelled GAG in the presence of 1.0 mM O_2N-Phe-Xyl (results not shown). In all of these experiments production of ^{3}H labelled hyaluronan (void volume fractions in Fig. 1d–f) was also observed.

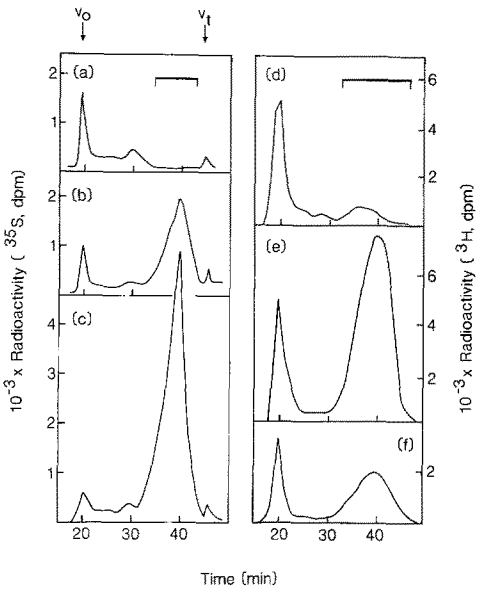

Figure 1. Gel chromatography on Superose 6 of radiolabelled proteoglycans and galactosaminoglycans. Fibroblast cultures were incubated with [^{35}S]sulfate (a–c) or [^{3}H]galactose (d–f) in the absence (a) or presence (b–f) of various concentrations of HO-Phe-Xyl. The concentrations used were: b, 0.1 mM; c, 0.5 mM; d, 0.05 mM; and (e, f), 1 mM. In f, 10 mM $NaClO_3$ was also added to the incubation medium. Polyanionic material was recovered from the culture media by ion exchange chromatography as described in the Materials and methods section and subjected to gel chromatography. Aliquots of the fractions were analysed for radioactivity by liquid scintillation spectrometry. Radiolabelled, free GAG chains were pooled as indicated by the bars. v_0, Void volume; v_t, total volume.

Table 1. Yield of ^{35}S labelled GAG initiated onto exogenous HO-Phe-Xyl or O_2N-Phe-Xyl[a].

Xyloside (mM)	[^{35}S]*Glycosaminoglycan* (10^{-6} counts min^{-1})	
	HO-Phe-Xyl	O_2N-Phe-Xyl
0.1	2.9	3.0
0.3	5.4	
0.5	7.0	
1.0	6.8	6.5
2.0	4.0	

[a] Cells were incubated with various concentrations of xyloside and radiosulfate as described in the Materials and methods section. ^{35}S Labelled GAGs were isolated from the culture medium after ion exchange and gel chromatography.

The various ^{3}H labelled GAGs were then ^{125}I labelled in their HO-Phe groups. These GAGs incorporated approx. 10^6 counts min^{-1} of ^{125}I/μg. The O_2N-Phe-Xyl-initiated GAG incorporated less than 10^4 counts min^{-1} μg^{-1}. The

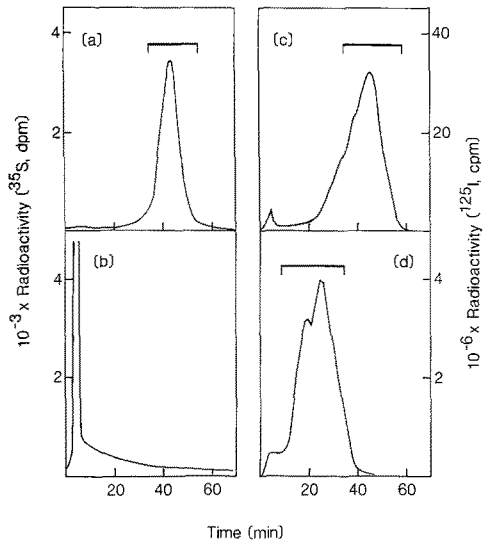

Figure 2. Ion exchange HPLC on MonoQ of radiolabelled GAGs before and after enzymic degradation. Free GAGs were isolated from the culture media after incubation with 0.5 mM (a,b) or 1 mM (c,d) HO-Phe-Xyl as described in the Materials and methods section (see also Fig. 1). In d, 10 mM $NaClO_3$ was also added to the incubation medium. In a,b, the GAGs were [^{35}S]sulfate labelled whereas in c,d, the GAGs were initially [^{3}H]galactose labelled (Fig. 1e, f) and then ^{125}I labelled in the HO-Phe group. The various GAGs were chromatographed directly (a,c,d) or after depolymerization with 5 mIU chrondroitin ABC lyase (b). The gradient starts with 0.3 M NaCl at 10 min and ends after 70 min with 1.2 M NaCl. Aliquots of the fractions were analysed for radioactivity by liquid scintillation (^{3}H) or by gamma radiation spectrometry (^{125}I). Material was pooled as indicated by the bars. Hyaluronan (the void volume material from Fig. 1e) eluted after 20 min and CS/DS-standards after 45 min.

^{125}I labelled HO-Phe-Xyl-initiated GAGs produced in the absence or presence of $NaClO_3$ were finally characterized by ion exchange HPLC on Mono Q (Fig. 2c,d). The sulfated GAG eluted in the same position as CS or DS (Fig. 2c) whereas the undersulfated (non-sulfated) product eluted much earlier (Fig. 2d).

The linkage-region of xyloside-initiated GAG

To examine whether the xylose of HO-Phe-Xyl-initiated GAG had been phosphorylated (substituted) at C-2/C-3 the ^{125}I labelled product was treated with periodate, which oxidizes unsubstituted xylose (and iduronic acid). After scission of susceptible sugars in alkali the reaction mixtures were subjected to gradient PAGE (Fig. 3). Untreated GAG (lane 1) migrated as a polymer of 30–60 disaccharide repeats corresponding to a molecular weight of 15 000–30 000. After oxidation with periodate for only 2 min followed by alkali treatment there were essentially no radioactive products in the electrophoretogram. These findings indicate that the xylose remained unsubstituted at C-2 (or C-3). The ^{125}I-labelled GAG was resistant to alkaline treatment alone (results not shown).

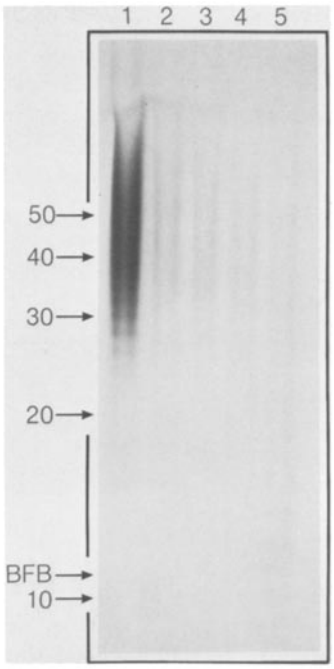

Figure 3. Gradient PAGE of xyloside-initiated ^{125}I labelled CS/DS after periodate oxidation alkaline elimination. Free GAG was isolated from the cell medium after incubation with [^{3}H]galactose and 1 mM HO-Phe-Xyl (see Fig. 1). After ^{125}I labelling of the HO-Phe group samples (10 μg) containing 4×10^6 counts min^{-1} ^{125}I were oxidized with $NaIO_4$ (final volume 20 μl) for the following periods of time: lane 1, 0 min; 2, 30 min; 3, 1 h; 4, 4 h; 5, 24 h. Oxidations were stopped by the addition of mannitol, and oxidized sugar rings were cleaved in alkali. Gradient PAGE, blotting and autoradiography were performed as described in the Materials and methods section. Arrows to the left indicate migration positions of oligosaccharides (degree of polymerization 10, 20, 30, 40 and 50) produced by exhaustive digestion with testicular hyaluronidase of pig skin DS preparation DS-36. BPB, bromophenol blue.

Longer segments comprising the GlcA-Gal-Gal linkage region between xylose and the main chain were obtained after digestion with chondroitin ABC or AC-I lyase. The former enzyme should produce the fragment ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I] whereas the latter enzyme removes also the last repeating disaccharide [8, 9]. Radio-iodinated GAGs initiated on HO-Phe-Xyl in the absence or presence of $NaClO_3$ were digested exhaustively with either of the two lyases and then chromatographed on Bio-Gel P-6 (Fig. 4). As expected, the major ^{125}I-labelled products obtained after treatment with the ABC lyase were larger (fraction 70–80 in Fig. 4a,c) than were those generated by the AC lyase (fraction 85–95 in Fig. 4b,d); the latter should have the structure ΔHexA-Gal-Gal-Xyl-Phe-OH[^{125}I] [8,9]. Furthermore, the products derived from fully sulfated or undersulfated GAGs eluted in the same positions (cf. Fig. 4a,b with c,d).

The radio-iodinated, fully sulfated GAG was also examined by gradient PAGE after both partial and complete digestion with chondroitin ABC lyase (Fig. 5a). After limited digestion a

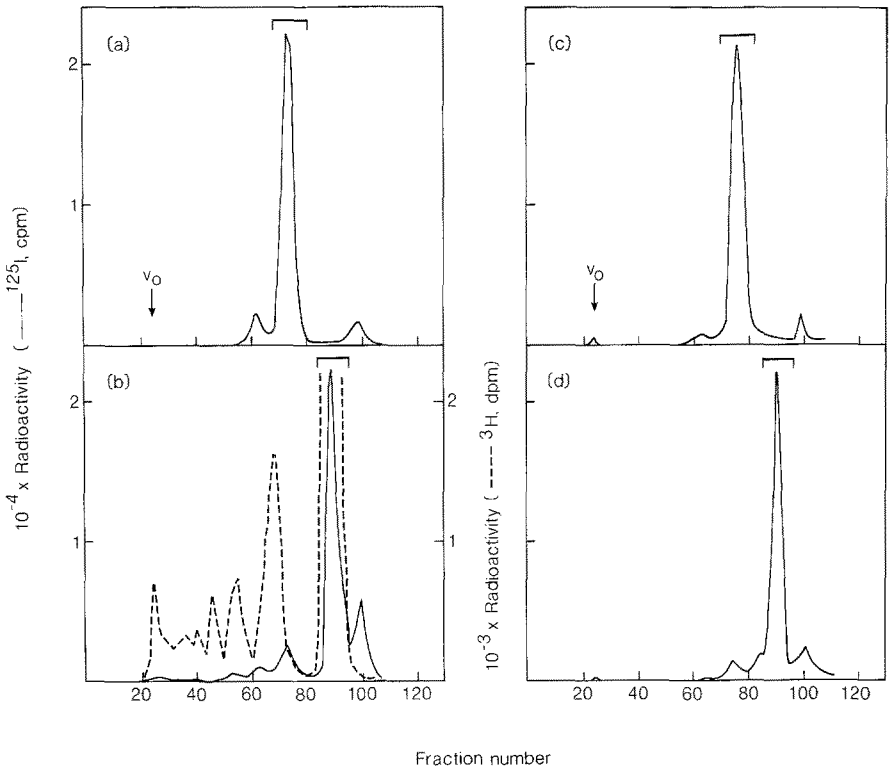

Figure 4. Gel chromatography on Bio-Gel P-6 of exhaustive chondroitin lyase digests of CS/DS. Free GAG was isolated from the cell medium after incubating cells with [^{3}H]galactose and 1 mM HO-Phe-Xyl. In (c,d) 10 mM $NaClO_3$ was also added to the incubation medium (see also Fig. 1). Portions of the various GAGs were then ^{125}I labelled in the HO-Phe group. Samples containing 5×10^6 counts min^{-1} ^{125}I (or 10^6 disintegrations min^{-1} ^{3}H) and 50 µg CS-carrier in 50 µl digestion buffer were incubated with 5 mIU chondroitin ABC (a,c) or AC-I lyase (b,d) at 37°C for 4 h. The digests were diluted with 1 ml elution buffer and chromatographed. In b, a separate run of the same sample before radio-iodination (dashed line) is included. Aliquots of the fractions were analysed for radioactivity by gamma radiation (^{125}I) or liquid scintillation spectrometry (^{3}H). Fractions were pooled as indicated by the bars. Standard disaccharide eluted around fraction 70. Note that, in b, ^{3}H-labelled material also appeared in the position of the expected linkage-region fragment ΔHexA-Gal-Gal-Xyl-Phe-OH (fraction 85–95). Apparently, HO-Phe-Xyl containing saccharides eluted in an unexpectedly retarded position on Bio-Gel P-6, possibly due to hydrophobic interactions between the HO-Phe groups and the polyacrylamide gel matrix. In this way, HO-Phe-tagged saccharides can be better resolved on this gel.

complex pattern of products emerged (lane 2) but after extensive digestion (lanes 3–5) there were essentially only two components left. However, as mentioned above, chromatography on Bio-Gel P-6 of a complete digest of the same material (Fig. 4a) yielded only one component. We therefore isolated the latter material and subjected it to gradient PAGE (Fig. 5b, lane 1). It is seen that the single component obtained after size-separation (Fig. 4a) was split into two after electrophoresis. Most likely, these two components had different negative-charge density. When undersulfated GAG was treated with the ABC lyase a major linkage-region component of the expected size was obtained (Fig. 4c). However, this component was homogeneous upon electrophoresis (Fig. 5b, lane 2) and emerged in the more retarded position. Hence, we conclude that the two components seen in Fig. 5b, lane 1 were ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I] with or without sulfate (presumably at the *N*-acetylgalactosamine moiety).

To examine the segment -GlcA-Gal-Gal-Xyl- radio-iodinated, fully sulfated GAG was treated with chondroitin AC-I lyase for various time periods and subjected to gradient PAGE (Fig. 6a). After partial degradation, several bands were observed (lanes 2 and 3), one of which became prominent after complete degradation (lanes 4 and 5). The latter component corresponded (Fig. 6b, lane 1, see arrow) to the major linkage-region fragment ΔHexA-Gal-Gal-Xyl-Phe-OH[^{125}I] isolated after chromatography on Bio-Gel P-6 of AC-I treated GAGs (Fig. 4b). Hence, there were no charge variants in the segment -GlcA-Gal-Gal-Xyl-. The corresponding radiolabelled component migrated relatively slowly upon electrophoresis (Fig. 6a, lanes 4 and 5) because of a low negative charge density (−1 per four monosaccharides as compared to −2 or −3 per six monosaccharides in the ABC-generated fragments, see Fig. 5). It should also be added that the results were the same when gels were autoradiographed directly. (In most experiments material were blotted onto membrane and then visualized.)

To confirm that the AC-generated linkage region fragment

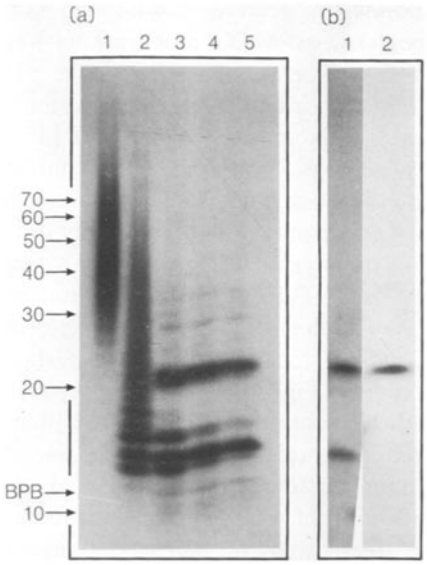

Figure 5. a, Gradient PAGE of xyloside initiated and ^{125}I-labelled CS/DS after partial or complete degradation with chondroitin ABC lyase. Free GAG was isolated from the cell medium after incubation with [^{3}H]galactose and 1 mM HO-Phe-Xyl (Fig. 1). After ^{125}I labelling of the HO-Phe group samples (3 μg) containing 10^6 counts min^{-1} ^{125}I and 10 μg of CS-carrier in 10 μl of digestion buffer were incubated with 1 mIU enzyme at 37°C for the following periods of time: lane 1, 0 min; 2, 2 min; 3, 15 min; 4, 30 min; 5, 1 h. Digestions were stopped by boiling for 2 min and the reaction mixtures were then electrophoresed. b, Gradient PAGE of ^{125}I-labelled linkage-region fragments derived from chondroitin ABC lyase-treated CS/DS that was initiated onto xyloside in the absence (lane 1) or presence (lane 2) of $NaClO_3$. The xyloside initiated GAGs were the preparations described in Fig. 1e,f. After radio-iodination and exhaustive digestion with chondroitin ABC lyase, the linkage-region fragments were isolated by gel chromatography on Bio-Gel P-6 (see Fig. 4a,c). Portions (10^5 counts min^{-1} ^{125}I) of the materials were lyophilized and electrophoresed. Arrows to the left of the panels indicate migration positions of standard DS oligosaccharides (see Fig. 3).

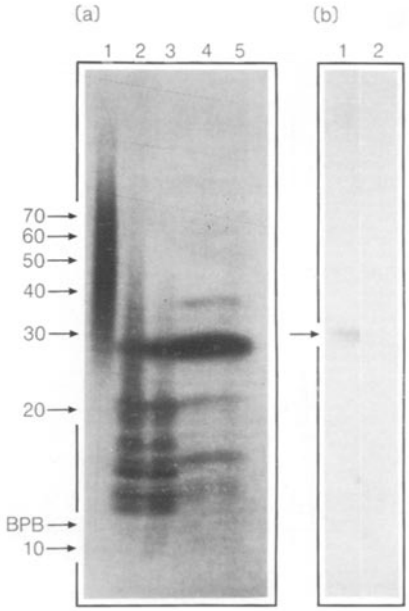

Figure 6. a, Gradient PAGE of xyloside initiated and ^{125}I-labelled CS/DS after partial or complete degradation with chondroitin AC-I lyase. The radiolabelled GAG was the same preparation as used in Fig. 5. Samples (3 μg) containing 10^6 counts min^{-1} ^{125}I and 10 μg of CS-carrier in 10 μl of digestion buffer were incubated with 1 mIU of enzyme at 37°C for the following periods of time: lane 1, 0 min; 2, 2 min; 3, 5 min; 4, 30 min; 5, 1 h. Digestions were stopped by boiling and the reaction mixtures were then electrophoresed. b, Gradient PAGE of ^{125}I-labelled linkage region fragment derived from chondroitin AC-I lyase-treated CS/DS before (lane 1) and after (lane 2) further degradation with $HgCl_2$. The xyloside-initiated GAG (Fig. 1e) was labelled with ^{125}I in the HO-Phe group, digested exhaustively with chondroitin AC-I lyase, and chromatographed on Bio-Gel P-6 (Fig. 4b). The smallest linkage-region fragment was recovered, lyophilized and portions (10^5 counts min^{-1} ^{125}I) were electrophoresed before and after removal of terminal ΔHexA residues. For further details see Figs 3 and 5.

contained only one negative charge (that of ΔHexA) the nonreducing terminal unsaturated sugar was eroded by treatment with mercuric salt [17]. When the reaction mixture was electrophoresed (Fig. 6b, lane 2) no charged products were seen. However, after gel chromatography of these products on fast-desalting Sephadex G-25 (Fig. 7), a radio-labelled component was recovered from the void volume fractions. This component was sensitive to digestion with *β*-galactosidase, resulting in the formation of a fragment eluting after standard disaccharide and near the total volume. Before treatment with $HgCl_2$ the AC-generated fragment was insensitive to *β*-galactosidase (results not shown). We therefore conclude that the segment -GlcA-Gal-Gal-Xyl- of HO-Phe-Xyl-initiated GAG contains no further negatively charged substituents, such as sulfate at C-6 of the second galactose [21] or phosphate at C-2 of xylose (see also Fig. 3). The first sulphate group to be encountered was located at the first *N*-acetylgalactosamine.

Structure of the main chain in xyloside initiated GAG

After the linkage region -GlcA-Gal-Gal-Xyl- is completed the main chain starts to grow by alternating additions of *N*-acetylgalactosamine and glucuronic acid to the nonreducing end of the terminal glucuronic acid. To examine the extent of conversion of glucuronic acid to iduronic acid in the fully sulfated product, [^{3}H]galactose-labelled and HO-Phe-Xyl-initiated GAG was digested with chondroitin AC-I lyase, which cleaves GalNAc-to-GlcA glycosidic bonds. Chromatography on Bio-Gel P-6 (Fig. 4b, dashed line) showed that disaccharide (fraction 63–73) but also higher saccharides were obtained. It was estimated that the IdoA/GlcA ratio was approx. 1:1. Hence, the polysaccharide precursor formed in the presence of 1 mM HO-Phe-Xyl can still be converted to DS (see also [9]).

To examine the copolymeric structure of the main chain further, xyloside primed DS was degraded partially with testicular hyaluronidase (which cleaves GalNAc-to-GlcA

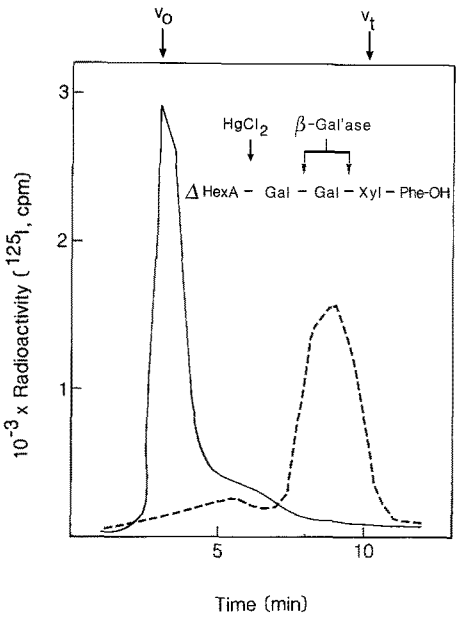

Figure 7. Gel chromatography on fast-desalting G-25 of the putative fragment Gal-Gal-Xyl-[^{125}I]HO-Phe before (———) and after (------) treatment with β-galactosidase. The starting material was the same xyloside initiated and radio-iodinated GAG as in Fig. 6 (see also Fig. 1e). The radiolabelled GAG was digested exhaustively with chondroitin AC-I lyase and the smallest linkage-region fragment (putative structure ΔHexA-Gal-Gal-Xyl-[^{125}I]HO-Phe) was isolated by gel chromatography on Bio-Gel P-6 (Fig. 4b). After removal of terminal ΔHexA by treatment with $HgCl_2$ the saccharide was chromatographed directly (———) and after further digestion with β-galactosidase (------). A standard disaccharide from CS eluted after 6–7 min.

glycosidic bonds in regions containing clustered GlcA-GalNAc repeats). Two GAG preparations were used, one produced in the presence of 0.05 and the other in 1 mM HO-Phe-Xyl. In the former case the extent of uronic acid C-5 epimerization is only marginally affected [11, 12]. As the above results (see Figs 5 and 6) indicated that linkage region fragments extended with a few additional disaccharide repeats migrated further on electrophoresis than did the shorter linkage region fragment itself, gel chromatography on Bio-Gel P-6 was used (Fig. 8) in combination with gradient PAGE (Fig. 9). The chains initiated at low xyloside concentration afforded mainly small fragments after partial degradation with hyaluronidase (Fig. 8a). The same preparation was also degraded with hyaluronidase for different time-periods and then subjected to gradient PAGE (Fig. 9a). Although an extensive banding pattern could be observed after very short digestion-times, most of the fragments appeared to be relatively short. Hence, there were no signs of a repeated, periodic appearance of clustered GlcA-GalNAc repeats as was previously observed in core protein-bound DS [9].

The various saccharide fragments isolated after gel chromatography (Fig. 8a, fractions a–e) were also separately analysed by gradient PAGE (Fig. 9b). As suspected, the shortest fragment (component e, lane 5) migrated more slowly than did fragments containing one or two additional disaccharide repeats (components d and c, lanes 4 and 3). In the case of fragments containing one repeat (component d, lane 4) and upwards (components a–c, lanes 1–3) there was a linear relationship between size and migration distance. Furthermore, the two shortest fragments (components d and e, lanes 4 and 5) were heterogeneous, suggesting that they

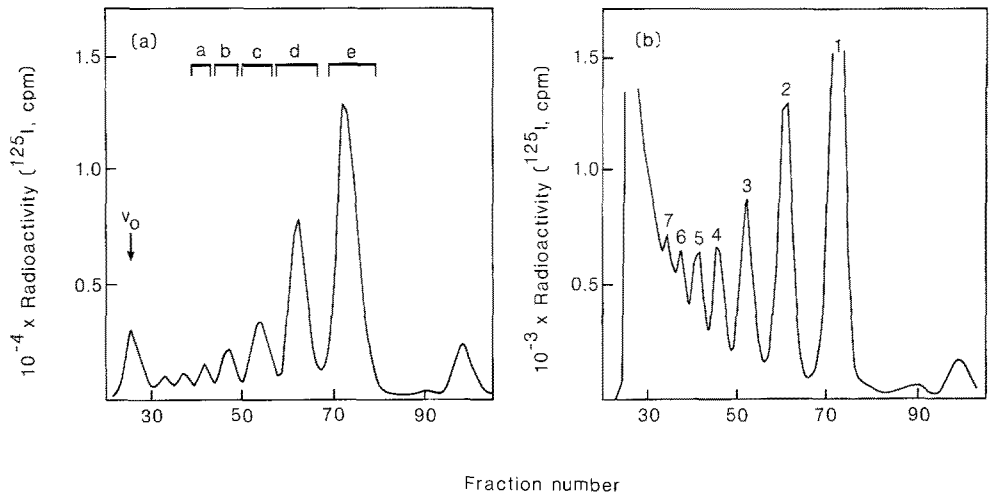

Figure 8. Gel chromatography of partial testicular hyaluronidase digests of CS/DS. The GAGs used were initiated in the presence of 0.05 mM (a) or 1 mM HO-Phe-Xyl (b) (see Fig. 1d,e) and then ^{125}I-labelled in their HO-Phe groups. Samples containing 5 × 10^6 counts min^{-1} ^{125}I (with 50 μg CS-carrier in b) in 50 μl digestion buffer were incubated with 2 mIU testicular hyaluronidase at 37°C for 5 min. After boiling for 2 min the digests were chromatographed. Fractions a–e were pooled as indicated by bars. For further details, see Fig. 4. The generated fragments have the general structure (HexA-GalNAc)$_n$-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I]. Those corresponding to n = 1–7 are indicated in b.

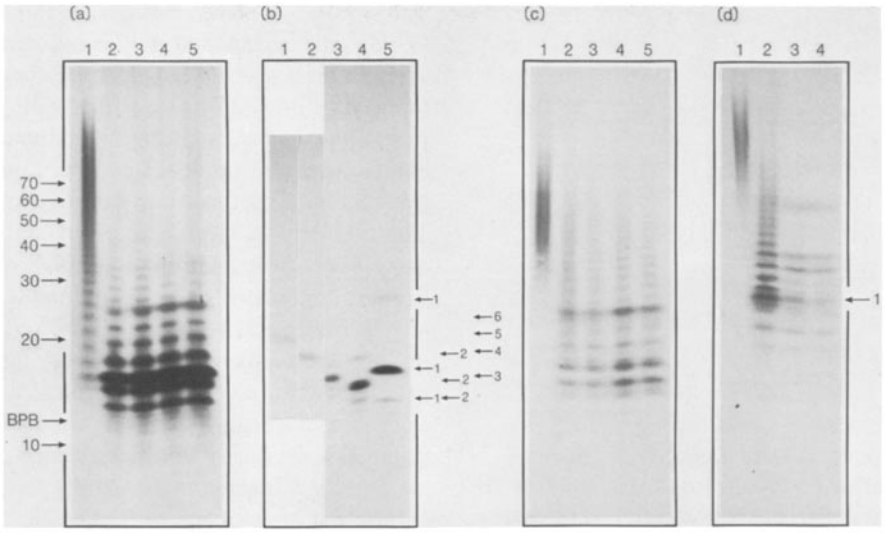

Figure 9. Gradient PAGE of xyloside-initiated and ^{125}I-labelled CS/DS after partial or complete degradation with testicular hyaluronidase. Free GAGs were isolated from the cell media after incubation with [^{3}H]galactose and 0.05 mM (a,b) or 1 mM HO-Phe-Xyl (c,d). In d, 10 mM $NaClO_3$ was also added to the incubation medium. After ^{125}I-labelling of GAGs in their HO-Phe groups they were subjected to the following treatments. a, Samples of CS/DS initiated in the presence of 0.05 mM HO-Phe-Xyl and containing 5×10^6 counts min^{-1} ^{125}I in 10 µl digestion buffer were incubated with 0.4 mIU enzyme at 37°C for 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 30 min (lane 4), and 1 h (lane 5). b, A 5-min digest of the same ^{125}I labelled CS/DS was first size-separated on Bio-Gel P-6 (Fig. 8a) into fractions a–e, lyophilized and then electrophoresed (lane 1, a; 2, b; 3, c; 4, d; 5, e). Note that the shortest linkage region fragment migrates more slowly than some of the longer fragments. This could be explained by a lower charge density in the linkage region fragment compared to fragments containing one or more repeating disaccharides. c, Samples of CS/DS initiated in the presence of 1 mM HO-Phe-Xyl and containing 10^6 counts min^{-1} ^{125}I and 10 µg CS-carrier were treated and electrophoresed as in a. d, Samples of GAGs initiated in the presence of 1 mM HO-Phe-Xyl and 10 mM $NaClO_3$ and containing 10^6 counts min^{-1} ^{125}I were incubated with the enzyme for 0 min (lane 1), 1 min (lane 2), 30 min (lane 3) and 1 h (lane 4). All digestions were stopped by boiling and the reaction mixtures were then electrophoresed. For further details, see Figs 3 and 5. The generated fragments have the general structure (HexA-GalNAc)$_n$-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I]. Those corresponding to $n = 1$–6 are indicated by arrows.

contained saccharides with different degrees of sulfation. The saccharide with one repeat (component e, lane 5) contained two major components which migrated to the same positions as did the linkage-region fragments generated by chondroitin ABC lyase (see Fig. 5b, lane 1). Hence, we conclude that the shortest hyaluronidase generated fragments were GlcA-GalNAc-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I] with or without sulfate on the *N*-acetylgalactosamine. Undersulfation appeared to be more prominent in DS produced at high xyloside concentration.

Also the DS-chains initiated at high xyloside concentration (1 mM) yielded short fragments after limited digestion with hyaluronidase (Fig. 8b, 9c) indicating that clustered GlcA-GalNAc repeats were concentrated to the vicinity of the linkage region (especially the first three repeats). However, in both cases, relatively long fragments remained after more extensive digestions (see lanes 3–5 in Fig. 9a,c). This suggested that there were chains which contained very few, if any, clustered GlcA-GalNAc repeats. The results were principally the same when digestions were carried out in the presence of an excess of β-glucuronidase (results not shown). Again, there were no signs of a repeated, periodic appearance of clustered GlcA-GalNAc repeats.

We also compared GAGs produced in the absence or presence of $NaClO_3$ which will suppress sulfation [13]. As expected the undersulfated GAG migrated more slowly upon electrophoresis (cf. Fig. 9c, lane 1 with d, lane 1). Furthermore, as efficient epimerization of glucuronic acid to iduronic acid requires concomitant sulfation of neighbouring *N*-acetylgalactosamine at C-4 [7], we did not expect to find any DS [13]. As shown in Fig. 9d, limited digestion with hyaluronidase of undersulfated GAG (lane 2) did produce a banding pattern. However, with time, only a few, sometimes widely spaced, bands remained (see lanes 3 and 4). One of the major bands seen in lane 2 (see arrow) appeared approximately in the same position as a linkage-region fragment containing one nonsulfated disaccharide repeat. This component and the 2 or 3 more retarded ones persisted throughout. The latter components could either represent a few chains with occasional IdoA-GalNAc repeats or they could be the result of transglycosylation of tetrasaccharides or higher ones onto the shortest linkage-region fragment. Undersulfated, xyloside-primed and end-labelled DS can be used as a substrate for C-5-uronosyl epimerase to identify the location of enzyme binding sites (A. Malmström and L.-Å. Fransson, unpublished results).

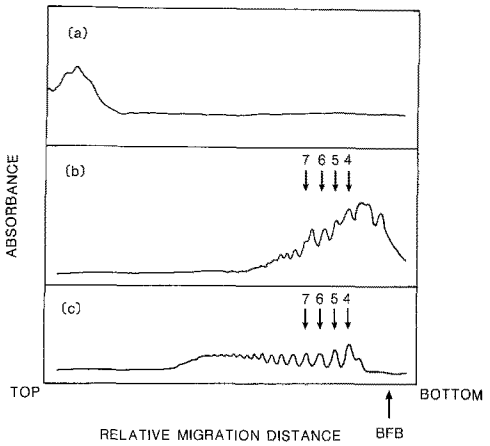

Figure 10. Densitometric scans of electrophorograms of enzyme-treated sulfated or undersulfated ^{125}I-labelled GAGs. Free GAGs were isolated from cell media after incubation with [^{3}H]galactose and 1 mM HO-Phe-Xyl. In a, 10 mM $NaClO_3$ was also added to the medium. After ^{125}I labelling of GAGs in their HO-Phe groups they were subjected to the following treatments. a,c, Samples containing 10^6 counts min^{-1} ^{125}I-labelled undersulfated (a) or sulfated (c) GAG together with 10 μg CS-carrier in 10 μl digestion buffer were incubated with 0.3 mIU chondroitin B lyase at 30°C for 1 h. b, A similar amount of sulfated GAG was treated with testicular hyaluronidase (as in Fig. 9c, lane 4). After electrophoresis and autoradiography the tracks were scanned by videodensitometry. The generated fragments have the general structure (HexA/ΔHexA-GalNAc)$_n$-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I]. Those corresponding to $n = 4$–7 are indicated by arrows. The ordinate is absorbance in arbitrary unit.

To examine more directly the location of iduronic acid residues, fully sulfated and undersulfated GAGs were degraded with chondroitin B lyase followed by gradient PAGE (the results are presented as densitometric scans in Fig. 10). Undersulfated GAG was essentially resistant to degradation by this enzyme (Fig. 10a), indicating that iduronic acid residues were indeed rare in this material. The fully sulfated GAG was degraded partially, and in parallel, by both testicular hyaluronidase (Fig. 10b) and chondroitin B lyase (Fig. 10c) followed by gradient PAGE. The fragments generated had the general structure (HexA/ΔHexA-GalNAc)$_n$-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I] and the positions of fragments corresponding to $n = 4$–7 are indicated in the scanning profiles. Whereas hyaluronidase generated substantial quantities of fragments shorter than $n = 4$ (Fig. 10b), the chondroitin B lyase afforded preferentially fragments with $n = 4$ or higher (Fig. 10c). We therefore conclude that glucuronic acid is common in the first three positions from the linkage region, whereas iduronic acid can appear in almost any position thereafter. There were no obvious signs of a repeated, periodic appearance of this residue.

Discussion

The procedure described here can be used to examine sequential arrangements of disaccharide repeats in xyloside initiated CS/DS. By using HO-Phe-Xyl the isolated free GAGs can be labelled at the reducing terminal with ^{125}I. Partial cleavage with selective chemical and enzymatic methods followed by gradient PAGE permits the identification of fragments extending from the radiolabelled reducing end to the point of cleavage. The fragments generated have the general structure (HexA-GalNAc)$_n$-GlcA-Gal-Gal-Xyl-Phe-OH[^{125}I], as indicated by the results of analyses described above. Gradient PAGE performed under the present conditions resolves fragments with $n = 1$ to almost $n = 20$. Fragments with $n = 1$–3 showed anomalous behaviour on gradient PAGE, because they appeared in the order $n = 2$, $n = 3$, $n = 1$. Saccharides with $n = 4$ or higher appeared in a regular fashion. The presence of undersulfated fragments produced additional bands. To avoid this complexity, short fragments ($n = 1$–4) can be resolved on Bio-Gel P-6 on the basis of size only. The electrophoretic mobility of the fragments studied here was generally slower than that of fragments obtained from core protein-bound DS derivatized in the serine moiety [9], because the latter ones contain an additional negative charge (the carboxylate of the serine).

It should also be discussed whether GAGs initiated onto exogenous xylosides are made in the same way (involving the same enzymes or regulated in the same manner) as GAGs that are attached to the appropriate core protein. Alternative pathways may exist because mutant cells defective in galactosyltransferase I (forming Gal-Xyl-R) can utilize O_2N-Phe-Xyl for the formation of GAG chains [22]. The xyloside concentration can also affect the extent and nature of polymer modification. As shown previously [11, 12], DS chains assembled on xyloside and chains attached to the core proteins of biglycan and decorin afford similar oligosaccharide maps provided the xyloside concentration is low.

In the present study we have shown that HO-Phe-Xyl can be used as an initiator of DS-synthesis. As the DS chain produced can be end-labelled with ^{125}I, sequence analysis can be performed. We have analysed DS chains primed both at low (0.05 mM) and high (1 mM) concentrations of HO-Phe-Xyl. In both cases we obtained chains without phosphate at C-2 of the xylose moiety. This is in agreement with results obtained by Greve and Kresse [23], who found that O_2-Phe-Xyl-initiated CS/DS incorporated very little [^{32}P]-phosphate. Furthermore, DS from pig skin contains non-phosphorylated xylose in this position [9]. The remainder of the linkage region segment -GlcA-Gal-Gal-Xyl- appeared identical in the HO-Phe-Xyl-initiated DS and in core protein-bound DS [9]. There were no indications of additional charged substituents (like Gal-6-SO_4, see [21]) in this region. The first 2 or 3 disaccharide repeats following the linkage region of core protein-bound DS are largely GlcA-GalNAc often lacking sulfate groups [9, 24]. Similarly, the HO-Phe-Xyl-initiated DS contained GlcA-GalNAc repeats in these positions.

However, partial degradations with either testicular hyaluronidase or chondroitin B lyase indicated that the

arrangement of the GlcA-GalNAc and IdoA-GalNAc repeats was different in DS-chains initiated onto xyloside (at low concentrations) compared to core protein-bound DS [9]. The latter has a repeated, periodic distribution of clustered GlcA-GalNAc repeats with peaks at positions 1–3, 8 or 9, and around 25. In contrast, DS assembled on xyloside has glucuronic acid in positions 1–3 but no obvious clustering elsewhere. Evidently, oligosaccharide mapping fails to detect such subtle differences. It is thus possible that the core protein is important for the orientation of the proteoglycan precursor in relation to the multi-enzyme complexes in the Golgi membranes and that this will affect the distribution of modified glycan-segments. The approach described here should be useful for further investigations of DS-biosynthesis and its regulation.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council, the National Board for Technical Development, and the Cancer Trust, from the Ministry of Education, Science and Culture, Japan, the GustafV:s80-ås fond, the Österlund, JA Persson, Bergvall, and Kock Foundations, and the Medical Faculty, University of Lund. We are grateful to Lena Åberg for technical assistance and to Birgitta Jönsson for art-work.

References

1. Fransson LÅ (1987) *Trends Biochem Sci* **12**:406–11.
2. Ruoslahti E (1988) *Annu Rev Biochem* **4**:229–55.
3. Heinegård D, Oldberg Å (1989) *FASEB J* **3**:2042–51.
4. Fransson LÅ (1989) In *Heparin* (Lane DA and Lindahl U, eds) pp. 115–33. London: Edward Arnold.
5. Gallagher JT, Lyon M (1989) In *Heparin* (Lane DA and Lindahl U, eds) pp. 135–58. London: Edward Arnold.
6. Lindahl U (1989) In *Heparin* (Lane DA and Lindahl U, eds) pp 159–89. London: Edward Arnold.
7. Malmström A (1984) *J Biol Chem* **259**:161–5.
8. Fransson LÅ (1985) In *The Polysaccharides* Vol 3 (Aspinall GO, ed) pp 337–415. Orlando: Academic Press.
9. Fransson LÅ, Havsmark B, Silverberg I (1990) *Biochem J* **269**:381–8.
10. Sobue M, Habuchi H, Ito K, Yonekura H, Oguri K, Sakurai K, Kamohara S, Ueno Y, Noyori R, Suzuki S (1987) *Biochem J* **241**:591–601.
11. Cöster L. Hernnäs J, Malmström A (1991) *Biochem J* **276**: 533–9.
12. Fransson LÅ, Schmidtchen A, Cöster L, Malmström A (1991) *Glycoconjugate J* **8**:108–15.
13. Greve H, Cully Z, Blumberg P, Kresse H (1988) *J Biol Chem* **263**:12886–92.
14. Farndale RW, Buttle DJ, Barrett A (1986) *Biochim Biophys Acta* **883**:173–7.
15. Lindblom A, Carlstedt I, Fransson LÅ (1989) *Biochem J* **261**:145–53.
16. Fransson LÅ, Carlstedt I (1974) *Carbohydr Res* **36**:349–58.
17. Ludwigs U, Elgavish A, Esko JD, Meezan E, Rodén L (1987). *Biochem J* **245**:795–804.
18. Fransson LÅ, Silverberg I, Carlstedt I (1985) *J Biol Chem* **260**:14722–26.
19. Turnbull JE, Gallagher JT (1988) *Biochem J* **251**:597–608.
20. Kyhse-Andersen J (1984) *J Biochem Biophys Meth* **10**:203–63.
21. Sugahara K, Yamashina I, De Waard P, Van Halbeck H, Vliegenhart JFG (1988) *J Biol Chem* **263**:10168–74.
22. Esko JD, Weinke JL, Taylor WH, Ekborg G, Rodén L, Anantharamaiah G, Gawish A (1987) *J Biol Chem* **262**:12189–95.
23. Greve H, Kresse H (1988) *Glyconconjugate J* **5**:175–83.
24. Fransson LÅ (1968) *Biochim Biophys Acta* **156**:311–16.